Chromatin assembly factors and heterochromatin organization during cell prolifertaion, tumorigenesis and in quiescence

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List of abbreviations

6,4-PP: 6-4 pyrimidine-pyrimidone adducts
Å: Angström
Asf1: Anti-silencing function 1
bp: base pairs
CAF-1: Chromatin Assembly Factor 1
CGH: Comparative Genomic Hybridization
CHD: Chromodomain
CHO: Chinese Hamster Ovarian
ChIP: Chromatin Immunoprecipitation
CPD: Cyclobutane Pyrimidine Dimers
CpG: Cytosine-phospho-Guanine
CS: Cockayne syndrome
CSD: Chromoshadowdomain
DNA: Deoxyribonucleic acid
DNMT: DNA Methyl Transferase
DSB: Double Strand Break
dsRNA: double stranded RNA
E(var): Enhancer of variegation
E2F: E2 promoting Factor
ER: Estrogen receptor
EZH2: Enhancer of Zeste Homolog 2
FISH: Fluorescent In Situ Hybridization
FRAP: Fluorescence Recovery After Photobleaching
GGR: Global Genome Repair
HAT: Histone Acetyl Transferase
HD: Histone Demethylase
HDAC: Histone Deacetylase
HER2: Human Epidermal Growth Factor Receptor-2
HIRA: Histone Regulator A
HMT: Histone Methyl Transferase
HP1: Heterochromatin Protein 1
IF: Immunofluorescence
KAP-1: KRAB associated protein 1
Kb: Kilobase
KO: Knock-out
KRAB: Kruppel-associated box
M: Molar
Mb: Megabase
MBD: Methyl Binding Domain
MCM: Minichromosome Maintenance
mRNA: messenger RNA
Nasp: Nuclear Autoantigenic Sperm Protein
NER: Nucleotide Excision Repair
ORC: Origin Recognition Complex
PCNA: Proliferating Cell Nuclear Antigen
PEV: Position Effect Variegation
PR: Progesteron Receptor
PRC: Polycomb Repressor Complex
Rb: Retinoblastoma
RNA: Ribonucleic Acid
RNAi: RNA interference
SAHF: Senescence Associated Heterochromatin Foci
siRNA: short interfering RNA
Su(var): Suppressor of Variegation
SV40: Simian Virus 40
TCR: Transcription-Coupled Repair
TSA: Trichostatin A
TTD: Trichothiodystrophy
UV: Ultra-Violet
XP: Xeroderma Pigmentosum

Abstract

In cancer cells, the organization of DNA in chromatin is frequently affected. Thus, it is crucial to understand how factors involved in chromatin organization contribute to tumorigenesis. Of particular interest in this context is the Chromatin Assembly Factor 1 (CAF-1). CAF-1 is a complex involved in chromatin assembly coupled to DNA synthesis, both during DNA replication and DNA repair. Two subunits of the CAF-1 complex are downregulated in quiescent cells and can be used as proliferation markers in cancer cells. In addition, CAF-1 has a role in the maintenance of the compact chromatin domains near the centromeres, called pericentric heterochromatin, through its interaction with Heterochromatin Protein 1 (HP1). Three HP1 isoforms (HP1α, β et γ) exist in mammalian cells, among which HP1α associates most specifically with pericentric heterochromatin regions.

During my PhD, I have addressed two main questions: First, **how do the three human HP1 isoforms relate to cell proliferation and tumorigenesis?** Using both cell line models and tumoral and healthy human tissue samples, I showed that the expression of HP1α isoform, but not HP1β ou γ, is proliferation-dependent. Downregulation of HP1α, specifically, results in mitotic defects. In addition, HP1α, but not HP1β ou γ, is overexpressed in several tumoral tissues compared to the corresponding healthy tissues. In breast cancer, the level of HP1α overexpression significantly correlates with global survival and the occurence of metastasis. Our results suggest that HP1α overexpression confers a growth advantage to tumor cells that is related to the organization of pericentric heterochromatin and the passage of mitosis. We put forward HP1α as new prognostic marker in breast cancer and potentially other cancers. This study gave rise to the deposition of a patent and a publication in EMBO Molecular Medecine.

The second main question I have addressed is: **how do quiescent cells, which express CAF-1 at very low levels, deal with chromatin assembly coupled to DNA repair?** This question is of particular interest since a differential repair capacity between tumoral (proliferative) cells and healthy (quiescent) cells will affect the efficiency and the toxicity of genotoxic cancer treatments, such as chemo- and radiotherapy Using ultra-violet (UV) irradiation as a means to induce DNA lesions, I was able to show that CAF-1 expression is not induced upon DNA damage and that the low levels of CAF-1 are sufficient for its recruitment to sites of UV lesions, suggesting that its function is conserved in quiescence. However, we observe a delayed repair of a specific type of UV lesions in quiescence. Our results suggest that the repair of these lesions might require additional chromatin dynamics, which would be the limiting step in quiescence, potentially due to the extremely low levels of CAF-1. These observations gave rise to a manuscript that is currently in preparation.

In these two major projects, I have demonstrated how factors involved in chromatin organization are related to cell proliferation, tumorigenesis and, more generally, genome stability. In addition, we have been able to put forward a new marker of clinical interest for breast cancer prognosis.

Key words: chromatin, cell proliferation, cancer, quiescence, DNA repair

Résumé

Dans les cellules cancéreuses, des défauts affectant l'organisation d'ADN en chromatine sont fréquemment observés. L'étude de facteurs impliqués dans cette organisation est donc essentielle pour mieux appréhender leur implication dans la tumorigénèse. Un facteur particulièrement intéressant dans ce contexte est le facteur d'assemblage de la chromatine, le complexe CAF-1 (Chromatin Assembly Factor 1). CAF-1 est impliqué dans l'assemblage en chromatine de l'ADN lors de la réplication et la réparation de l'ADN. Deux sous-unités de CAF-1 sont sous-exprimés dans les cellules non-proliférantes (quiescentes) et constituent des marqueurs de prolifération dans le cancer. De plus, CAF-1 a un rôle au niveau des régions de chromatine dense proches des centromères, l'hétérochromatine péricentrique, par son interaction avec les protéines HP1 (Heterochromatin Protein 1). Il existe trois isoformes de HP1 dans les cellules mammaires (HP1α, β et γ), dont HP1α est le plus spécifiquement associé aux régions d'hétérochromatine péricentrique, impliqués dans la répression des gènes et la ségrégation des chromosomes.

Pendant ma thèse, je me suis penchée sur deux questions majeures: Premièrement, **est-ce que l'expression des isoformes de HP1 est régulée d'une façon dépendante de la prolifération et de la tumorigénèse ?** En combinant des modèles de lignées cellulaires et des échantillons de tissu humain, j'ai pu montrer que l'expression de l'isoforme HP1α, mais pas HP1β ou γ, est dépendante de la prolifération. La déplétion de HP1α, spécifiquement, affecte le passage de la mitose. De plus, HP1α, mais pas HP1β ou γ, est surexprimé dans de nombreux types de cancer comparé aux tissus sains correspondants. La surexpression de HP1α dans le cancer du sein est corrélée de façon significative à la survie des patientes et la formation de métastases. Ces résultats révèlent HP1α comme un marqueur pronostique dans le cancer du sein et potentiellement dans d'autres types de cancer. Nous proposons que la surexpression de HP1α présente un avantage sélectif pour les cellules cancéreuses, liée à l'organisation de l'hétérochromatine péricentrique et le passage de la mitose. Ces résultats ont donné lieu à un brevet et à une publication dans EMBO Molecular Medecine.

La seconde question à laquelle je me suis intéressée est : **comment des cellules quiescentes, qui expriment peu de CAF-1, gèrent l'assemblage de la chromatine couplé à la réparation de l'ADN ?** En effet, une capacité de réparation différente entre cellules proliférantes (tumorales) et quiescentes (saines) aura un impact majeur sur l'efficacité et la toxicité des traitements génotoxiques comme la chimio- et la radiothérapie. J'ai pu montrer que, dans les cellules quiescentes, les irradiations aux ultra-violets (UV) n'induisent pas l'expression de CAF-1. De plus, la faible quantité de CAF-1 est recrutée aux sites des lésions d'UV, suggérant que sa fonction dans la réparation est conservée hors du cycle cellulaire. Cependant, en quiescence, nous observons une réparation retardée d'un type spécifique de lésions, qui reflète potentiellement une difficulté des cellules quiescentes à gérer la prise en charge de ces lésions au sein de la chromatine. Ces résultats font l'objet d'un manuscrit actuellement en préparation.

Dans ces deux projets majeurs, j'ai mis en avant comment des facteurs de l'organisation de la chromatine peuvent être impliqués dans la prolifération, la tumorigénèse et la réparation d'ADN suite à des traitements génotoxiques. De plus, nous avons pu proposer un nouvel outil d'intérêt médical pour le pronostique des cancer du sein.

Mots-clés: chromatine, prolifération cellulaire, cancer, quiescence, réparation de l'ADN

Preface

How to read beyond the genetic code?

The Human Genome Project (1990 – 2006) had for goal to sequence the human genome in order to reveal the secrets and the complexity of human beings. Although sequencing and analysis is still ongoing, it has become clear that the DNA sequence alone was not able to reveal all secrets of life. A human genome is estimated to contain fewer genes than those of many plants and human genes show very high similarity (up to 98%) with their homologues in apes. So what accounts for the differences that, as we like to think, would make us superior to apes and plants?

Even more intriguing are twins. Monozygotic twins possess the same genome; they are true clones of each other. Yet, they do show differences in phenotype, character and sensitivity to certain diseases. So what accounts for these differences that are not encoded in the genome?

Even within a single organism, most cells contain the same genome, which is formed from a paternal and a maternal set of chromosomes upon fertilization. Yet, many different cells with specialized functions and distinct tissue organizations can be formed out of one genome.

In conclusion, In addition to the genetic code, other sources of information are needed to account for these observations. This extra information would determine how to read the information encoded by DNA.

The term “epigenetics” has been put forward by Conrad Waddington in 1942 to designate “interactions between the genome and the environment that brings the phenotype into being”. It was based on the concept of epigenesis, put forward by Aristotle to explain how the complexity of an organism arises during development. More recently, the definition of epigenetics has been refined and is most often used to denote all those *somatically heritable changes in gene expression that do not involve changes in DNA sequence.* The concept of heritability is of major importance and distinguishes epigenetics from temporary gene regulation by signaling pathways. It implies that

epigenetic changes can be transmitted through cell division and potentially even from one generation to the next.

Until recently, cancer was considered as a genetic disease: an accumulation of mutations, deletions or translocations that affect oncogenes and tumorsuppressor genes and that can ultimately lead to tumorigenesis (Hanahan & Weinberg, 2000). The American Society of Cancer actually still states: "Cells become cancer cells because of damage to DNA" (http://www.cancer.org). Yet, only a few types of cancer harbor mutations in the same genes among different patients (with the possible exception of the tumor suppressor gene p53) and genetic alterations alone cannot predict cancer prevalence or patient outcome. Thus, mechanisms that alter the usage of the genome, without affecting the genetic sequence, are thought to contribute to cancer initiation and progression. As an example, colon cancer is often characterized by a specific series of genetic alterations, affecting among others the gene MLH1. However, at least two cases have been identified in which this gene was not mutated but silenced by epigenetic mechanisms (Suter et al, 2004).

In this thesis, I will focus on epigenetic mechanisms in the context of cell proliferation, DNA damage and tumorigenesis, particularly breast cancer. Breast cancer is a heterogeneous disease, both clinically and genetically. Unlike colon cancer, many different genetic alterations can be found among breast cancer patients, and the correlation with the clinical characteristics are not evident. Several recent studies have compared in a genome-wide fashion the genomic alterations and the changes in gene expression in breast cancer samples (Valladares et al, 2006; Vincent-Salomon et al, 2008; Yao et al, 2006). They show that relatively few changes in gene expression can be attributed to genomic alterations. The recent development of high-throughput DNA sequencing techniques might allow the detection of single nucleotide polymorphisms or point mutations associated with familial breast cancer (reviewed in Stratton et al, 2009). Yet, the vast majority of familial and sporadic breast cancer is not related to mutations in high-risk genes and the contribution of epigenetic changes could thus to be of particular importance. How such changes arise and how they contribute to cancer development remains largely unknown.

In order to address these issues in a comprehensive manner, I will first introduce the best-known candidate for the transmission of epigenetic information: the

organization of DNA into chromatin. Next, I will discuss how chromatin organization can be affected in breast cancer and what remains to be learnt. I will then present in more detail two factors involved in chromatin organization: Heterochromatin Protein 1 (HP1) and Chromatin assembly factor 1 (CAF-1). This background information, provided in the introduction, will bring me to the questions that have intrigued me during my PhD: (i) Are the Heterochromatin Protein 1 isoforms related to cell proliferation and to breast tumorigenesis? and (ii) Are the chromatin dynamics that accompany DNA repair dependent on cell proliferation? Next, I will present published and unpublished results obtained during my PhD, which I will then discuss and put into a broader context in the discussion.

I. Introduction

1. Chromatin: a vector of information

1.1 Organization of DNA into chromatin

Walther Flemming used the term "chromatin" as early as 1882 to describe the structures that he observed in the nuclei of cells stained with basic dyes (Flemming, 1882). However, it wasn't until the 20th century, with the exciting discovery that DNA was the genetic material (Avery et al, 1944; Watson & Crick, 1953), that scientific attention focused on the molecular structure of the nucleus and the organization of DNA in chromatin in the nucleus. Chromatin is composed of DNA and a multitude of other molecules, among which are the histone proteins, and serves to organize, compact, and regulate DNA. Chromatin organization, as depicted in Figure 1, comprises multiple steps of compaction, which help to confine 2 meters of DNA in a cell nucleus of about 10 microns. How these different levels of chromatin organization are achieved will be the first focus of this chapter. Secondly, I will discuss the biological implications of this organization in normal cells and the changes encountered in cancer cells.

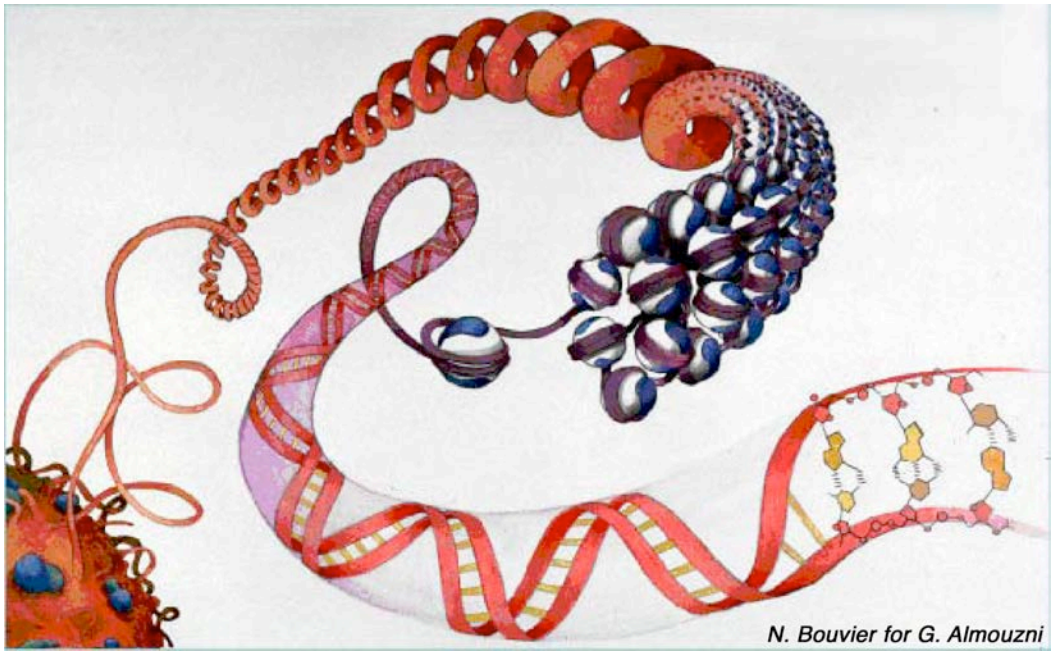

Figure 1: Artistic representation of chromatin organization. DNA is wrapped around histone proteins (blue and white) to form the repeated unit of chromatin, the nucleosome. Nucleosomal arrays can adopt higher order levels of organization corresponding to nuclear domains in interphase nuclei (bottom left). Watercolour by N. Bouvier for G. Almouzni.

1.1.1 The nucleosome: basic unit of chromatin

The first level of chromatin organization and genome compaction consists of the formation of the basic repeated subunit of chromatin, the nucleosome (Kornberg, 1977; Oudet et al, 1975). The nucleosome consists of a core particle, in which 146bp of DNA turn 1.6 times around a histone octamer, and of linker DNA, which interconnects two nucleosomes. The length of the linker DNA varies depending on cell type and chromatin compaction levels (Kornberg, 1977).

The octamer of histone proteins is composed of an $(H3\text{-}H4)_2$ tetramer flanked by two H2A-H2B dimers. Histone proteins are small, highly basic proteins with a high affinity for acidic DNA. They are characterized by the presence of histone fold domains that are highly conserved and form α-helices (Arents & Moudrianakis, 1995). The minimal histone fold domain comprises three α-helices connected by two loops. The 2.8Å resolution of the crystal structure of the core particle (Davey et al, 2002; Luger et al, 1997) revealed the detailed interactions both between different histones proteins and between histones and DNA (Figure 2).

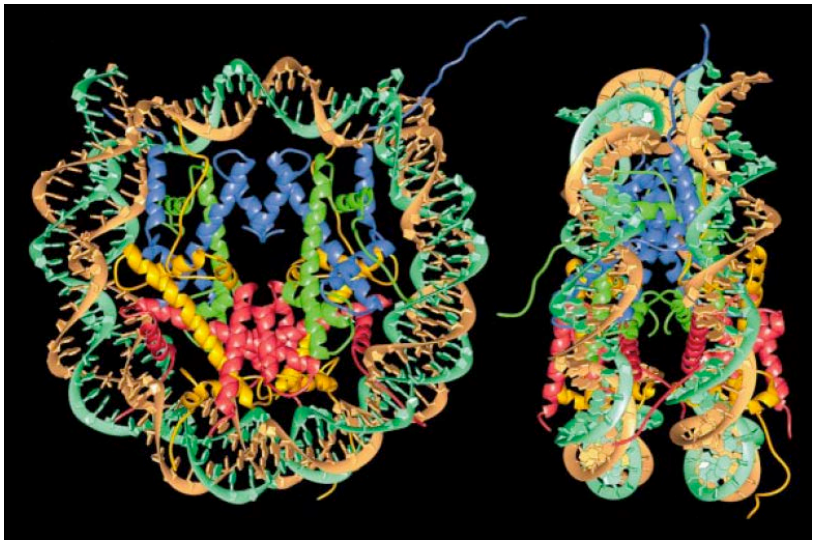

Figure 2: Crystal structure of the nucleosome core particle at a resolution of 2.8Å. A human repetitive DNA fragment and recombinant core histones were crystallized and their structure analyzed by X-ray crystallography. DNA: brown and turquoise; histone H3: blue; histone H4: green; histone H2A: yellow; histone H2B: red). Left panel: front view. Right panel: side view. Note that unstructured N-terminal tails of the histones protrude from the core particle and are thus accessible for protein interactions. Image from Luger et al, 1997.

In addition to the four histone proteins found in the core particle, a fifth type of histone protein can bind to the linker DNA. This family of linker histones, of which histone H1 is the best-known example, seems to bind a stretch of 15 to 20 nucleotides of DNA at the exit of the core particle, but is also in contact with DNA that is located within the nucleosomal core particle (Figure 3) (reviewed in Happel & Doenecke, 2009). The presence of histone H1 has been associated with the formation of a regularly spaced, higher order organization of nucleosomes (see below). Different variants of histone H1 exist, which are differentially regulated throughout differentiation and development (reviewed in Godde & Ura, 2008).

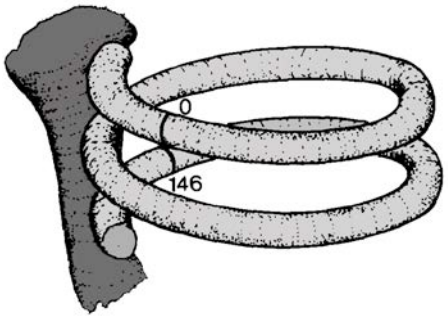

Figure 3: Schematical model of the binding of linker histone H1 to nucleosomal DNA. DNA is represented in light grey. The 146bp that are part of the core particle are indicated. Histone H1, which binds the linker DNA that exits and enters the core particle, is depicted in dark grey. Image adapted from Thoma et al, 1979.

1.1.2 Higher order chromatin structure

Arrays of nucleosomes, observed as "beads on a string" at low ionic strength using atomic force or electron microscopy (Figure 4) (Olins & Olins, 1974; Olins et al, 1980; Oudet et al, 1975), need to be further compacted for nuclear organization. The first level of compaction is promoted by the binding of linker histones. Indeed, in presence of linker histones, arrays of nucleosomes fold into a fiber with an approximate diameter of 30nm (Figure 4) (Thoma et al, 1979).

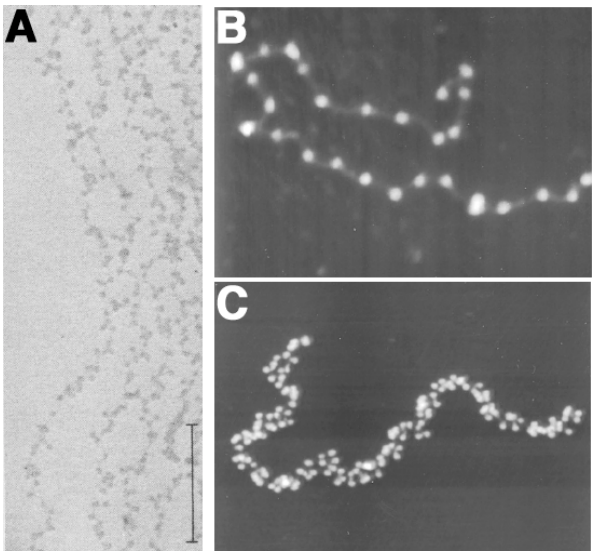

Figure 4: Nucleosomal arrays visualized as beads on a string. A. Chromatin fibers from rat thymus nuclei, stained with phosphotungstic acid and ethanol, as observed by electron microscopy. Scale bar: 0.2 μm. Image from Olins & Olins, 1974. **B&C.** A glutaraldehyde-fixed chromatin fiber prepared from chicken erythrocytes as observed by scanning force microscopy. Linker histones have been removed (**B**) or not (**C**) with 0.35M NaCl. Images from Bustamante et al, 1997.

How the nucleosomes are exactly arranged within this fiber is a highly debated subject. Two models have been proposed (Figure 5) (reviewed in Robinson & Rhodes, 2006): (i) the one-start model, in which the array of nucleosomes forms a left-hand helix (Robinson et al, 2006), and (ii) the two-start model, in which the linker DNA connects nucleosomes back and forth in a zigzag manner (Dorigo et al, 2004). Recent studies using reconstituted nucleosome arrays suggest that both structures can be formed, depending on the length of the linker DNA (Kruithof et al, 2009; Routh et al, 2008). Indeed, shorter linker DNA length favors the formation of the more compact two-start model, while longer linker DNA lengths seem more compatible with the formation of a one-start helix (Kruithof et al, 2009; Routh et al, 2008). However, it should be kept in mind that the structures of reconstituted chromatin fibers as observed by microscopy are variable and highly dependent on the preparation techniques and the fixation method. The extent to which 30nm fibers can be formed *in vivo* remains unclear. An extreme view is that there may not be any fiber formation and that the nucleosomes instead form a disorganized "sea of nucleosomes" *in vivo* (Eltsov et al, 2008).

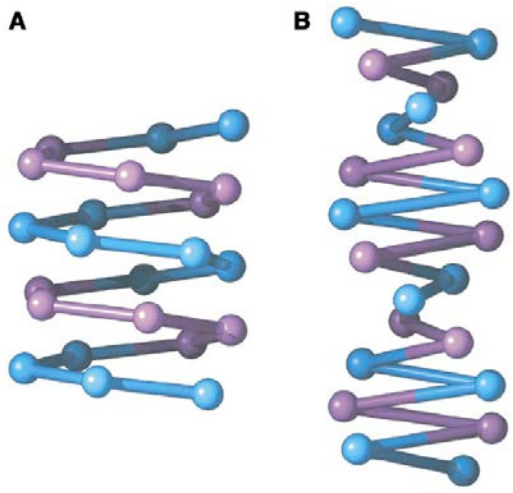

Figure 5: Schematic representation of the two models for the 30nm fiber folding. A. The one-start model, in which the array of nucleosomes forms a left-handed helix. **B.** The two-start model, in which the linker DNA connects nucleosomes back and forth in a zigzag manner. Image from Robinson & Rhodes, 2006.

Further compaction levels ultimately lead to the most condensed form of chromatin found in mitotic chromosomes. The existence of long chromatin loops has been put forward in the context of DNA replication (Berezney et al, 2000), gene transcription (van Driel & Fransz, 2004) and DNA recombination (Kantidze & Razin, 2009), in order to explain the observed spatial distribution of these processes. Domains of approximately 1Mb would fold into ~100kb loops and help to compact and organize chromatin (Bode et al, 2003; Kantidze & Razin, 2009). Yet, to what extent such loops exist *in vivo* and contribute to higher order chromatin organization remains an open issue.

1.1.3 Nuclear chromatin organization

Many organisms display a non-random organization of chromatin in the interphase nucleus. Both in mammalian (reviewed in Cremer et al, 2006) and in yeast (Berger et al, 2008) nuclei, each chromosome occupies a distinct location (Figure 6). Although the localization of each chromosome is highly variable from one cell to another and seems to depend on cell type and proliferation status (Bolzer et al, 2005), a general picture is emerging in which chromosomes can be organized based on their size or their gene density. However, this organization is dynamic, and particularly highly expressed genes can be found to protrude outside of their chromosome territory (e.g. Mahy et al, 2002, Chambeyron et al, 2005 and Chambeyron & Bickmore, 2004). Thus, a certain degree of intermingling of chromosomes is thought to occur (Branco & Pombo, 2007). In addition, sub-nuclear compartments can be distinguished that contribute to gene

regulation and cell functioning. Cells from several types of cereals show a Rabl-chromosome configuration, with centromeres at one side of the nucleus and telomeres at the other side (Shaw et al, 2002). In yeast, the telomere sequences are also localized at the nuclear envelope, where they form clusters (Gotta et al, 1996). In eukaryotes, the nuclear membrane is often associated with compact, repetitive and transcriptionally silent chromatin, called constitutive heterochromatin. Tethering a chromosomal region to the nuclear periphery can indeed modulate transcriptional regulation, both in terms of gene activation and gene repression (reviewed in Ruault et al, 2008). Thus, a picture emerges in which the nuclear organization seems to impact on transcriptional regulation and vice versa. Whether these effects are direct or indirect, and whether they follow a general rule, is a current subject of investigation.

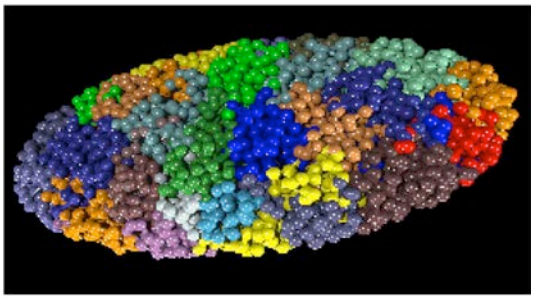

Figure 6: Chromosome territories in a human fibroblast. Computer simulation of the distribution of all chromosomes in the nucleus of a human quiescent fibroblast, based on a chromatin painting experiment in which all chromosomes were visualized by fluorescent in situ hybridization. Adapted from Bolzer et al, 2005.

1.2 Chromatin dynamics: histone chaperones and histone variants

Histones are not permanently associated with DNA. Newly synthesized histones have to be transported to the nucleus and targeted to the required location, whereas old or damaged histones have to be discarded. In addition, cellular processes involving DNA can require transient histone eviction and replacement (Loyola & Almouzni, 2004). As histones are highly basic proteins, their presence in the cell could lead to deleterious effects. Thus, when histones are not in association with DNA, they are bound to dedicated proteins called histone chaperones (De Koning et al, 2007, see appendix 1). These escort proteins, defined as "factors that associate with histones and stimulate a reaction involving histone transfer, without being part of the final product", help to control histone supply and incorporation into chromatin. This implies that histone chaperones are implicated in all cellular processes that operate on chromatin, like transcription, replication and repair (Corpet & Almouzni, 2009; Groth et al, 2007b; Polo & Almouzni, 2006). They interact with an intricate network of different partners to regulate histone traffic (Figure 7).

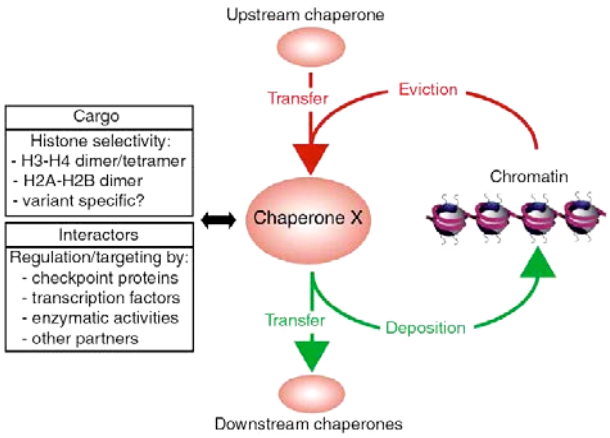

Figure 7: Schematic representation of the relationship between a given histone chaperone and its network of interacting partners. A given chaperone X will show specificity towards certain histone dimers, or potentially tetramers, which it can receive from, or transfer to, other histone chaperones. Alternatively, it can contribute to histone eviction from or deposition on DNA. Its activity can be regulated by interacting partners involved in various cellular processes. Image from De Koning et al, 2007.

To identify factors that can promote histone deposition, cell-free systems have been developed. The first system allowing the study of chromatin assembly was derived from *Xenopus* egg extracts (Laskey et al, 1977). These egg extracts contain all factors needed for chromatin assembly during the rapid rounds of DNA replication that occur in early development. Incubation of these extracts with circular template DNA from the Simian Virus 40 (SV40) (Laskey et al, 1977) or with circular single stranded

bacteriophage DNA (Almouzni & Mechali, 1988a; Almouzni & Mechali, 1988b) results in chromatin assembly independent from or coupled to DNA synthesis, respectively. This model system allowed the identification of the most abundant histone H2A-H2B chaperone in *Xenopus* eggs, nucleoplasmin (Laskey et al, 1978). The further development of a system using a combination of human cytosolic and nuclear extracts and biochemical fractionation (Stillman, 1986) helped to identify Chromatin Assembly Factor 1 (CAF-1), a chaperone promoting chromatin assembly coupled to DNA synthesis (Smith & Stillman, 1989).

Histone chaperones can be distinguished based on their specificity towards distinct histones or histone variants. We have proposed a classification of currently known histone chaperones, according to their histone preference and whether they act alone, within a chaperone complex or within an enzymatic complex (De Koning et al, 2007, appendix 1). A first level of specificity is based on whether they bind either to H3-H4 or H2A-H2B, with several chaperones preferentially binding to specific isoforms (variants) of histones H3 or H2A. Indeed, among the histone proteins, we can distinguish replicative histone variants, which are expressed and deposited mainly during S-phase, and replacement variants, which are constitutively expressed and incorporated in a replication independent fashion. As an example, CAF-1 shows specificity towards the replicative variant H3.1, while the histone chaperone histone regulator A (HIRA) shows specificity for the replacement variant H3.3. Elegant studies using Xenopus egg extracts and *in vitro* chromatin assembly have shown that whilst CAF-1 promotes the deposition of H3.1-H4 in a manner coupled to DNA synthesis, HIRA does so for H3.3-H4 independently of DNA synthesis (Tagami et al, 2004) (figure 8). Anti-silencing function 1 (Asf1), a factor that cannot promote histone deposition on its own *in vivo* (Mello et al, 2002; Ray-Gallet et al, 2007) but rather acts as a histone donor for CAF-1 and HIRA in histone deposition (Polo & Almouzni, 2006), can interact with both H3.1-H4 and H3.3-H4. However, only the Asf1a isoform can interact with HIRA (Tagami et al, 2004; Zhang et al, 2005), thus providing an indirect specificity for H3.3-H4 deposition. However, it should be kept in mind that the chaperone-histone interaction is not always a strict specific interaction, as exemplified by Nuclear Autoantigenic Sperm Protein (Nasp). Nasp was first reported to co-purify with the linker histone H1 (Richardson et al, 2000; Wang et al, 2008a), but was more recently found to interact with H3-H4 (Tagami et al, 2004). This may not be so surprising since Nasp is the homologue of the H3-H4

chaperone N1/N2 in Xenopus. Thus, histone binding preferences should be taken with caution and may be subject to regulation, depending on the cellular context and binding partners.

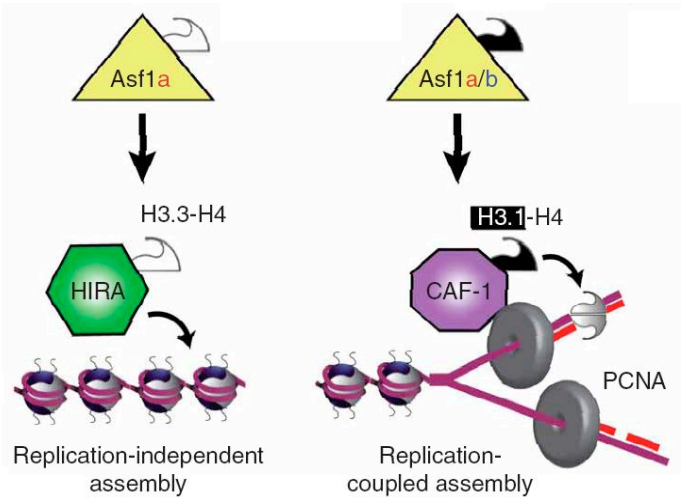

Figure 8: Chromatin assembly in a replication-dependent or -independent fashion. CAF-1 deposits H3.1-H4 in a replication-coupled fashion, while HIRA specifically deposits H3.3-H4 in a manner independent from DNA synthesis. Asf1 serves as a histone donor for both CAF-1 and HIRA and can interact with both H3.1 and H3.3. However, only the Asf1a isoform interacts with HIRA, while both Asf1a and b can interact with CAF-1. Image from De Koning et al, 2007.

1.3 Chromatin organization as a source of information

In the previous chapters, we have discussed how DNA is organized into chromatin and how the basic unit of this organization, the nucleosome, is formed. Here, I will address how this organization can be a possible source of epigenetic information, i.e. contribute to a heritable status of gene silencing and expression that contributes to cell identity and behavior.

Chromatin organization is dynamic and can show a large repertoire of molecular characteristics, or marks, which contribute to different levels of organization (Figure 9). The combination of these marks, rather than the presence or absence of a single mark, is thought to constitute the motif that can, for example, impact on the accessibility and dynamics of a given part of the genome.

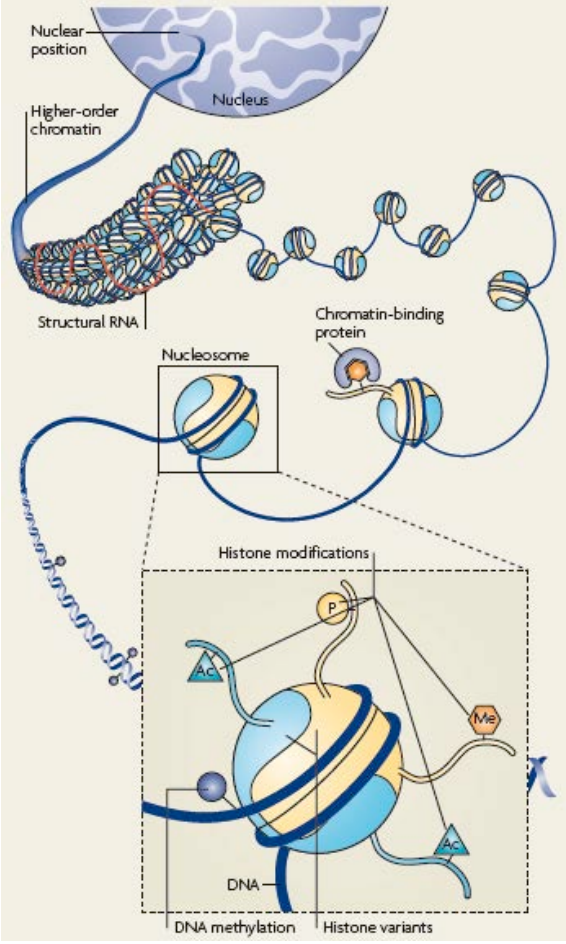

Figure 9: Schematic representation of potential epigenetic marks. At all levels of chromatin organization, form the DNA up to the nuclear position, marks and variants can change and contribute to the accessibility and dynamics of the genome. Image from Probst et al, 2009.

1.3.1 DNA methylation

First, the DNA component of chromatin can be methylated. In mammalian cells, this methylgroup is almost exclusively found on the symmetrical CpG (cytosine-phospho-guanine) dinucleotides. These CpG dinucleotides are not distributed uniformly across the genome, but are grouped into so-called CpG islands, which often correspond to gene promoter regions. A high fraction of methylated CpGs within such islands is generally associated with gene silencing. Specific enzymes, named DNA Methyl Transferases (DNMTs) ensure the establishment and maintenance of DNA methylation. Several types of DNMTs are found in mammalian cells. In general, Dnmt3a and b, together with their co-factor Dnmt3L, are involved in *de novo* DNA methylation, while Dnmt1 is involved in the maintenance of the methylation mark through S-phase (Figure 10). Indeed, upon DNA replication, the newly synthesized strand is unmethylated, thus resulting in a hemi-methylated CpG dinucleotide. For transmission of the mark, the new strand needs to acquire the same marks as the mother strand. Dnmt1 can be recruited to the DNA replication fork through its interaction with the polymerase accessory factor Proliferating Cell Nuclear Antigen (PCNA) (Chuang et al, 1997). However, the dominant manner of Dnmt1 recruitment seems to be through its interaction with UHRF1, a protein that binds specifically hemi-methylated DNA and is crucial for the maintenance of methyl marks (reviewed in Prokhortchouk & Defossez, 2008) and (Probst et al, 2009). Since the methyl mark can be transmitted from mother to daughter cells, DNA methylation is considered as a *bona fide* epigenetic mark.

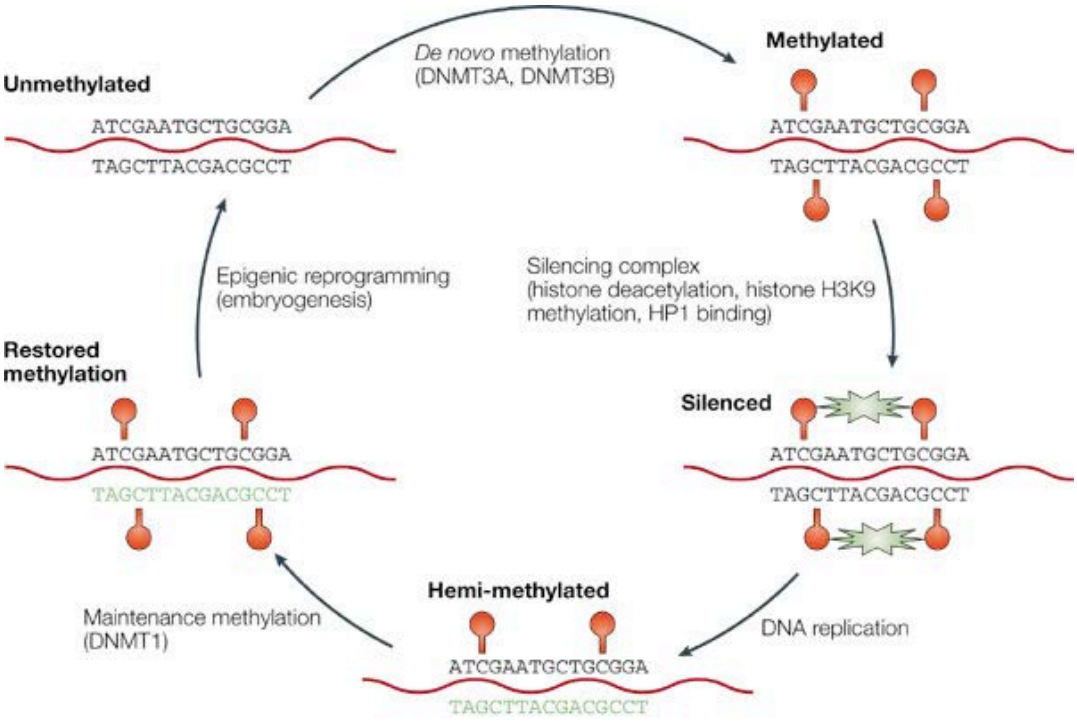

Figure 10: Schematic representation of DNA methylation in mammalian cells. Dnmt3a and b are *de novo* methyl transferases, which can establish a silent chromatin status. DNA replication results in hemi-methylated DNA. Dnmt1 can restore metylation patterns by methylating the newly synthesized strand. Image from Issa, 2004.

In the absence of Dnmt1 activity, passive DNA demethylation would occur when cells go through multiple cell divisions. For a long time, this was thought to be the only mechanism to erase DNA methyl marks in mammalian cells. Recently, however, mechanisms have been proposed that might enable the removal of DNA methylation *in vivo*, which involve the transformation of the methylated cytosine into a mark that can be recognized and excised by DNA damage repair pathways (Gehring et al, 2009; Ma et al, 2009). Thus, DNA methylation in mammals might be a less stable mark than initially thought. Interestingly, in *Drosophila*, DNA methylation seems restricted to a short time window in early development and does not necessarily correlate with gene silencing (reviewed in Mandrioli & Borsatti, 2006).

1.3.2 Histone variants

At the level of the nucleosome, different isoforms of histones H3 and H2A, called variants, can be incorporated (reviewed in Loyola & Almouzni, 2007; Polo & Almouzni, 2006; Pusarla & Bhargava, 2005). Two types of histone variants can be distinguished (Table I) (Osley, 1991). Replicative histone variants, of which the synthesis and the deposition peak in S-phase, supply the required amount of histones to ensure chromatin

assembly during DNA replication. Replacement variants, which are expressed at steady state levels throughout the cell cycle and in quiescence, provide a constitutive source for histone turnover or dynamics. The centromere specific histone H3 variant CENPA (Centromere Protein A) is an exceptional case, since its expression peaks in G2 (Shelby et al, 2000) and its deposition occurs during a short time window at the exit of mitosis in mammalian cells (Dunleavy et al, 2009; Foltz et al, 2009; Jansen et al, 2007).

Table I: Somatic Mammalian Histone Variants

Histone type		Variant	Specificity/function
H2A	replicative	H2A.1 H2A.2	Not determined
	replacement	H2AX	DNA repair signaling
		H2AZ	Regulation of transcription
		macroH2A	Inactivation of one X chromosome in female
		H2ABbd	Active chromatin, nulceosome destabilization
H3	replicative	H3.1	deposition coupled to DNA synthesis
		H3.2	deposition coupled to DNA synthesis, generally associated with repressive chromatin
	replacement	H3.3	deposition independent of DNA synthesis, generally associated with active chromatin
		CENPA	centromere structure and function

Histone variants often show high similarity in amino acid sequence, as exemplified by histone H3.1 and H3.3, which only differ by 5 amino acids. Nevertheless, the choice of the histone variant is of importance for genome function and structure. Indeed, most histone variants are not distributed uniformly over the genome, but show specificity for certain chromatin regions, as summarized in Table I. For example, H3.3 dynamics are most pronounced at promoters, enhancers and transcriptionally active genes (Mito et al, 2005) and the presence of H3.3 seems to facilitate gene expression by forming unstable nucleosomes that can be easily disrupted (Jin & Felsenfeld, 2007). In addition to the somatic histone variants presented in table I, tissue specific histone variants have been identified, such as the testis-specific variant H3.1t (Witt et al, 1996) that differs in four amino acids from canonical H3.1. The presence of such variant might reflect an adaptation to particular cellular demands in terms of histone levels and dynamics during rapid proliferation and/or development.

Interestingly, the presence of multiple histone variants has arisen during evolution. The yeast *S. cerevisiae* contains only one histone H3 variant, which is most closely related to mammalian H3.3. In organisms such as plants, flies and frogs, an

additional replication-dependent variant, similar to H3.2, has evolved. Only mammals express two different replication-dependent variants, H3.1 and H3.2. The increasing number of histone variants throughout evolution is thought to parallel the increasing nuclear complexity, required to optimize and diversify genome usage.

1.3.3 Histone modifications

An important source of information is provided by an extensive panel of post-translational modifications of histone proteins (Figure 11). These modifications include acetylations, methylations, phosphorylations and ubiquitinations and mainly concern amino acids in the N-terminal tails of histone proteins, which protrude from the nucleosome and are therefore most accessible.

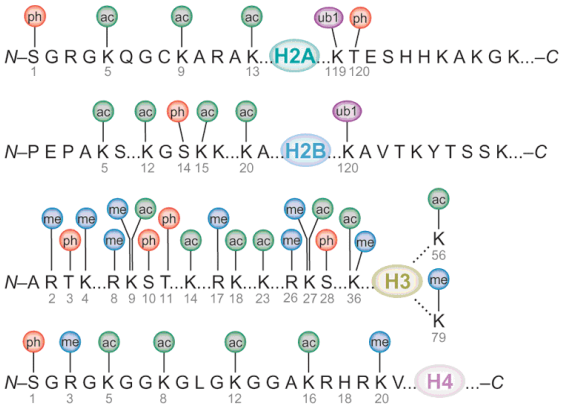

Figure 11: Scheme of histone modifications. Histones modifications include acetylation (ac), methylation (me), phosphorylation (ph) and ubiquitination (ub1). Most modifications occur on the N-terminal tails of histones, with some exceptions involving the C-terminal histone tails or their globular domains, here depicted as colored ovals. Image from Bhaumik et al, 2007.

It has been proposed that histone modifications could be involved both in short-term cell signaling and in hereditary long-term regulations. Only in the latter case, histone modifications can be considered as true epigenetic marks (Turner, 2002; Turner, 2007) (Figure 12). How specific histone modifications can possibly be transmitted through DNA replication and mitosis is currently an important question in the field (Corpet & Almouzni, 2009; Probst et al, 2009).

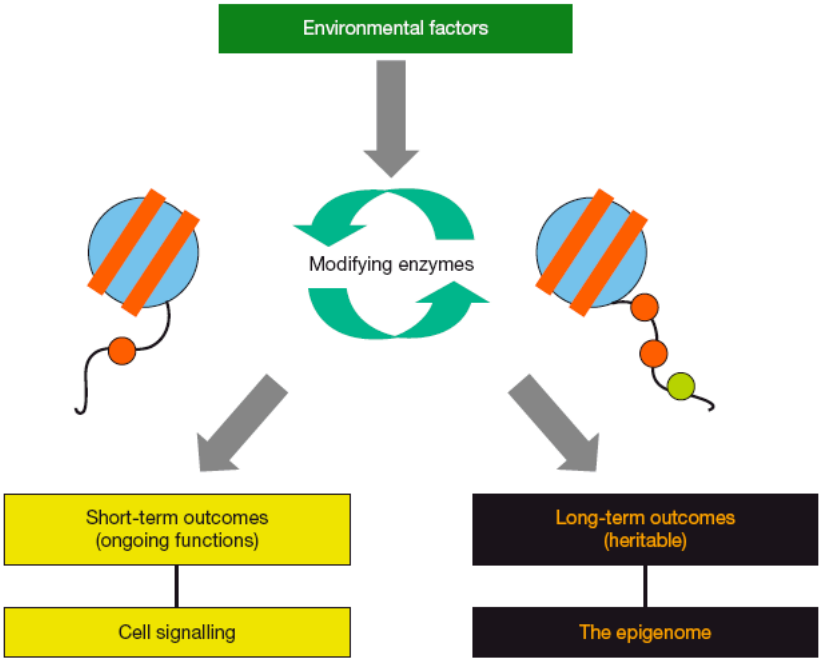

Figure 12: Histone modifications and epigenetics. Signals from the environment may induce certain histone modifications. These modifications can have short-term outcomes in ongoing processes, such as transcription, and thus serve in cell signalling. Alternatively, these modifications can have long-term, hereditary, effects on chromatin organization and thus contribute to the epigenetic state of a cell. Scheme from Turner, 2007.

While particular combinations of modifications have been associated with activation of gene transcription, others are implicated in gene silencing. Different histone variants are associated with different post-translational modifications, of which some are already partially present before histone incorporation into chromatin (reviewed in Loyola & Almouzni, 2007). Importantly, it is the combination of multiple histone modifications within one or several nucleosome(s), rather than one signal modification, that will be decisive for gene activity. This view has been put forward as the "histone code" (Jenuwein & Allis, 2001; Turner, 2000; Turner, 2002). In line with this view, several modifications are known to "cross-talk", either within the same histone or between different histones (Suganuma & Workman, 2008). For example, the presence of phosphorylation on serine 10 of histone H3 (H3S10P) seems to render inaccessible the trimethylation of the neighboring lysine 9 of histone H3 (H3K9me3) (Duan et al, 2008; Fischle et al, 2005; Hirota et al, 2005), while H3K9me3 has been proposed to be required for the establishment of trimethylation on lysine 20 of histone H4 (H4K20me3), at least in established mouse embryonic fibroblasts cell lines (Schotta et al, 2004).

Histone modifications are established and removed by a series of enzymes, including histone acetyl transferases (HAT), histone deacetylases (HDAC), histone methyl transferases (HMT) and histone demethylases (HD). In each group of enzymes, different members often show specificity toward a certain histone residue. The most diverse group is the histone deacetylases, which contains 18 members in mammalian cells (reviewed in Witt et al, 2009). This group is further divided into four classes, based on sequence homology with their yeast orthologues. HDACs of class I (HDAC1, 2, 3 & 8) are expressed in a ubiquitous manner, while HDACs of classes II, III and IV are tissue specific and might thus contribute to development and cell identity. Many HDACs not only deacetylate histone proteins, but also other nuclear or cytoplasmic proteins such as the tumor suppressor protein p53 (Luo et al, 2000) or tubulin (Zhang et al, 2003a). It is noteworthy that histone methylation, which can occur on lysine and arginine residues, was until recently considered as a very stable mark with low turnover (Byvoet et al, 1972). The discovery of Lysine-Specific Demethylase 1 (LSD1) (Shi et al, 2004), which demethylates H3K4me2 though an oxidation reaction, therefore constituted a landmark in our understanding of chromatin dynamics. Since then, a large family of other histone demethylases, which share a so-called Jumonji C catalytic domain, has been described (reviewed in Agger et al, 2008). The reversibility of all histone modifications underscores the dynamic nature of chromatin organization and raises again the question of which marks could be considered as epigenetic, and how these can be transmitted through cell division.

1.3.4 Chromatin binding proteins and higher order organization

Both DNA methylation and histone modifications mainly affect chromatin organization through the recruitment of "effector proteins", which recognize a specific mark. DNA methylation is recognized by Methyl Binding Domain proteins (MBD1-4), the Methyl-CpG-binding Protein 2 (MeCP2) and zinc finger proteins like Kaiso (reviewed in Klose & Bird, 2006; Prokhortchouk & Defossez, 2008). Histone acetylation is specifically recognized by proteins containing a so-called bromodomain (reviewed in Mujtaba et al, 2007), while histone methylation forms a binding site for proteins with a chromodomain (reviewed in Brehm et al, 2004). Many proteins are known to contain such domains,

and, importantly, many of such proteins also interact with each other and with the proteins that establish or remove the modifications. As an example, the Heterochromatin Protein 1 (HP1), which will be discussed in more detail later, binds to H3K9me3 (Bannister et al, 2001; Lachner et al, 2001), but also to the enzyme that establishes this modification, Suv39h1 (Aagaard et al, 1999). In addition, HP1 interacts with the methyl binding protein MeCP2 (Agarwal et al, 2007), but also with the DNA methyl transferase Dnmt1 (Fuks et al, 2003). In conclusion, one can imagine a process in which an "effector" protein, the reader of a mark, communicates with the protein that establishes the mark, the writer, in order to perpetuate the mark (Figure 13) or, on the contrary, inhibit additional modifications. These networks can ensure the maintenance of a higher order chromatin organization, once established.

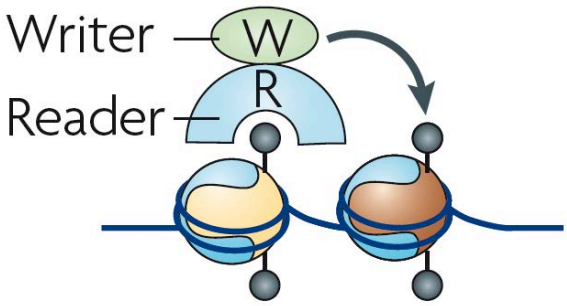

Figure 13: The reader-writer model. A chromatin mark, represented by a grey ball, is recognized by a specific binding protein, the reader, which in turn recruits the modifying enzyme, the writer. This model explains how chromatin marks can be copied to an adjacent molecule, in this case an adjacent nucleosome. Image from Probst et al, 2009.

1.4 Heterochromatin: the "other type of chromatin"

In 1928, the botanist Emile Heitz (1892-1965) observed that certain regions of the genome stain intensely with basic dyes during interphase, while other regions seem to decondense when cells exit mitosis (Heitz, 1928) (Figure 14). He termed the intensely stained chromatin "heterochromatin" (literally: "the other chromatin"), as opposed to the decondensed "euchromatin" ("true chromatin"). He noticed that heterochromatin was mostly localized near the nuclear periphery and surrounding the nucleoli, and proposed that heterochromatin contains passive, inactive chromatin. More recently, molecular studies have shown that, although exceptions do exist, heterochromatin is indeed generally gene-poor and transcriptionally silent, as opposed to euchromatin, which is rich in protein-coding genes that can be transcriptionally active (reviewed in Craig, 2005; Delcuve et al, 2009).

The repressive features of heterochromatin have been extensively studied in *Drosophila*, where the positioning of a gene near heterochromatin results in its silencing in a fraction of cells, while staying active in other cells (reviewed in Girton & Johansen, 2008). This phenomenon of position effect variegation (PEV) is due to spreading of the heterochromatic state into the gene. Taking advantage of this phenomenon, by using a gene with a visible phenotype, such as eye color, it has been possible to identify genes that, when they are mutated, inhibit or promote heterochromatin spreading. So-called "suppressors of variegation" (Su(var)) are factors in which a mutation impairs variegation and that therefore normally promote heterochromatin formation. On the other hand, Enhancers of variegation (E(var)) are factors in which a mutation promotes variegation and that therefore normally prevent heterochromatin formation.

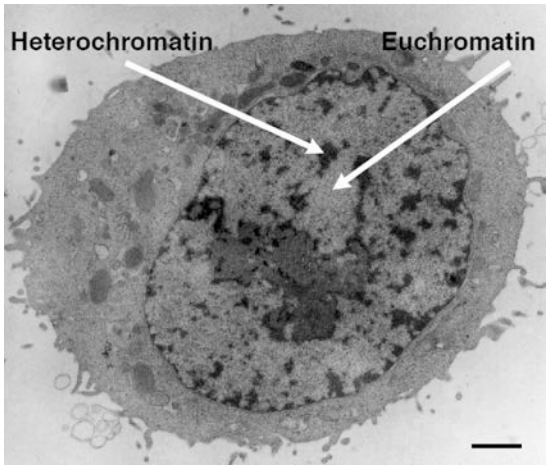

Figure 14: Human HeLa cell observed by electron microscopy. Heterochromatin (dark regions) and euchromatin (bright regions) are visible within the nucleus. Image adapted from « *Principles of Nuclear Structure and Function » by P.R. Cook.*

1.4.1 Facultative and constitutive heterochromatin

Among heterochromatic regions, S. Brown distinguished two distinct types (Brown, 1966): constitutive and facultative heterochromatin (reviewed in Trojer & Reinberg, 2007). Brown defined these regions as: « In constitutive heterochromatization both the homologous chromosomes, one maternal, the other paternal, respond in the same way during development. In facultative heterochromatization, the two homologous chromosomes differ; one becomes heterochromatic during development, and the other remains euchromatic ». The best-described example and study model of facultative heterochromatin is the transcriptional inactivation of a large fraction of one of the two X chromosome in female mammals, in order to obtain dosage compensation with regard to male cells that only contain one X chromosome (reviewed in Heard & Disteche, 2006; Wutz & Gribnau, 2007).

As a study model for constitutive heterochromatin in mammals, mouse cell lines have proven extremely useful. Mouse cells contain large blocks of constitutive heterochromatin near the centromeres, the so-called pericentric heterochromatin, which are organized into large clusters, called chromocenters (Guenatri et al, 2004; Maison et al, 2002). These clusters of pericentric heterochromatin are easily identifiable cytologically (Hsu et al, 1971) (Figure 15) and facilitate the study of constitutive heterochromatin. In human cells, such clusters do not exist since (peri-) centromeric regions are more dispersed.

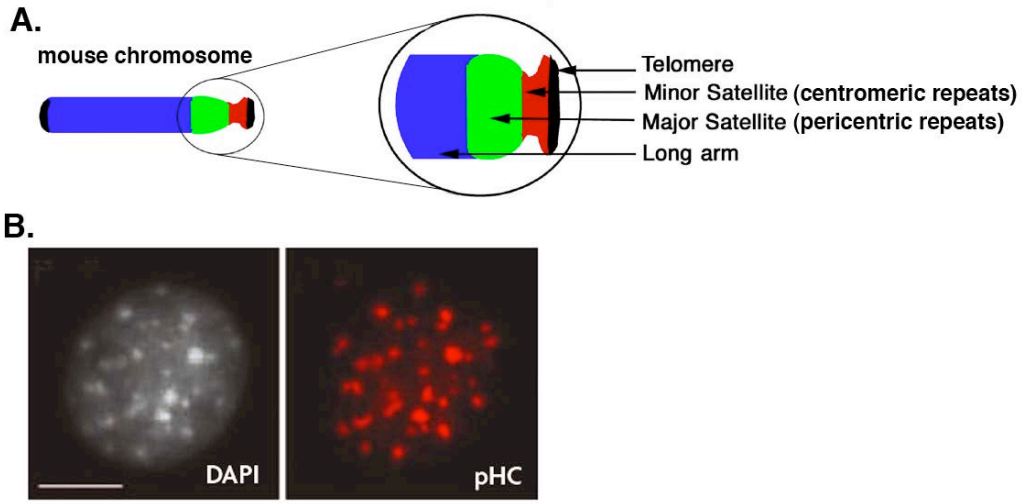

Figure 15: Organization of pericentric heterochromatin into chromocenters in mouse cells. A. Schematic representation of a murine chromosome. Pericentric heterochromatin is positioned next to the centromere and is composed of repetitive sequences, the so-called major satellite repeats. **B.** Clusters of pericentric heterochromatin (pHC) can be visualized by DNA staining (left picture) or by DNA Fluorescent In Situ Hybridization (FISH) using a probe for pericentric repeats (right picture). Each spot comprises the pericentric regions of several chromosomes. Images adapted from Guenatri et al, 2004 and Probst et al, 2009.

1.4.2 Heterochromatic components

Heterochromatic regions are characterized by the presence of a specific combination of factors. These include specific features of the DNA, modifications of histone proteins and the recruitment of specific proteins, of which many have been identified as suppressors or enhancers of variegation in *Drosophila*.

The *DNA* within regions of constitutive heterochromatin is often methylated and is enriched in repetitive elements. This is particularly striking at centromeres and pericentric regions, which are composed of megabase-long arrays of repeated elements (reviewed in Plohl et al, 2008). In human cells, the repeated elements at the centromere, designated as α-satellites, have a length of 171 bp and are organized in a head-to-tail fashion. Pericentric regions are composed of other types of repeats, including satellites I, II and III and β- and γ-satellites in human cells. Furthermore, telomeres are also associated with a juxtaposed stretch of repeated sequences of 6 bp each, which is constitutively heterochromatic. Finally, other types of repetitive elements, such as mini- and microsatellites and transposable elements, can be found dispersed along the chromosomes and are also organized into heterochromatic structures. While these repetitive sequences were considered for a long time as inert "junk" DNA, recent work has highlighted the importance of these blocks in the structural and functional organization of the genome (Plohl et al, 2008). The fact that these sequences are often affected in cancer cells has largely contributed to this view.

A combination of specific *histone modifications* is a characteristic of constitutive heterochromatin in mammalian cells (Maison & Almouzni, 2004). These include a global hypoacetylation of the histones (Jeppesen et al, 1992) and an enrichment in H3K9me3 (Peters et al, 2001) and H4K20me3 (Schotta et al, 2004). The H3K9me3 mark is established by the enzymes Suv39h1 and Suv39h2 (Peters et al, 2001), and the H4K20me3 mark by the enzymes Suv4-20h1 and Suv4-20h2 (Schotta et al, 2004) (Figure 16). In line with the localization of heterochromatin close to the centromeres and the telomeres, mice deficient for both Suv4-20h1 and Suv4-20h2 are unviable and show telomere deficiencies and chromosomal aberrations (Benetti et al, 2007; Schotta et al, 2008). Suv39h1 and Suv39h2 knockout mice show impaired viability and the

corresponding mouse embryonic fibroblasts show chromosomal instabilities and mitotic defects (Guenatri et al, 2004; Peters et al, 2001). In addition, the histone modification H3K27me3, generated by the Polycomb Repressor Complex PRC2, is specifically associated with the establishment of facultative heterochromatin (reviewed in Schwartz & Pirrotta, 2008; Trojer & Reinberg, 2007).

The heterochromatic marks can recruit *interacting proteins*, of which the best studied are the Heterochromatin protein 1 (HP1) isoforms. Mammalian cells express 3 HP1 isoforms, HP1α, β and γ, which will be discussed in detail in chapter 2. HP1 proteins bind specifically to H3K9me3 (Bannister et al, 2001; Lachner et al, 2001; Nielsen et al, 2002). Although this interaction occurs with moderate affinity (K_d= ~70nM), it is thought to constitute their major binding activity in constitutive heterochromatin (Figure 16). Indeed, in cells derived from Suv39h1/2 knockout mice embryos, HP1 is no longer concentrated on pericentric heterochromatin (Lachner et al, 2001; Maison et al, 2002).

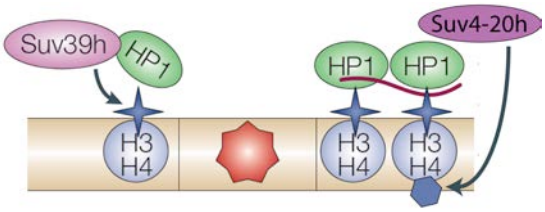

Figure 16 : Schematical representation of pericentric heterochromatin in mammalian cells. Blue stars represent trimethylation on H3K9, blue hexagones represent trimethylation on H4K20. Image adapted from Maison & Almouzni, 2004.

Finally, an *RNA component* is of importance for the organization of (pericentric) heterochromatin. In mammalian cells, the presence of RNA is required to stabilize HP1 binding at pericentric heterochromatin (Maison et al, 2002; Muchardt et al, 2002). Indeed, treatment of cells with RNase results in the dispersion of HP1 (Maison et al, 2002). The nature and the origin of this RNA component is still an open question, and it has been tempting to make the connection with the RNA interference (RNAi) pathway. Well described in the yeast *S.pombe*, but also in plants, flies and worms, this pathway contributes to gene silencing and heterochromatin maintenance through the processing of non-coding RNAs (reviewed in Djupedal & Ekwall, 2009). Long double stranded RNAs derived from centromeric regions are processed by Dicer (Dcr1) into small interfering

RNAs (siRNA) and loaded on Argonaute (Ago1). Through sequence homology between RNA and DNA, Argonaute can establish heterochromatin in the DNA from which the siRNA originates, potentially trough the recruitment of histone modifiers and HP1 homologues (Volpe et al, 2002). In mouse cells, transcripts from centromeric regions have been detected as early as in 1968 (Gaubatz & Cutler, 1990; Harel et al, 1968; Rudert et al, 1995); reviewed in Eymery et al, 2009), suggesting that a similar mechanism could operate in mammals. Although mammalian cells seem to lack an RNA-dependent RNA polymerase, which initiates or amplifies dsRNA production in other organisms (Djupedal & Ekwall, 2009), transcription from both sense and antisense strands or from the head-to-tail oriented satellite repeats might give rise to dsRNA. Strikingly, depletion of Dicer from a chicken-human hybrid cell line resulted in the accumulation of long α-satellite transcripts and decreased HP1 concentration at (peri)centromeric regions (Fukagawa et al, 2004). Yet, a functional link between satellite transcripts, the RNAi machinery and heterochromatin formation remains to be demonstrated in mammals.

1.5 Chromatin organization and tumorigenesis

1.5.1 Current prognostic tools

Defects in chromatin organization have been explored in the context of cancer (reviewed in Jones & Baylin, 2007). Such defects could contribute to the deregulation of a cells' response to signaling pathways and could potentially be transmitted through cell division. An epigenetic alteration that results in a cellular growth advantage could be selected for during tumorigenesis. In many types of cancer, the correlation between identified genomic mutations, gene expression and disease outcome remains poor. As discussed in the preface, genetic alterations alone seem insufficient to explain the prevalence and the heterogeneity of a cancer like breast cancer. In these types of cancer, defects in chromatin organization are likely to contribute to the disease. I will present here the clinical value of current prognostic factors and the potential contribution of aberrant chromatin organization, in the context of breast cancer.

With more than 1 million new cases per year worldwide, of which more than 50,000 are in France, breast cancer (Figure 17) is the most frequent cancer among women in developed countries. Although survival rates have improved since 1990, probably due to earlier detection and improved therapies, it remains the most lethal cancer for women both in France and worldwide (source: Institut National du cancer at *www. www.e-cancer.fr*). The lethality of breast cancer is mainly due to the formation of metastasis in vital organs, such as lungs and bone marrow. Predicting the risk of metastasis formation of a tumor is therefore a key challenge in the clinic, and crucial to determining the treatment of the patient. Indeed, only 20 to 30% of all patients treated for breast cancer will eventually develop metastasis.

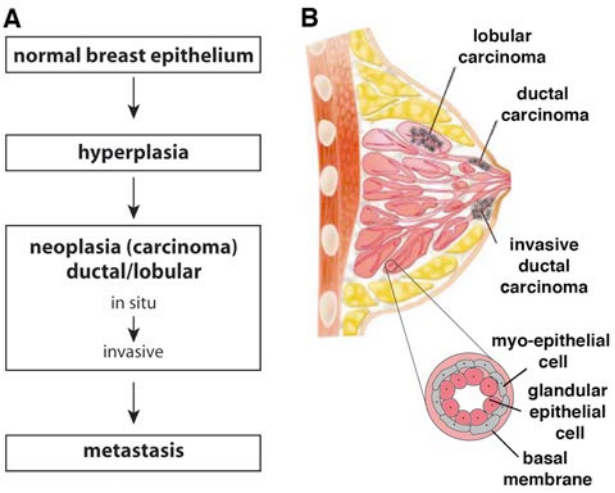

Figure 17: Breast cancer tumorigenesis. A. Scheme of the subsequent steps of breast tumorigenesis, from benign hyperplasia to the formation of metastasis. **B.** Histological characteristics of breast cancer. Breast cancer can form either in the mammary ducts or in the lobules. A close-up of a lobule, in which the epithelial cells line the lumen and are surrounded by myo-epithelial cells (or basal cells) and the basal membrane, is illustrated at the bottom. Image adapted from *www.sanofi-aventis.com.*

In order to predict the disease outcome, several methods of breast cancer classification have been used, now compiled on the website www.adjuvantonline.com to assist the clinicians in their decisions. First, the Nottingham Prognostic index (Galea et al, 1992) is a staging procedure to indicate how advanced the tumor is, based on clinical observations. It includes the size of the tumor (T), the lymphe node involvement (N) and the presence of metastasis (M) and can be applied to all tumors. Second, the tumor grade classifies tumors according to their histological characteristics. The most widespread grading system was proposed by Elston and Ellis (Elston & Ellis, 1991) and takes into account the level of cell differentiation, the nuclear morphology and the percentage of mitotic cells (Figure 18). Additional proliferation markers, such as Ki67 (Barnard et al, 1987), are used routinely. Although significantly associated with disease outcome in general, individual prognoses based on these systems remain too imprecise and do not take into account the biology of the tumor.

Grade	Description
Grade 1 (lowest)	Well-differentiated breast cells; cells generally appear normal and are not growing rapidly; cancer arranged in small tubules.
Grade 2	Moderately-differentiated breast cells; have characteristics between Grade 1 and Grade 3 tumors.
Grade 3 (highest)	Poorly differentiated breast cells; Cells do not appear normal and tend to grow and spread more aggressively.

Figure 18: The Elston & Ellis grading system in breast cancer. The grading system takes into account the differentiation of the cells (organization in tubules), the nuclear morphology and the mitotic index of the tumor. Table adapted from *www.imaginis.com*.

The search for a more molecular approach of breast cancer prognosis started with the discovery that tumor cells can be dependent or not on specific signaling pathways, notably hormones. The tumoral expression of the estrogen (ER) and progesterone (PR) receptor in premenopausal women and the overexpression of the Human Epidermal Growth Factor Receptor-2 (HER2 or ERBB2) are crucial prognostic factors. In addition, they gave rise to new therapies: the monoclonal antibody trastuzumab binds and inactivates HER2 (Hudis, 2007), while tamoxifen binds and blocks the estrogen receptor (Lee et al, 2008).

With the arrival of microarray-based technologies, the search for more precise, genome-wide, prognostic classifications of breast cancer intensified, with the hope to individualize administered therapies based on tumor biology. Several studies have shown that, based on microarray mRNA expression profiles, breast cancers can be divided into four main groups: (i) the luminal breast cancers, predominantly ER positive and further divided into luminal A (low grade) and B (high grade); (ii) basal-like breast cancers, predominantly ER, PR and HER2 negative; and (iii) the HER2 overexpressing cancers (Perou et al, 2000; Sorlie et al, 2001; Sorlie et al, 2003; Sotiriou et al, 2003, reviewed in Sotiriou & Piccart, 2007). Although of major importance for our understanding of breast cancer sub-types, these genome-wide approaches are mainly based on already known markers (grade, ER, HER2). Several companies have taken advantage of these new technologies to create tests based on a selection of genes (Geyer et al, 2009; Pusztai et al, 2007), as exemplified by the MammaPrint® test (Agendia, The Netherlands), based on the expression of 70 genes, or the Oncotype DX test (Genomic Health, USA), based on the expression of 21 genes. Yet, these tests remain very expensive and their added value to classical analysis uncertain. Thus, there is a need for new prognostic markers that can be analyzed in an easy, cheap and fast manner. Much hope has been put on new generation sequencing techniques (Stratton et al, 2009), which could reveal point mutations or single nucleotide polymorphisms that were not detectable by comparative genomic hybridization (CGH). However, these techniques are unlikely to be applied to each patient on a day-to-day basis and will rather be used to detect new mutations in the context of unexplained familial breast cancers cases. In sporadic breast cancer, only a small fraction of aberrant gene expression can be

attributed to genomic alterations (Valladares et al, 2006; Vincent-Salomon et al, 2008; Yao et al, 2006). Defects in chromatin organization, affecting gene expression without changing the DNA sequence, could be a source of potent prognostic marks.

1.5.2 Chromatin organization in breast cancer

The best described epigenetic aberrations in cancer cells concern DNA methylation (reviewed in Jones & Baylin, 2007). Cancer cells generally display a diminished global DNA methylation level, which is thought to contribute to genome instability. On the other hand, the promoters of certain tumor suppressor genes locally gain methylation and thus become epigenetically silenced. In breast cancer, this is not any different and many genes involved in cell proliferation, DNA repair, cell adhesion or apoptosis were found to be either hypo- or hypermethylated (reviewed in Agrawal et al, 2007 and Hinshelwood & Clark, 2008). These aberrant methylation profiles seem to be, at least partially, the consequence of an aberrant expression of Dnmt1 and Dnmt3b (reviewed in Esteller, 2007).

Histone modifications in cancer cells have been less extensively studied, but seem to follow similar rules as DNA methylation. Specific genes become silenced through the loss of active histone modifications, such as H3K4me2 and H3K9Ac, and a gain in repressive marks like H3K9me2 (Ballestar et al, 2003; Fahrner et al, 2002). However, on a global level, DNA hypomethylation associates with decreased levels of the repressive marks H4K20me3 and H4K16Ac in several types of cancers (Fraga et al, 2005), among which is breast cancer (Tryndyak et al, 2006). Interestingly, Suv39h1/2 double knockout mice develop B cell lymphomas at very high rates (Peters et al, 2001), suggesting that the loss of a repressive histone modification can indeed be associated with tumorigenesis. Again, aberrant expression levels of several histone-modifying enzymes, including HDAC1 and HDAC2 (reviewed in Weichert, 2009), Suv4-20h2 (Van Den Broeck et al, 2008) and the polycomb protein Enhancer of Zeste Homolog 2 (EZH2) (reviewed in Simon & Lange, 2008), have been reported and could contribute to the observed changes in histone modifications.

The DNA methylation and histone modifications are thought to affect gene expression through the binding of specific interacting proteins, which interpret the

signal and contribute to higher order chromatin organization. Thus, these "reader" proteins are crucial to translate epigenetic marks and might constitute an additional pathway to affect chromatin organization in cancer cells. However, until now, little is known about how these proteins are regulated and whether they are affected in cancer cells. Tumoral overexpression of certain DNA methyl binding proteins, such as MeCP2 (reviewed in Esteller, 2007) and Kaiso (Prokhortchouk et al, 2006), has been reported. BMI1, a subunit of the Polycomb repressor complex 1 that binds to H3K27me3, is generally considered as an oncoprotein (Sparmann & van Lohuizen, 2006), although its expression was recently associated with a better prognosis in breast cancer (Choi et al, 2009; Pietersen et al, 2008). For the 3 human isoforms of Heterochromatin Protein 1 (HP1), the best-studied "reader" protein that specifically binds H3K9me3 and is involved in heterochromatin formation, even less is known about its correlation with tumorigenesis, as will be discussed in chapter 2.

In conclusion, although DNA methylation and histone modifications are affected in cancer cells, in many cases the correlation with breast cancer prognosis remains to be studied. Genome-wide analysis, using micro-array based approaches, are currently being developed to investigate these marks in patient samples (Esteller, 2007). However, as discussed above, these marks mainly participate in the first level of chromatin organization and much remains to be learnt about how their readout is affected. In addition, how they these marks can be transmitted from tumor cell to tumor cell, and how they could modulate the response to cancer treatment, remains largely an open question.

2. Heterochromatin Protein 1

2.1 HP1 proteins in different species

HP1 proteins were identified more than 20 years ago in Drosophila embryos (James & Elgin, 1986), where they were described as non-histone proteins that associate with heterochromatic loci on polytene chromosomes. A few years later, a mutation in Drosophila HP1 was shown to abolish position effect variegation (Eissenberg et al, 1990). This provided proof for its functional role in heterochromatic gene silencing. A search for homologous sequences showed that homologues of the protein exist in human and mice (Singh et al, 1991). Importantly, this study also suggested for the first time that different isoforms of HP1 proteins were present, at least in mice. The authors described M31 and M32, later known as HP1β and γ, respectively (see Table II for nomenclature).

Since then, HP1 homologues have been described in many organisms (Figure 19). While the yeast *S. pombe* and *Arabidopsis* contain only one HP1 homologue (Swi6 in yeast and Like Heterochromatin Protein 1 (LHP1) in *Arabidopsis*), *C. elegans* presents two HP1-like proteins. Flies express three constitutive HP1 proteins, HP1a, b and c, in addition to two tissue specific isoforms (HP1d/rhino in ovaries and HP1e in testes). Vertebrate cells express three HP1 isoforms, named HP1α, β and γ in mouse and human. The high conservation is underlined by rescue experiments in *Drosophila*, where expression of human HP1α rescues the lethality of homozygous HP1a mutants (Norwood et al, 2004). Murine and human HP1 proteins are highly similar (Figure 19B) both in structure and in function, and antibodies raised against murine HP1 isoforms generally recognize the human proteins and vice versa.

Table II: HP1 nomenclature. Different names for each mammalian isoform		
- HP1α - chromobox homolog 5 (Cbx5)	- HP1β - chromobox homolog 1 (Cbx1) - M31 - Modifier 1 (MOD1)	- HP1γ - chromobox homolog 3 (Cbx3) - M32

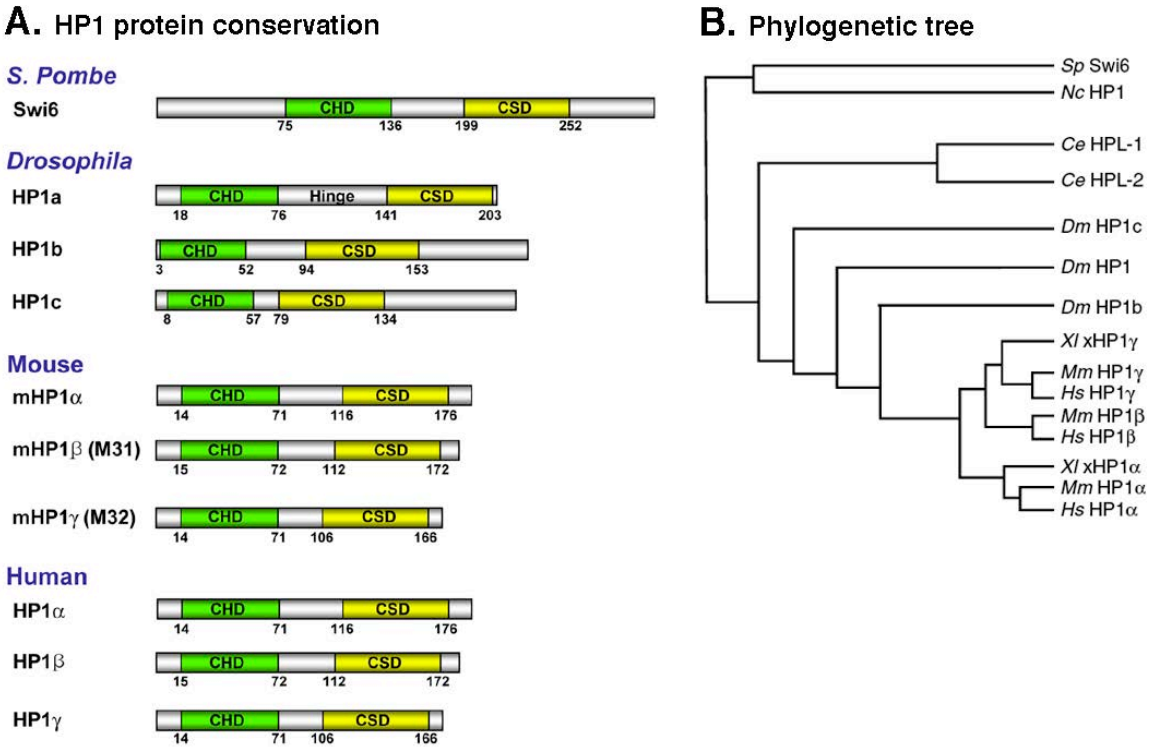

Figure 19: Constitutively expressed HP1 homologues in various species. A. Schematic representation of *S. pombe*, *Drosophila*, mouse and human HP1 proteins. Highlighted are the conserved N-terminal chromodomain (CHD) and the C-terminal chromoshadowdomain (CSD). The hinge region connects the two domains. Image adapted from Kwon & Workman, 2008. **B.** Phylogenetic tree of HP1 proteins in different species. Sp: *Schizosaccharomyces pombe*; Nc: *Neurospora crassa*; Ce: *Caenorhabditis elegans*; Dm: *Drosophila melanogaster*; Xl: *Xenopus laevis*; Mm: *Mus musculus*: Hs: *Homo sapiens*. Image adapted from Lomberk et al, 2006b.

Although the three mammalian HP1 isoforms, HP1α, β and γ, globally show high similarity in the conserved structural domains (Figure 20), several indications suggest that that they may not carry out identical functions. First, they show differences in their nuclear pattern of distribution. The overall nuclear staining of HP1α marks strongly pericentric heterochromatin, while HP1γ shows less specificity for these regions (Gilbert et al, 2003; Minc et al, 1999; Nielsen et al, 2001a; Taddei et al, 2001). In addition, the three isoforms show different chromatin-binding dynamics. Indeed, HP1 proteins distribute in different nuclear fractions, distinguished by their capacity to resist extraction with high salt concentrations or with Triton X-100 detergent. The salt- or detergent-resistant pool of HP1 is considered as the active, chromatin-bound pool of HP1 and represents less than 10% of total HP1 in human and rodent cells, as determined by Western Blot quantification (Taddei et al, 2001) or Fluorescence

Recovery After Photobleaching (FRAP) (Cheutin et al, 2003; Dialynas et al, 2007; Festenstein et al, 2003). HP1γ shows the highest mobility among the three isoforms, both in murine and human cells (Cheutin et al, 2003; Dialynas et al, 2007), suggesting that it is less tightly associated with (hetero-) chromatin (Taddei et al, 2001). Furthermore, despite their high similarity, the three isoforms are not always present together and can interact with different binding partners (see below). Finally, all three HP1 isoforms can be extensively modified, not only by phosphorylation but also by acetylation, sumoylation and ubiquitination (Lomberk et al, 2006a; Minc et al, 1999). Some of these modifications seem to be isoform-specific and may further diversify their functions. In the chapters below, isoform-specificity will be indicated, when known. However, most often functions or interactions have not been tested for all three HP1 isoforms. Thus, the common or divergent characteristics among the isoforms remain largely unknown.

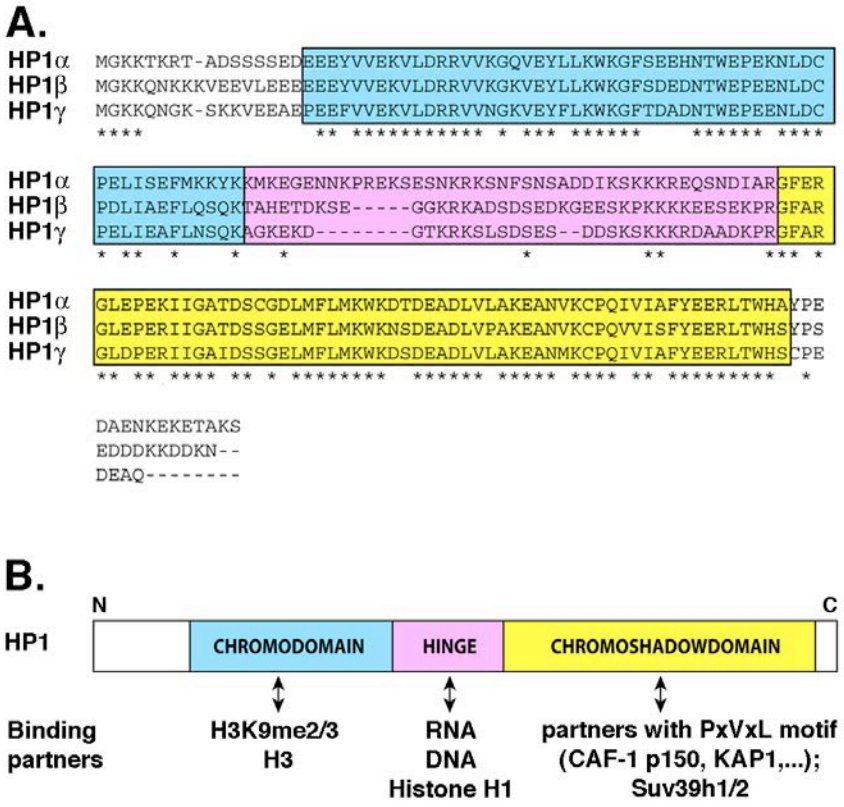

Figure 20: The three HP1 isoforms in human cells. A. Amino acid sequences of human HP1α, β and γ. Asterisks indicate identical residues within the three isoforms. The chromodomain is highlighted

in blue, the hinge region in pink and the chromoshadow domain in yellow. **B.** Schematical representation of HP1 protein domains and binding partners. Colours as in A. Image adapted from Maison & Almouzni, 2004.

2.2 Structural organization of HP1 proteins

HP1 proteins are characterized by several conserved structural features, which have facilitated the search for homologues (see also Figure 19 and 20). The chromodomain (CHD) was the first identified conserved domain. This domain is located N-terminally and consists of a three-stranded β-sheet folded close to an α-helix (Figure 21A) (Ball et al, 1997). Chromodomains are found in many proteins involved in chromatin organization, such as chromatin remodeler factors and Polycomb repressor proteins (Jones et al, 2000). In HP1 proteins, the chromodomain provides a specific binding site for di- and trimethylated H3K9 (Figure 21B) (Bannister et al, 2001; Lachner et al, 2001; Nielsen et al, 2002). Its structure is slightly different in polycomb proteins, where it preferentially binds to trimethylated H3K27 (Fischle et al, 2003).

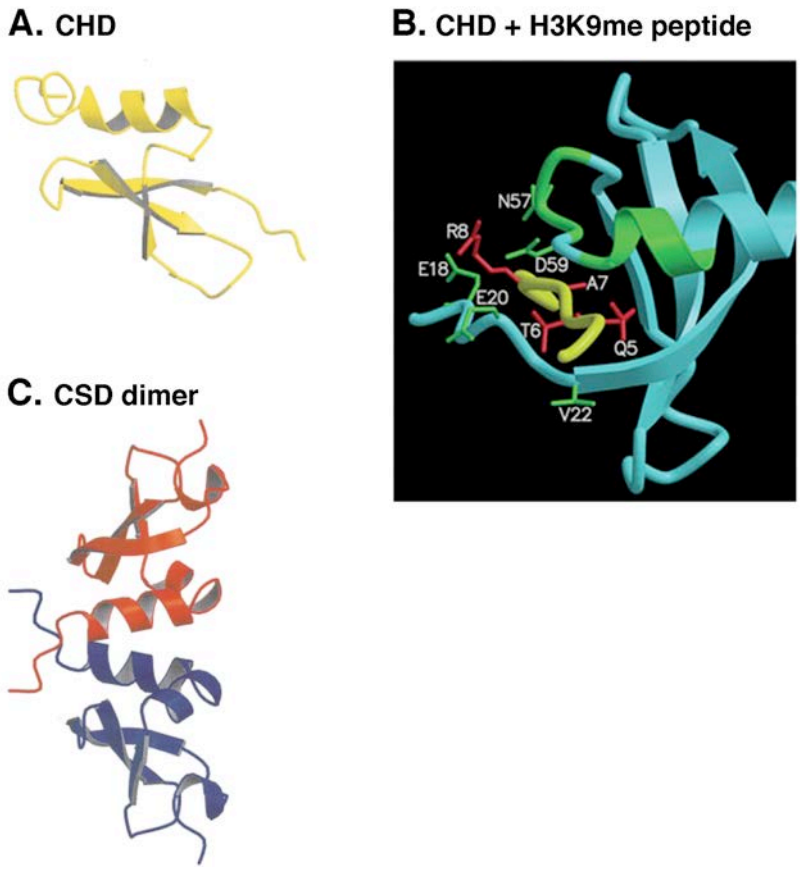

Figure 21: Schematical representation of the structural domains of HP1 proteins. A. Schematical representation of the chromodomain (CHD) of murine HP1β. Image from Brasher et al, 2000. **B.** Interface between the chromodomain of murine HP1β (blue and green) and a dimethylated peptide corresponding to residues 1-13 of histone H3 (yellow and red). Interacting regions in the CHD are depicted in green. Image from Nielsen et al, 2002. **C.** Schematical representation of a chromoshadowdomain (CSD) dimer of murine HP1β. Image from Brasher et al, 2000.

The second conserved domain is the C-terminally located chromoshadow domain (CSD) (Aasland & Stewart, 1995). This domain is specific for HP1 proteins and resembles the chromo domain, except that a small additional α-helix is present between the large α-helix and the β-sheet (Figure 21C) (Brasher et al, 2000; Cowieson et al, 2000). The chromoshadow domain provides the interface for homo-and heterodimerization of HP1 proteins (Brasher et al, 2000; Cowieson et al, 2000). In addition, this domain is required for the interaction of HP1 with many different binding partners, characterized by the presence of a conserved motif consisting of a PxVxL sequence (Nielsen et al, 1999; Smothers & Henikoff, 2000).

A hinge region that is poorly conserved, not only between species but also between the different isoforms, separates the chromodomain and the chromoshadow domain. The hinge region binds not only to chromatin templates in a sequence-independent manner (Meehan et al, 2003), but also to RNA (Muchardt et al, 2002) and to histone H1 (Nielsen et al, 2001a). It has been proposed that the hinge domain, due to its poor conservation, modulates and diversifies the interactions of each HP1 isoform (Smothers & Henikoff, 2000). Furthermore, this unstructured region is thought to allow the two conserved domains to move independently from each other. This would allow HP1 to be a bridging molecule between chromatin and methylated H3K9 on the one hand and interacting partners on the other. A mechanism for recruiting factors to heterochromatic regions can thus be envisaged. This is exemplified by the interaction of the CSD with the H3K9 methyltransferase enzymes Suv39h1/2 (Aagaard et al, 1999). HP1 proteins could bind to methylated H3K9 by the CHD and recruit Suv39h1/2 through their CSD (Schotta et al, 2002; Yamamoto & Sonoda, 2003), resulting in additional methylation of H3K9 and the recruitment of more HP1 proteins (Bannister et al, 2001; Lachner et al, 2001). In this manner, the heterochromatic state could be maintained and self propagate. Interestingly, studies on chromatin templates assembled in *Xenopus* oocytes indicated that the stable binding of HP1 proteins to methylated H3K9 also required the presence of the Suv39 enzymes (Stewart et al, 2005), suggesting a tightly regulated mechanism. However, it remains unclear how HP1 proteins bind to Suv39 enzymes, which lack the classical PxVxL sequence. Both the N-terminal region and the C-terminal catalytic SET-domain of Suv39h1 were reported to interact with

HP1α (Schotta et al, 2002; Stewart et al, 2005; Yamamoto & Sonoda, 2003), but the individual importance and the regulation of these two binding sites are yet to be defined.

2.3 HP1 partners and functions

HP1 proteins interact with an impressive list of proteins (Table III), and new interacting partners are still regularly described. The binding partners of HP1 proteins give an insight into their potential roles, revealing functions beyond the one initially described as a non-histone structural component of constitutive heterochromatin (Grewal & Jia, 2007; Kwon & Workman, 2008). Instead, HP1 proteins could constitute a platform to integrate a variety of signals involved in many cellular processes, dependent on cell cycle and developmental stage.

2.3.1 Heterochromatin formation

The targeting of exogenous HP1α or β to a transgene region that is particularly decondensed in Chinese hamster ovarian (CHO)-derived cells results in the condensation of this region (Verschure et al, 2005). The compacted region becomes enriched in H3K9 methylation through the activity of the HMT SET Domain Bifurcated 1 (SetDB1), and endogenous HP1 proteins are recruited in parallel. In addition to their interaction with Suv39h1/2, the three HP1 isoforms were also documented to associate with another H3K9 methyltransferase, G9a (Chin et al, 2007). Interestingly, the Suv39h1/2 enzymes are thought to methylate H3K9 mainly in (pericentric) heterochromatin regions, while G9a activity seems restricted to euchromatic regions (Tachibana et al, 2002). Thus, HP1 proteins could be involved both in the maintenance of the heterochromatic state and in the silencing of euchromatic sites. Furthermore, all three HP1 isoforms interact with the H4K20 methyltransferase Suv4-20h2 and correct localization of HP1 is necessary for the recruitment and the activity of this enzyme (Schotta et al, 2004). Thus, sequential steps of heterochromatin formation or maintenance can be identified, with H3K9 methylation and HP1 binding preceding H4K20 methylation. In addition, at least the HP1β isoform was also reported to interact with the DNA methyl transferases Dnmt1 and Dnmt3a/b (Fuks et al, 2003). As discussed earlier, while Dnmt1 is involved in the duplication of DNA methylation during replication, Dnmt3 is a *de novo* histone methyltransferase. The interaction of HP1 with

both types of methyltransferases suggests that HP1 might contribute both to the establishment and the maintenance of heterochromatin. The recruitment of DNA methyltransferase activity could ensure the full heterochromatization of the target region and the formation of a repressive state that is transmitted through mitosis (Ayyanathan et al, 2003). Finally, at least the HP1α isoform interacts with the Suppressor of Zeste 12 (Suz12) subunit of the Polycomb Repressive Complex 2 (PRC2) (Yamamoto et al, 2004). Depletion of Suz12 in human cells

Table III: Major interacting partners of *Drosophila* and mammalian HP1 proteins and functional implications

Protein family/function	Partner protein	Organisms	HP1 variants	HP1 domain	Proposed associated function	Key reference(s)
Histones	**Histone H1**	Dm, Mm	HP1a, HP1α	Hinge	heterochromatin formation	Nielsen et al., 2001
	Histone H3	Dm, Mm	HP1a, HP1α, HP1β, HP1γ	CHD	heterochromatin formation?	Nielsen et al., 2001
	Histone H4	Dm, Mm	HP1a, HP1α	CSD	heterochromatin formation?	Polloudaki et al., 2001
	H3K9me3	Dm, Mm, Hs	HP1a, HP1b, HP1c, HP1α, HP1β, HP1γ	CHD	heterochromatin formation	Lachner et al., 2001; Bannister et al., 2001
H3K9 methyltransferases	**Suv39h1**	Dm, Mm, Hs	HP1a, HP1β	CSD	heterochromatin formation	Aagaard et al., 1999
	G9a	Hs	HP1α, HP1γ	nd	gene silencing?	Chin et al., 2007
H4K20 methyltransferase	**Suv4-20h1/2**	Mm	HP1α, HP1β, HP1γ	nd	heterochromatin formation	Schotta et al., 2004
H3K36demethylase	**dKDM4A**	Dm	HP1a	CSD	gene expression	Lin et al., 2008
Histone deacetylases	**HDAC4**	Mm	HP1α	Hinge	gene silencing/heterochromatin formation	Zhang et al., 2002
	HDAC5	Mm	HP1α	Hinge	gene silencing/heterochromatin formation	Zhang et al., 2003
DNA methyltransferases	**Dnmt1**	Hs	HP1β	nd	heterochromatin formation	Fuks et al., 2003
	Dnmt3a	Hs	HP1β	nd	heterochromatin formation	Fuks et al., 2004
Methylated DNA-binding factor	**MeCP2**	Hs	HP1γ	CSD	heterochromatin formation	Agarwal et al., 2007
Polycomb Group protein	**Suz12**	Hs	HP1α	CSD	heterochromatin formation	Yamamoto et al., 2004
Chromatin assembly	**CAF-1 p150**	Hs, Mm	HP1α, HP1β, HP1γ	CSD	heterochromatin maintenance	Murzina et al, 1999; Quivy et al., 2004
DNA replication	**ORC1-6**	Dm, Mm, Hs	HP1a, HP1α	CHD + CSD	heterochromatin maintenance/replication timing?	Pak et al., 1997
	Suur	Dm	HP1a	CSD + Hinge	replication timing	Pindyurin et al., 2008
	Ki67	Hs	HP1α, HP1β, HP1γ	CSD	heterochromatin maintenance/replication timing?	Scholzen et al., 2002
Gene transcription regulation	**KAP1/Tif1β**	Hs, Mm	HP1α, HP1β, HP1γ	CSD	gene silencing/DNA repair	Le Douarin 1996
	Tif1α	Mm	HP1α, HP1β, HP1γ	CSD	gene silencing?	Nielsen et al. 1999
	Rb	Hs	HP1α, HP1β, HP1γ	CSD	gene silencing/ heterochromatin formation?	Williams & Grafi, 2000
	SP100b	Hs	HP1α, HP1β, HP1γ	CSD	gene silencing?	Lehming et al., 1998
	STAT92E	Dm	HP1a	nd	gene silencing?	Shi et al., 2006
	BCL11b/CTIP2	Hs	HP1α	nd	gene silencing?	Rohr et al., 2003
Transcription-associated protein	**Taf4a/TafII130**	Hs	HP1α, HP1γ	CSD	gene silencing?	Vassallo & Tanese, 2002
	RNA polymerase II	Hs	HP1γ	nd	active gene transcription	Vakoc et al., 2005
SWI/SNF chromatin remodeler	**ATRX**	Mm	HP1α	CSD	gene regulation/chromatin remodeling?	Lechner et al., 2005
Serine/threonine kinase	**Pim-1**	Hs	HP1γ	CSD	gene silencing?	Koike et al., 2000
Double strand break repair	**Ku70**	Hs	HP1α, HP1γ	CSD	DNA repair/telomere maintenance	Lomberk et al., 2006; Song et al., 2001
Telomere binding factors	**Hip**	Dm	HP1a	CSD	gene silencing/telomere maintenance	Schwendemann et al.,2007
	Umbrea	Dm	HP1a	CSD	telomere folding/ maintenance	Joppich et al., 2009
	Hoap	Dm	HP1a	CSD + Hinge	telomere folding/ maintenance	Shareef et al., 2001; Badagu et al., 2003
Centromere/kinetochore components	**INCENP**	Hs	HP1α, HP1γ	Hinge	faithful mitosis	Ainsztein et al., 1998
	Mis12	Hs	HP1α, HP1γ	nd	faithful mitosis	Obuse et al., 2004
Nuclear architecture	**Lamin B receptor**	Hs	HP1α, HP1β, HP1γ	CSD	peripheral heterochromatin localization	Ye & Worman, 1996

Table III : Major interacting partners of Drosophila and mammalian HP1 proteins and functional implications. Inspired by Kwon & Workman, 2008**.**

leads to reduced H3K9 methylation and altered HP1α distribution (de la Cruz et al, 2007). This suggests that, although generally considered as two separate repressive pathways, HP1 and Polycomb-dependent silencing do show cross talk that could contribute to HP1 functioning in heterochromatic regions.

How HP1 proteins are targeted to specific genomic sites is still largely unknown. The two distinct types of heterochromatin, constitutive and facultative heterochromatin, potentially rely on two distinct pathways for HP1 targeting. As discussed in the introduction, the RNA interference pathway might be involved in the propagation and maintenance of constitutive heterochromatin. *S.pombe* centromeric repeats rely highly on this process for their enrichment in H3K9me and Swi6 and their silent state. In mammalian cells, the process might be conserved (Eymery et al, 2009; Morris et al, 2004). Indeed, RNA degradation by RNase treatment on permeabilized mouse cells delocalizes HP1 from pericentric heterochromatin (Maison et al, 2002) and at least the HP1α isoform interacts with multiple RNA binding proteins (Ameyar-Zazoua et al, 2009). In addition, depletion of factors involved in RNAi (Argonaute, Dicer) results in the accumulation of centromere-derived RNAs and reduced levels of H3K9me3 and HP1 proteins at centromeres (Fukagawa et al, 2004; Kim et al, 2006). However, these associations could be indirect, since the mutation of Dicer results in reduced expression levels of Dnmt1 and Dnmt3a/b (Benetti et al, 2008). Thus, the importance of RNAi for mammalian centromere organization and stability remains unclear.

For the silencing of genes localized in euchromatic regions, HP1 targeting seems to rely on specific binding partners. The tumor suppressor Retinoblastoma protein (Rb) interacts with HP1 proteins (Nielsen et al, 2001b; Williams & Grafi, 2000) and recruits both Suv39h1/2 and HP1 to induce silencing of cell cycle genes, such as Cyclin E (Nielsen et al, 2001b). In addition, HP1 interacts with the transcriptional repressor KAP-1, also called Tif1β (Ryan et al, 1999). KAP-1 serves as a co-repressor protein for a large number of zinc finger proteins that contain a Kruppel-associated box (KRAB) motif. The genomic targets of KAP-1-mediated repression include a wide variety of genes involved in crucial cellular pathways (O'Geen et al, 2007). KAP-1 induces silencing of its target genes by recruiting the H3K9 methyltransferase SETDB1 and HP1 proteins (Sripathy et al, 2006). At least on a stably integrated transgene, this repressed state is stably maintained for over more than 40 mitoses (Schultz et al, 2002), suggesting that silencing of a euchromatic region can be mitotically heritable. Importantly, this also suggests that

silencing of a euchromatic regions by H3K9me3 and HP1 recruitment may be mainly restricted to genes requiring long-term silencing. Indeed, Suv39h1/2 enzymes are crucial for silencing of S-phase genes in the context of muscle cell differentiation (Ait-Si-Ali et al, 2004).

A highly specific type of heterochromatin is formed in the context of cellular senescence. This permanent cell cycle exit is accompanied by the formation of Senescence Associated Heterochromatin Foci (SAHF), which are enriched in H3K9me3 and all three HP1 isoforms (Narita et al, 2003; Zhang et al, 2005). These domains are thought to contain many genes involved in cell proliferation, such as target genes of the E2 promoting Factor (E2F) (Narita et al, 2003), thus ensuring their stable silencing. Interestingly, in this case, HP1 proteins are not involved in the initiation of the heterochromatin formation, since dense regions of DNA appear before HP1 enrichment (Zhang et al, 2005). In addition, expression of a dominant negative truncated HP1β, which traps endogenous HP1 isoforms and prevents their recruitment, does not impair the formation of SAHF (Zhang et al, 2007). Therefore, it was proposed that HP1 proteins might be involved in the stabilization, rather than the formation, of these heterochromatic structures and would thus contribute to the irreversibility of the cell cycle exit (Adams, 2007).

2.3.2 Heterochromatin maintenance during DNA replication

Links between HP1 and factors involved in DNA replication have provided interesting insights into heterochromatin replication and maintenance. Both human and murine HP1α and β interacts with several subunits of the Origin Recognition Complex (ORC) (Auth et al, 2006; Lidonnici et al, 2004; Prasanth et al, 2004). This complex, initially implicated in the formation of pre-replication complexes at replication origins, was found to colocalize with HP1 proteins at centromeres (Prasanth et al, 2004; Prasanth et al, 2002). Interestingly, the depletion of the ORC2 subunit leads to the delocalization of HP1 and mitotic defects (Prasanth et al, 2004) that are similar to those observed after HP1 depletion (Auth et al, 2006; Obuse et al, 2004; Serrano et al, 2009). In contrast, perturbation of HP1 localization, by prolonged treatment with the HDAC inhibitor trichostatin A (TSA) or by RNase treatment, does not affect the ORC1 localization (Lidonnici et al, 2004). It has been put forward that HP1 recruitment by ORC

could provide a connection between replication origin firing and heterochromatin and might thus contribute to the late replication timing of heterochromatin (Sasaki & Gilbert, 2007). How exactly this interaction could affect replication timing was not discussed. According to Leatherwood and colleagues, the interaction between ORC and HP1 might facilitate the replication of heterochromatic domains, through the enhanced recruitment of initiation complexes (Leatherwood & Vas, 2003). Indeed, replication of heterochromatin requires access to the compact domains, but also maintenance and duplication of the heterochromatic components (reviewed in Wallace & Orr-Weaver, 2005). In this respect, an interesting connection between replication and heterochromatin maintenance has been revealed for the chromatin assembly factor 1 (CAF-1). The CAF-1 complex is involved in chromatin assembly coupled to DNA synthesis and localizes to sites of DNA synthesis through the interaction of its largest subunit p150 with the polymerase accessory factor PCNA. However, CAF-1 p150 was also found to bind the chromoshadow domain of all three HP1 homologues *in vitro* (Murzina et al, 1999). Interestingly, the PxVxL sequence required for the interaction is located in the N-terminal part of CAF-1 p150 that is dispensable for chromatin assembly, suggesting two distinct functions. This was confirmed by the detailed analysis of CAF-1 and HP1 during replication of pericentric heterochromatin clusters (chromocenters) in mouse cells (Quivy et al, 2008; Quivy et al, 2004). These studies showed the *in vivo* association of CAF-1 p150 with HP1α and γ in a complex that did not contain histones, again pointing to an independent function distinct from chromatin assembly (Quivy et al, 2004). Moreover, depletion of CAF-1 p150 leads to an arrest in S-phase. This arrest is due an impaired replication of HP1-rich pericentric heterochromatin, specifically (Quivy et al, 2008). This phenotype can be rescued by a wild-type p150, but not by a mutant p150 that cannot bind to HP1. Furthermore, in Suv39h1/2 deficient cells, where HP1 is not enriched at pericentric domains, the depletion of CAF-1 p150 no longer affects S-phase progression (Quivy et al, 2008). Thus, the interaction of CAF-1 p150 with HP1 is crucial for replication of HP1-rich chromocenters. Interestingly, the replication of these domains takes place at their periphery, and a rim of CAF-1 can be observed surrounding the chromocenters during their replication. This CAF-1 is associated with a fraction of HP1 that is not dependent on the presence of H3K9me3 or RNA (Quivy et al, 2004). Thus, a model was proposed in which CAF-1 p150 accepts, sequesters and provides HP1 proteins during replication of pericentric heterochromatin, which occurs at the

periphery of the chromocenters (Quivy et al, 2008; Quivy et al, 2004). More recently, the histone methyltransferase SetDB1 was also identified as a component of the CAF-1 – HP1 complex (Loyola et al, 2009). SetDB1 preferentially monomethylates H3K9 before histone deposition onto DNA and localizes to pericentric heterochromatin during replication (Loyola et al, 2009). The created H3K9me1 constitutes the main substrate for further H3K9 methylation by Suv39h1/2 (Loyola et al, 2006) and downregulation of SetDB1 indeed diminishes the levels of H3K9me3 within heterochromatin (Loyola et al, 2009). Thus, a picture emerges in which CAF-1 coordinates the propagation of pericentric heterochromatin, by orchestrating both SetDB1-dependent H3K9 methylation and HP1 dynamics. Whether these findings also apply to human cells, where pericentric regions are not organized in dense chromocenters, remains to be addressed.

2.3.3 Cell division

In *S.pombe*, it is well established that the HP1 homologue Swi6 is involved in mitotic segregation. The protein localizes to centromeric regions and its mutation gives rise to mitotic defects including lagging chromosomes (Table IV) (Ekwall et al, 1995). Swi6 promotes proper chromosome segregation by stimulating cohesin recruitment to centromeres (Bernard et al, 2001). Furthermore, Swi6 is involved in the initial recruitment of the centromeric histone H3 variant CENP-A^{Cnp1} (Folco et al, 2008), to promote *de novo* formation of centromeric chromatin.

Table IV: Phenotypes associated with depletion of HP1 proteins

Species	Protein	Approach	Phenotype	References
S.pombe	Swi6	mutant	- Mating type switching deficiencies - Silencing defects - Mitotic defects	Breeden & Nasmyth, 1987 Lorentz et al., 1992 Ekwall et al., 1995
C.elegans	HPL1	RNAi	None	Couteau et al., 2002
	HPL2	RNAi	- Sterile - Vulval development deficiencies - Silencing defects	Couteau et al., 2002
	HPL1 & HPL2	KO	- Larval lethality - Somatic gonad development deficiencies	Schott et al., 2006
D.melanogaster	HP1a	mutant	- Developmentally lethal - Suppression of PEV	Eissenberg & Hartnett, 1993

			- Chromosome missegregation - Telomere fusions	Eissenberg et al., 1990 Kellum & Alberts, 1995 Fanti et al., 1998
	HP1a	RNAi	- Proliferation defects - Chromosome missegregation	De Lucia et al., 2005
M.musculus	HP1α	RNAi	- Mitotic defects	Auth et al., 2006
	HP1α	RNAi	- Differentiation defects	Panteleeva et al., 2007
	HP1β	KO	- Perinatal lethality - Genomic instability	Aucott et al., 2008
	HP1α & HP1β	KO	Not documented*	Dialynas et al., 2007
H.sapiens	HP1α & HP1γ	RNAi	- Chromosome missegregation (HeLa cells)	Obuse et al., 2004 Serrano et al., 2009
	HP1α & HP1β	Dom. Neg. HP1βΔN	- Chromosome missegregation (HeLa cells)	Inoue et al., 2008

Abbreviations: RNAi: RNA interference; KO: knock-out; Dom.Neg.: Dominant Negative mutant; PEV: Position effect variegation.
**The authors use KO embryonic stem cells to introduce tagged exogenous HP1; they mention that the phenotype of the mice will be described elsewhere.*

In mammalian cells, the connection with chromosome segregation seems conserved (Table IV), but the exact role of HP1 proteins in this process is still a matter of debate. The presence of three different isoforms, that might have overlapping functions, has also complicated research in this direction. Mammalian HP1 proteins interact with proteins that localize to the kinetochore complex and the centromere, such as Minichromosome Instability 12 (Mis12) (Obuse et al, 2004) and Inner Centromere Protein (INCENP) (Ainsztein et al, 1998; Wheatley et al, 2001). Downregulation (Auth et al, 2006; Inoue et al, 2008; Obuse et al, 2004) or mislocalization of HP1 isoforms, due to the absence of H3K9me3 (Guenatri et al, 2004) or to treatment with the histone deacetylase inhibitor TSA (Taddei et al, 2001), result in mitotic defects. Intriguingly, recent reports indicate that, in contrast to Swi6 in *S. pombe*, the correct localization of HP1 is not required for the recruitment of cohesins to centromeric regions (Koch et al, 2008; Serrano et al, 2009). The HP1α isoform might instead help to stabilize the Shugoshin protein upon prolonged metaphase arrest, which protects cohesins from degradation and thus prevents chromosome segregation (Yamagishi et al, 2008). However, this observation could not be reproduced under less extreme cell cycle arrest conditions (Serrano et al, 2009). Interestingly, during mitosis, phosphorylation of H3S10 by Aurora B induces the release of HP1 proteins from pericentric chromatin during mitosis (Fischle et al, 2005; Hirota et al, 2005). Yet, the HP1α isoform can be visualized on mitotic chromosomes, where it is associated with centromeric regions (Guenatri et al, 2004; Hayakawa et al, 2003; Minc et al, 2001; Schmiedeberg et al, 2004). In conclusion,

distinct pools of HP1 proteins might exist and their exact roles in mitosis remain to be elucidated in mammals.

2.3.4 DNA repair

The first evidence that HP1 proteins might also play a role in DNA repair came from the observation that HP1α interacts with the protein Ku70 (Song et al, 2001). Together with Ku80, Ku70 is involved in the repair of double strand breaks (DSB) by the non-homologous end-joining (NHEJ) pathway. However, like many repair proteins, Ku70 also has an important role in the protection of telomeres (reviewed in Fisher & Zakian, 2005). Since HP1 proteins localize to telomere-associated heterochromatin (Minc et al, 1999), the interaction between Ku70 and HP1α has been explained as a manner to ensure telomere stability (Song et al, 2001). Indeed, overexpression of HP1α or β impairs the telomeric recruitment of the catalytic subunit of telomerase (hTERT) and induces telomere instability (Sharma et al, 2003).

Recently, more direct evidence for a role of HP1 proteins in DNA repair has emerged. Using local laser irradiation, Ayoub and colleagues showed that the induction of DSB within chromocenters of mouse cells results in the local spreading of HP1β (Ayoub et al, 2008). This dispersion is associated with the phosphorylation of HP1β, which prevents its binding to H3K9me3. The authors put forward that this apparent decompaction is necessary for the efficient accumulation of γ–H2AX foci and thus DNA repair signaling. These observations would suggest that chromatin relaxation is crucial for the DNA damage response to occur. Goodarzi and colleagues (Goodarzi et al, 2008) came to similar conclusions but propose a distinct mechanism. They showed that the repair of DSB within chromocenters, specifically, requires the activity of the Ataxia Telangiectasia Mutated (ATM) damage response protein and that downregulation of HP1, HDAC1+2 or KAP-1 alleviates this need for ATM. They suggest that, upon DSB induction, ATM phophorylates KAP-1, which would diminish the affinity of KAP-1 for heterochromatin and promote the accessibility of chromocenters for the repair machinery. It is indeed conceivable that chromatin relaxation is required to gain access to the dense chromocenters. However, it is also tempting to speculate that the repair might occur at the periphery of these domains, as is the case for DNA replication (Quivy et al, 2008; Quivy et al, 2004).

More recently, three groups have reported an accumulation of HP1 proteins on DNA damage sites (Ayoub et al, 2009; Luijsterburg et al, 2009; Zarebski et al, 2009). According to Ayoub and colleagues (Ayoub et al, 2009), this accumulation, observed in both euchromatin and heterochromatin, occurs after the initial HP1 dispersion that they documented earlier (Ayoub et al, 2008). However, Luijsterburg and colleagues (Luijsterburg et al, 2009) were not able to reproduce this dispersion and document a rapid and long-lasting recruitment of all three HP1 isoforms to Ultra-Violet (UV) - induced damage or DSB. This recruitment is independent of H3K9me3 and requires the chromoshadow domain of HP1. Furthermore, HP1 can be recruited even in the absence of certain DNA damage detection and signaling factors, suggesting that HP1 is recruited independently of functional DNA repair (Luijsterburg et al, 2009). The rapid recruitment of HP1 proteins in both euchromatin and heterochromatin was confirmed by Zarebski and colleagues (Zarebski et al, 2009). In conclusion, this topic and in particular the dispersion of HP1 remains controversial. A current aim will be to determine how HP1 proteins are recruited to DNA damage sites and what could be their functional role in DNA repair.

2.3.5 Regulation of transcription

While HP1 has been considered for a long time as a silencing protein, an increasing number of studies, mostly in *Drosophila*, also involve HP1 in active transcription. Several genes that are naturally located within heterochromatin actually require the presence of HP1 for their transcription (Clegg et al, 1998; Hearn et al, 1991; Lu et al, 2000). Subsequent studies evidenced that HP1 can indeed directly promote gene transcription. First, HP1 was found to localize to so-called puffs on polytene *Drosophila* chromosomes (Piacentini et al, 2003), which are euchromatic regions of intense transcription. Chromatin Immunoprecipitation (ChIP) analysis demonstrated that HP1 was indeed associated with several genes that showed decreased expression when HP1a levels were reduced (Cryderman et al, 2005). Furthermore, high resolution mapping of HP1 binding sites on *Drosophila* chromosomes 2 and 4 revealed that the association with actively transcribed genes is a general phenomenon that is not restricted to the promoter region, but concerns all gene exons (de Wit et al, 2007). A more direct role for HP1 in the positive regulation of transcription was reported for the *Drosophila* HP1c isoform, of which the targeting to a reporter gene resulted in enhanced

transcription instead of silencing (Font-Burgada et al, 2008). Furthermore, it was recently shown that HP1a interacts with and stimulates the H3K36 demethylase dKDM4A (Lin et al, 2008), thus providing for the first time a molecular mechanism linking HP1 to positive gene regulation.

Interestingly, H3K9me3 and HP1γ are also associated with actively transcribed genes in mammalian cells, in a manner that requires transcription elongation, and are rapidly lost upon transcriptional inactivation (Vakoc et al, 2005). Again, this enrichment is more prominent in the coding region than in the gene promoter. Similarly, HP1γ is recruited upon activation of a viral gene integrated in human cells, where it replaces repressive HP1β (Mateescu et al, 2008). These observations suggest that the requirement of HP1 for the transcription of certain genes could be conserved in mammalian cells and that HP1γ might have evolved as the isoform involved in gene activation, while HP1α would be more specific for gene silencing. Also, the enrichment associated with positive gene regulation seems to concern the entire coding region, with a role in elongation, while a concentration at the promoter region is more characteristic for gene silencing. How HP1 proteins can efficiently choose between gene silencing and promoting transcription is an open issue and probably depends on the chromatin environment, binding partners and cell status. Thus, a picture emerges in which HP1 proteins might constitute a platform of interactions to influence transcription in various ways.

2.4 HP1 proteins in the context of tumorigenesis

When considering the multiple functions of HP1 proteins described above, including gene silencing, gene expression and DNA repair, their involvement in tumorigenesis can be readily imagined. Surprisingly, very little has been reported in this context, possibly because of the diverse functions and the presence of three different isoforms, which do not facilitate the task in deciphering how HP1 isoforms impact on cell transformation. A first indication that HP1 proteins might play a role in tumorigenesis came from the construction of mice deficient for Suv39h1 and Suv39h2 (Peters et al, 2001). These double null mice show a dispersed localization of HP1 proteins, a high degree of chromosomal instability and develop B cell lymphomas.

Reduced H3K9 methylation can also be observed upon overexpression of the H3K9me2/3 histone demethylase GASC1 (Cloos et al, 2006), as observed in esophageal cancer (Yang et al, 2000; Yang et al, 2001). In addition, the finding that HP1 proteins interact with the tumor suppressor protein Rb (Nielsen et al, 2001b; Williams & Grafi, 2000) and contributes to Rb-dependent silencing of cell cycle genes, such as Cyclin E (Nielsen et al, 2001b), also suggest an implication of HP1 in tumorigenesis. Kirschmann and colleagues provided a more direct link between HP1α and the behavior of transformed cultured cells (Kirschmann et al, 2000). They showed that a high *in vitro* invasive potential is associated with low HP1α expression levels and vice versa. Downregulation of HP1α increased the *in vitro* invasive potential, while HP1α overexpression led to decreased invasive potential, without affecting proliferation rates (Norwood et al, 2006). Immunohistochemistry staining on breast cancer tissues shows low HP1α expression levels in 9 metastatic tissues compared to 6 primary invasive breast cancers. The authors therefore suggest that HP1α levels are low in the cells that are found after formation of metastasis, which they consider as the most invasive ones. However, these observations have not been validated by other laboratories and the mRNA levels coding for HP1α, β or γ were not associated with the occurrence of bone marrow metastasis in 37 breast cancer patients (Abreu et al, 2008). Moderate overexpression of exogenous HP1α and β reduced the tumorigenicity of human embryonic kidney cells when used in a xenograft mouse model (Sharma et al, 2003). Finally, intense HP1γ staining was observed in 26 human tumor samples (Takanashi et al, 2009), without any comparison to corresponding normal tissues nevertheless. In conclusion, thorough analysis of how levels and dynamics of the three HP1 isoforms are altered in different human tumors and how this contributes to the process of tumorigenesis remains unclear.

This has led me to one of the main questions that I have addressed during my PhD: how is the expression of the three human HP1 isoforms regulated in the context of cell proliferation and (breast) tumorigenesis? Is the expression of one or several isoforms cell cycle dependent or altered in cancer cells? What could be the clinical impact of such altered expression and to which function of HP1 could it be related? I will discuss this question, and the results we obtained, in more detail in the results (section II).

3. The CAF-1 complex: at the crossroad between replication, repair and heterochromatin

In the previous chapters, the CAF-1 complex has been mentioned mainly in the context of heterochromatin maintenance through its interaction with HP1. However, CAF-1 was initially discovered for its role in chromatin assembly coupled to DNA synthesis (Smith & Stillman, 1989). Indeed, it was known that, while cytosolic cell extracts are proficient for the replication of viral SV40 DNA, the addition of nuclear cell extracts is required to induce replication-coupled chromatin assembly (reviewed in Stillman, 1986). Fractionation of these nuclear extracts led to the identification of the CAF-1 complex as the single crucial component for replication-coupled chromatin assembly (Smith & Stillman, 1989). Here, I will first discuss the conservation and the structural features of the CAF-1 complex. Next, I will address in more detail the different cellular functions of the CAF-1 complex.

3.1 The CAF-1 complex: structure and conservation

CAF-1 is a complex composed of three subunits, which have been named according to their molecular weight: p150, p60 and p48 in human cells (Smith & Stillman, 1991b). Since their characterization, CAF-1 complexes have been identified in many different organisms, from *S.cerevisiae* to human (Table V). Each of the three subunits shows conserved structural characteristics among species, as shown in Figure 22.

Table V: subunits of the CAF-1 complex in different species

Species	Large subunit	Intermediate subunit	Small subunit	Key reference
H. sapiens	p150	p60	p48/RbAp48	(Smith & Stillman, 1989)
M. musculus	p150	p60	p48	(Murzina et al, 1999)
X. laevis	p150	p60	p48	(Quivy et al, 2001)
D. melanogaster	p180	p105/p75*	p55	(Kamakaka et al, 1996)
A. thaliana	Fas1	Fas2	MSI1	(Kaya et al, 2001)

S. pombe	Pcf1	Pcf2	Pcf3	(Dohke et al, 2008)
S. cerevisiae	Rlf2/Cac1	Cac2	Msi1/Cac3	(Kaufman et al, 1997)
**In Drosophila, the 75-kDa polypeptide of dCAF-1 is a C-terminally truncated form of p105.*				

3.1.1 CAF-1 p150

The largest subunit, p150 in mammals, contains large regions of acidic residues (including the KER and ED domains) (Kaufman et al, 1995b), which are thought to facilitate its interaction with basic histone proteins. The interaction with p60 has been mapped to the C-terminal region of p150 (Kaufman et al, 1995b). In line with the formation of a complex involved in histone deposition, the region that is crucial for the nucleosome assembly activity of p150 overlaps the binding sites for both p60 and histones (Kaufman et al, 1995b). In addition, two binding sites have been identified for the polymerase accessory factor PCNA (Krawitz et al, 2002; Moggs et al, 2000), which permit the recruitment of CAF-1 to sites of DNA synthesis (Shibahara & Stillman, 1999). It is interesting to note that p150 interacts with the HP1 proteins through a Mod1-Interacting Region (MIR) that is located outside of the region required for chromatin assembly (Murzina et al, 1999), pointing towards a function in heterochromatin maintenance that is separated from chromatin assembly (see also chapter 2.3.2) (Quivy et al, 2008; Quivy et al, 2004). Furthermore, the p150 subunit can dimerize through a region that is juxtaposed to the domain required for the binding to p60 (Quivy et al, 2001). Finally, p150 contains a so-called PEST domain, a region that is characteristic of proteins with a short half life (Rogers et al, 1986).

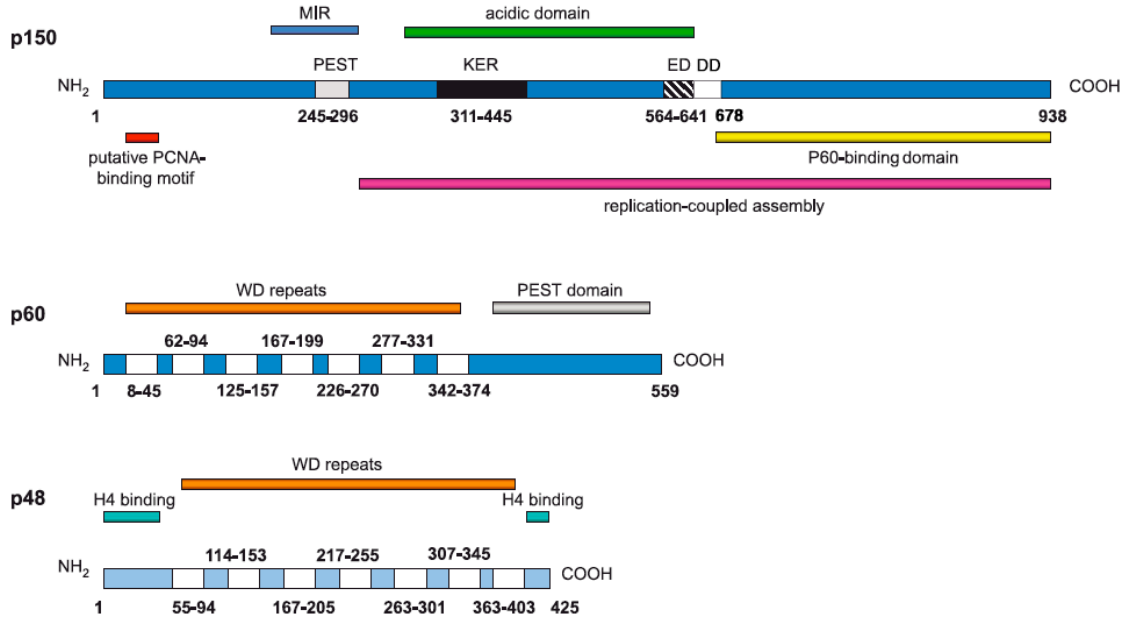

Figure 22: Conserved domains within the subunits of CAF-1. Depicted are the human proteins.

Abbreviations: MIR: MOD1-interacting region; DD: dimerization domain; PEST: proline (P), glutamic acid (E), serine (S) and threonine (T)-rich domain; KER: lysine (K), glutamic acid (E) and arginine (R)-rich domain; ED: glutamic acid (E) and aspartic acid (D)-rich domain; WD: tryptophan (W) and aspartate (D)-rich repeats; DD: Dimerization domain. Image from Ridgway & Almouzni, 2000.

3.1.2 CAF-1 p60

The intermediate subunit, p60 in mammals, is characterized by the presence of seven tryptophan-aspartate (WD) repeats (Kaufman et al, 1995b), which are generally implicated in protein-protein interactions. Like p150, the p60 subunit contains a PEST domain, suggesting a potential co-regulation of protein stability. Indeed, the two subunits show a similar regulation in protein levels throughout the cell cycle and in quiescence (Polo et al, 2004).

3.1.3 CAF-1 p48

The smallest subunit, p48, is not strictly specific for the CAF-1 complex and can be found in several histone-remodeling and histone-modifying enzymatic complexes, such as the ISWI and Mi-2/CHD chromatin-remodeling families and the histone acetyl transferase HAT1 (reviewed in Ridgway & Almouzni, 2000 and De Koning et al, 2007). Indeed, only a small fraction of available human p48 coprecipitates with the two other CAF-1 subunits (Verreault et al, 1996). Like the p60 subunit, p48 contains seven WD repeats, which can serve for the binding of its partners (Verreault et al, 1996). Indeed, p48 binds to histone H4 through a conserved binding pocket that mainly consists of the β-propeller structure formed by the WD repeats (Song et al, 2008). Within the HAT1 complex, this binding pocket of p48 is crucial for the histone acetyl transferase activity, suggesting that p48 could provide H4 binding capacity to HAT1. The contribution of the p48 subunit to the function of the CAF-1 complex remains largely unknown, but it could provide additional histone binding capacity or cross talk between multiple chromatin-associated complexes.

3.2 Functions of the CAF-1 complex

To gain insight into the functions of the CAF-1 complex, depletion and deletion approaches have been undertaken in multiple organisms (Table VI). The phenotypes resulting from these approaches show the importance of CAF-1 for cell proliferation, gene silencing and, at least in *S.cerevisiae* and *Drosophila*, recovery from DNA damage. These loss of function studies have paved the road for further cellular and biochemical analyses. Together, these have allowed the clarification of the roles of CAF-1 in these processes, which are depicted in figure 23 and will be discussed in more detail below.

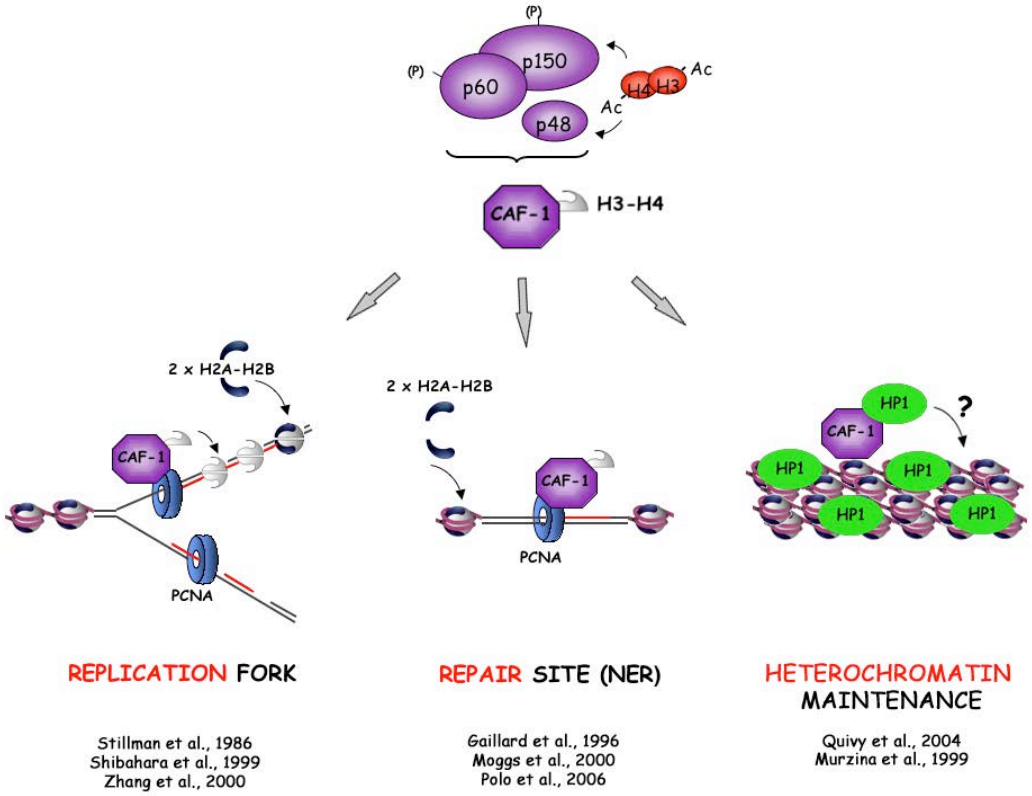

Figure 23: Cellular functions of the CAF-1 protein complex. CAF-1 is composed of three subunits: p150, p60 (Kaufman et al, 1995a) and p48 (Verreault et al, 1996) (upper panel). p150 and p60 can be phosphorylated (Smith & Stillman, 1991a), and p150 and p48 interact with acetylated newly synthesized histones H3 and H4 (Kaufman et al, 1995a). The complex is implicated in chromatin assembly during replication (left panel) and during repair (middle panel), both through its interaction with Proliferating Cell Nuclear Antigen (PCNA) (Shibahara & Stillman, 1999). Furthermore, CAF-1 has a less well-understood function in heterochromatin maintenance (right panel), possibly through maintenance and/or deposition of Heterochromatin Protein 1 (HP1) on these chromatin regions. Image adapted from Mello & Almouzni, 2001.

3.2.1 CAF-1 functions during DNA replication

CAF-1 was initially discovered for its role in chromatin assembly coupled to DNA replication (Smith & Stillman, 1989). The molecular link between CAF-1 and DNA replication was provided by the finding that CAF-1 p150 directly interacts with the polymerase accessory factor PCNA (Moggs et al, 2000; Shibahara & Stillman, 1999). CAF-1 indeed colocalizes with PCNA at sites of DNA synthesis (Krude, 1995; Shibahara & Stillman, 1999). In addition, depletion of PCNA from cell extracts abolished CAF-1 chromatin assembly activity coupled to DNA synthesis, both in the context of DNA replication (Shibahara & Stillman, 1999) and DNA repair (Moggs et al, 2000). The interaction of p150 with PCNA can be cell cycle regulated. The S-phase kinase Cdc7-Dbf4 phosphorylates p150, which abolishes p150 dimerization and promotes its interaction with PCNA (Gerard et al, 2006a). Thus, the monomerization of p150 at the onset of S-phase can facilitate its recruitment. The p150 dimerization on the other hand could be important in later steps of the reaction. Indeed, a dominant negative form of p150 that cannot dimerize impairs *Xenopus* development and is unable to promote chromatin assembly *in vitro* (Quivy et al, 2001). In conclusion, continuous switching between monomeric to dimeric states of p150 might be required to regulate histone supply and deposition during DNA replication.

Interestingly, both in *S.cerevisiae* and *S.pombe*, the inactivation of the three CAF-1 subunits does not impair cell viability or proliferation under normal conditions (Table VI). In multicellular organisms, DNA replication is affected only when the p150 subunit is depleted, while depletion of p60 does not notably affect cell proliferation. This suggests that the CAF-1 function in nucleosome assembly is not required *per se* for DNA replication and cell proliferation. This is exemplified in mouse cells, where the role of the p150 subunit in replication is distinct from its involvement in histone deposition and has been linked to the propagation of pericentric heterochromatin. Indeed, CAF-1 p150 orchestrates both SetDB1-dependent H3K9 methylation and HP1 dynamics during the replication of these regions (Loyola et al, 2009; Quivy et al, 2008; Quivy et al, 2004), as described in detail in chapter 2.3.2. This role of CAF-1 p150 seems to be highly conserved, since in all organisms the depletion or mutation of at least the p150 homologue gives rise to defects in heterochromatic silencing or organization (Table VI).

Thus, the p150 subunit might coordinate HP1 dynamics and replication fork progression, independently from the other two subunits.

Table VI: Phenotypes of CAF-1 loss of function

Species	Approach	Phenotype	Key references
S.cerevisiae	Deletion of Cac1, Cac2 & Cac3	- Silencing defects (impaired recruitment of Sir silencing proteins) - Sensitivity to UV damage	(Kaufman et al, 1997)
S.pombe	Deletion of Pcf1 & Pcf2	- Silencing defects (loss of Swi6 recruitment)	(Dohke et al, 2008)
A.thaliana	Mutation of Fas1 & Fas2	- Misorganization of apical meristems - Misregulation of gene expression - Defects in heterochromatin content and silencing - Increased homologous recombination	(Kaya et al, 2001) (Ono et al, 2006) (Schonrock et al, 2006) (Kirik et al, 2006) (Endo et al, 2006)
D.melanogaster	Mutation of p180	- Larval lethality - Heterochromatic gene silencing defects - Sensitivity to γ-irradiation - Reduced double strand break repair - Replication defects in endocycling cells	(Song et al, 2007) (Klapholz et al, 2009)
X.laevis	Dominant negative p150	- Arrest in early development	(Quivy et al, 2001)
M.musculus	RNAi p150 RNAi p60 KO mice p150	- Arrest in early S-phase - Impaired HP1 maintenance during heterochromatin replication - Deficient nucleosomal organization of replicated DNA - Impaired replication of (peri-)centromeric DNA - Slightly delayed cell cycle progression - Deficient nucleosomal organization of replicated DNA - Embryonic lethal - Misorganization of pericentric heterochromatin	(Quivy et al, 2004) (Quivy et al, 2008) (Houlard et al, 2006)
H.sapiens	Dominant negative p150 RNAi p60	- Arrest in early S-phase - Slightly delayed cell cycle progression	(Hoek & Stillman, 2003; Ye et al, 2003) (Polo et al, 2006)

3.2.1.1 CAF-1 as a marker for cell proliferation of interest for human oncology

In line with a major role of the CAF-1 complex during DNA replication, CAF-1 p150 and p60 expression levels are massively downregulated in non-proliferating, quiescent cells (Polo et al, 2004). The p48 subunit does not exhibit any detectable

change in expression. Upon exit from quiescence, CAF-1 p150 and p60 subunits are upregulated just before the onset of DNA replication (Polo et al, 2004). Since aberrant cell proliferation is a major characteristic of cancer cells, CAF-1 expression was further analyzed in human cancer samples. The detection of the CAF-1 p60 protein by immunostaining in breast cancer tissue samples correlates with the grade, the size and the proliferation index of the tumors (Polo et al, 2004) (Figure 24). These and additional observations led to the proposition that CAF-1 p60 could be used as a proliferation marker in breast cancer. These observations have been validated in tongue and prostate cancer (Staibano et al, 2009; Staibano et al, 2007). However, no data is available yet to validate whether CAF-1 p60 levels can predict the outcome of breast cancer, or if it would be a better prognostic marker than Ki67, the most widely used proliferation marker for clinical purposes. In addition, whether p150 expression parallels p60 expression in human breast cancer samples remains to be explored.

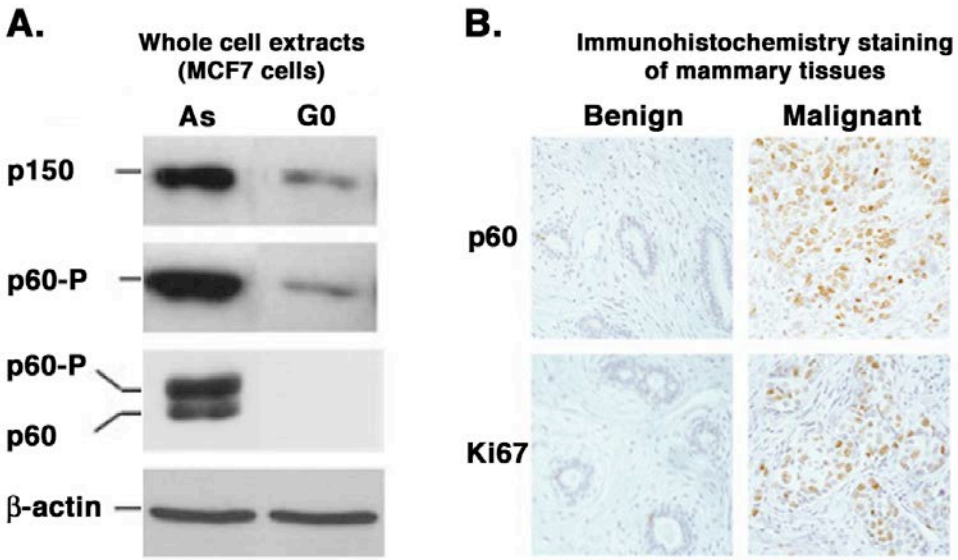

Figure 24: CAF-1 levels are downregulated in quiescence and upregulated in breast cancer cells. A. Levels of CAF-1 p150, p60 and phosphorylated p60 (p60-P) in whole cell extracts from asynchronously growing (As) or quiescent (G0) MCF7 cells. β-actin is used as a loading control. **B.** Immunostaining for CAF-1 p60 and the proliferation marker Ki67 in benign and malignant breast tumors. Image adapted from Polo et al, 2004.

3.2.2 CAF-1 function in DNA repair

The human genome is constantly exposed to endogenous and exogenous sources of genotoxic stress. To repair the induced DNA damage, a wide variety of repair machineries has evolved (Hoeijmakers, 2001). Many of these repair mechanisms operate through the removal of the stretch of damaged DNA and subsequent DNA re-synthesis (gap filling). As will be discussed in detail below, CAF-1 is recruited to these sites of DNA synthesis, through its interaction with PCNA, and promotes DNA synthesis-dependent histone deposition (Gaillard et al, 1996; Green & Almouzni, 2003b; Martini et al, 1998; Moggs et al, 2000; Polo et al, 2006). In line with a role for CAF-1 in DNA repair, *S.cerevisiae* and *Drosophila* show an increased sensitivity to DNA damage upon loss of function of CAF-1 (Kaufman et al, 1997; Song et al, 2007). While *Drosophila* shows reduced repair of DNA double strand breaks, *S.cerevisiae* is particularly sensitive to ultra-violet (UV) irradiation. The role of CAF-1 has been studied mostly in the context of the repair of UV-induced lesions. Therefore, I will first introduce the nature of these lesions and their repair by the Nucleotide Excision Repair (NER) pathway. Then, the role of CAF-1 in DNA repair will be discussed.

3.2.2.1 UV-induced lesions

Several types of UV irradiation can be distinguished based on their wavelength and their absorption by DNA. UV-A consists of longer wavelengths (400 nm–320 nm) that penetrate deeply in the skin and mostly generate reactive chemical intermediates, which in turn can cause DNA damage. Direct DNA damage is predominantly induced by the shorter wavelengths of UV-B (320 nm–280 nm) and UV-C (280 nm–100 nm). Although UV-C is absorbed by the ozone layer and does not reach earth, research laboratories widely use UV-C irradiation for its highly efficient induction of DNA lesions. In addition to some oxidative stress, UV-B and UV-C mainly create covalent bonds between two adjacent thymine or cytosine bases on the same DNA strand, thus creating a pyrimidine dimer. Two types of covalent bonds can be distinguished, giving rise to either Cyclobutane Pyrimidine Dimers (CPDs) or 6,4 pyrimidine-pyrimidone adducts (6,4-PPs) (Figure 25).

Interestingly, the two types of lesions do not have the same consequences for the cell and are not dealt with in the same way. First, 6,4-PP lesions are almost 4 times less abundant than CPDs in irradiated human cells (Suquet et al, 1995). Furthermore, 6,4-PP lesions induce a stronger local distortion of the DNA molecule. They induce a bending of 44°, against only 9° for a CPD lesion (Kim & Choi, 1995). Several factors involved in NER preferentially bind to distorted DNA (Batty & Wood, 2000), which could explain why 6,4-PP lesions are repaired much faster than CPDs, even when different UV doses are applied in order to start with similar amounts of each lesion (Suquet et al, 1995). Concerning their localization within chromatin, CPDs can be found both in nucleosomal DNA and linker DNA, while 6,4-PP lesions almost exclusively occur in nuclease-sensitive linker DNA (Gale & Smerdon, 1990; Mitchell et al, 1990; Suquet et al, 1995). The sterical conformation of DNA within a nucleosome is probably incompatible with the formation of a 6,4-PP lesion.

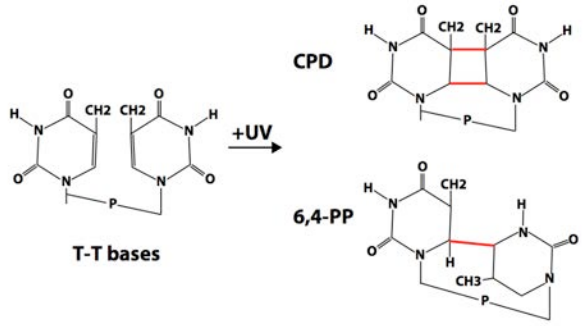

Figure 25: Structures of CPD and 6,4-PP lesions. Formation of Cyclobutane Pyrimidine Dimers (CPDs) or 6,4 pyrimidine-pyrimidone adducts (6-4 PPs) between two adjacent pyrimidines (here: two thymines). The created covalent bond is indicated in red.

3.2.2.2 The Nucleotide Excision Repair pathway

Two distinct sub-pathways exist within the NER pathway: the global genome repair (GGR) and the transcription-coupled genome repair (TCR) (reviewed in Costa et al, 2003 and Fousteri & Mullenders, 2008). TCR is initiated by RNA polymerase II encountering a lesion and thus removes lesions mainly in transcribed strands. The two pathways only differ in their way of detecting the lesion and subsequently use the same factors for DNA unwinding, lesion excision and DNA synthesis (Figure 26). The important DNA distortion induced by 6,4-PPs makes these lesions more amenable to be repaired by the global genome repair pathway, while CPD lesions rely more on transcription-coupled detection. TCR might thus help to minimize the toxic effects of

CPD lesions in actively transcribed essential genes (Fousteri & Mullenders, 2008). Indeed, CPD lesions contribute more significantly than 6,4-PPs to cell death after exposure to UV irradiation (de Lima-Bessa et al, 2008).

Our understanding of the NER pathway and the role of each of its components has greatly benefited from the combined use of *in vitro* reconstituted systems (Aboussekhra et al, 1995) and *in vivo* cellular model systems. These latter include the study of cells from patients that are deficient for one of the components. Indeed, mutations in genes coding for specific components of the NER pathway leads to Xeroderma pigmentosum (XP), Cockayne syndrome (CS) or trichothiodystrophy (TTD) syndromes, dependent on which factor is affected (reviewed in Cleaver, 2005 and Lehmann, 2003). XP patients show a defect in one of the factors involved in GGR and are characterized by a high photosensitivity and the development of skin cancers. CS patients on the other hand are specifically affected in TCR and do not display increased photosensitivity, but instead suffer from developmental and neurological symptoms. TTD patients display deficient helicase activity of the general transcription factor IIH (TFIIH), which is also involved in transcription, and show a wide variety of symptoms including growth retardation, neurological abnormalities and brittle hair and nails. Exposure of cells derived from these different patients to UV irradiation has allowed the dissection of the kinetics of the NER pathways.

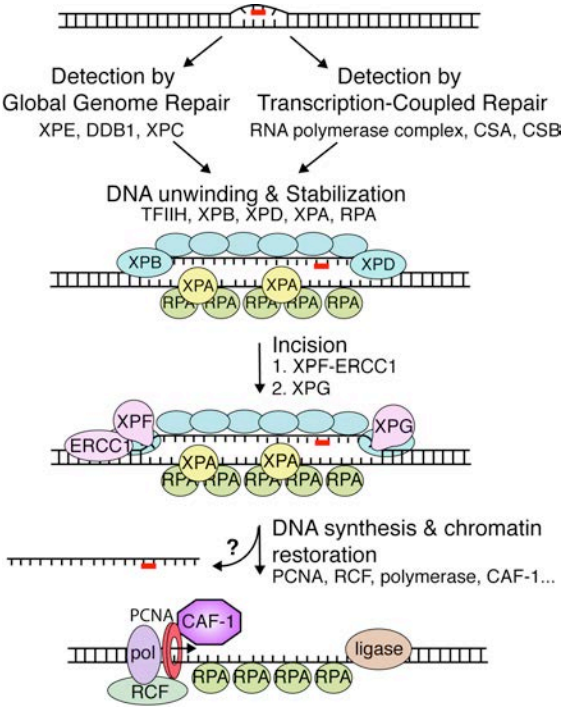

Figure 26: Schematical representation of the Nucleotide Excision Repair (NER) pathway. Lesions can be detected either in a transcription-coupled manner or by global genome surveying mechanisms. Detection is followed by DNA unwinding and stabilization of the single stranded DNA. A total length of about 30 nucleotides is excised, followed by DNA synthesis and chromatin restoration. Whether the damaged strand is released before or after completion of gap filling is not clear. Image inspired by Fousteri & Mullenders, 2008.

3.2.2.3 CAF-1 and repair of UV lesions in a chromatin context

The repair of DNA lesions does not occur on naked DNA, but should be considered in the context of chromatin organization. *In vitro* studies showed that the efficiency of DNA repair is reduced up to 10-fold when the DNA is assembled into nucleosomes, compared to repair on naked DNA (Araki et al, 2000; Hara et al, 2000; Liu & Smerdon, 2000; Sugasawa et al, 1993; Wang et al, 1991), and this even when the lesion is localized in linker DNA (Ura et al, 2001). Furthermore, *in vivo*, heterochromatic regions seem to be refractory to DNA repair at least in the case of double strand breaks (Cowell et al, 2007; Goodarzi et al, 2008; Kim et al, 2007). Thus, for efficient repair to occur, factors permitting access to the damaged DNA, including chromatin remodeling and modifying factors, are thought to be required (reviewed in Gong et al, 2005 and Gontijo et al, 2003). This in turn implies that after repair has occurred, the chromatin organization needs to be restored in order to maintain genome function and integrity.

The role of CAF-1 in chromatin assembly coupled to DNA repair was first demonstrated *in vitro* using UV-irradiated plasmids and extracts from human cells or *Xenopus* eggs (Gaillard et al, 1996). These approaches showed that CAF-1 dependent nucleosome assembly on the plasmid occurs in a manner coupled to the DNA synthesis step of DNA repair. The recruitment of CAF-1 to damaged DNA is dependent on the presence of the DNA lesions and PCNA (Moggs et al, 2000). In addition, the histone chaperones Asf1a and b, which interact directly with the p60 subunit of CAF-1, promote CAF-1-dependent histone deposition coupled to DNA repair (Mello et al, 2002). Since Asf1 proteins are not recruited to damaged DNA, they were proposed to act as histone donors for CAF-1. Asf1 and CAF-1 thus seem to form an assembly network of histone chaperones to regulate the supply of histones to required sites of incorporation (De Koning et al, 2007; Loyola & Almouzni, 2004; Polo & Almouzni, 2006).

These *in vitro* approaches have paved the way for exploring the role of CAF-1 in DNA repair *in vivo* using cellular model systems. First, global UV irradiation markedly increases the fraction of CAF-1 p150 and p60 associated with chromatin in human HeLa cells (Martini et al, 1998). The fraction of p60 that is recruited after UV irradiation is highly phosphorylated, which seems to stimulate nucleosome formation (Martini et al, 1998). Next, the development of a method to induce UV damage only in a small fraction of the nucleus, by irradiation through a filter with pores of a few μm in diameter (Volker et al, 2001), allowed to follow the recruitment of CAF-1 and PCNA to the site of UV lesions and showed a local rather than a global CAF-1 response (Green & Almouzni, 2003b) (Figure 27). Applying this method to cells derived from XP patients showed that CAF-1 is not recruited if factors involved in the early stages of the NER pathway (XPC, XPA) are absent. In addition, both endonucleases, XPF and XPG, need to be present in order to recruit PCNA and CAF-1 (Green & Almouzni, 2003b; Miura et al, 1996; Mocquet et al, 2008). More recently, Staresincic and colleagues showed that CAF-1 can be recruited if XPF has cut at the 5' end of the lesion and if XPG is present but unable to incise the DNA (Staresincic et al, 2009). Thus, the DNA lesion has to be recognized, both endonucleases have to be present and at least one side of the lesion should be incised for PCNA and CAF-1 recruitment.

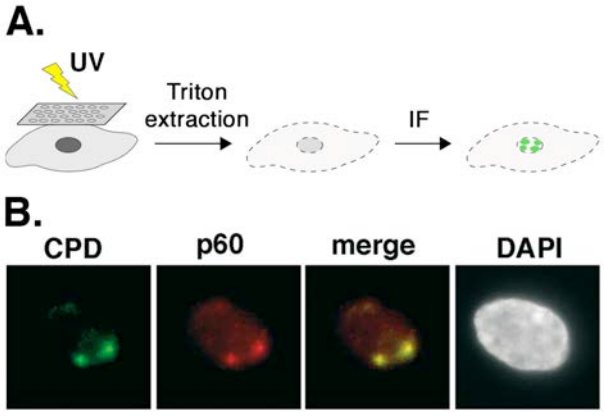

Figure 27: Local UV irradiation and CAF-1 recruitment. A. Scheme of the experimental approach. Cells are irradiated with UV-C through a filter and extracted with Triton X-100 to remove soluble protein fractions. Immunofluorescence (IF) reveals the NER patches. **B.** Recruitment of CAF-1 p60 to local sites of UV damage, revealed by staining for CPD lesions, in human cells. Image adapted from Polo et al, 2006.

Importantly, CAF-1 is dispensable for the removal of DNA lesions in both yeast and human cells (Game & Kaufman, 1999; Kim & Haber, 2009; Polo et al, 2006). In yeast, the absence of both CAF-1 and Asf1 does not affect the repair process of double strand breaks but impairs recovery from the cell cycle checkpoint that is induced by the DNA damage, resulting in cell death (Kim & Haber, 2009). In human cells, the recruitment of

CAF-1 is crucial for the local deposition of newly synthesized tagged histone H3.1 at sites of NER in human cells (Polo et al, 2006). These observations demonstrate the occurrence of a chromatin restoration step that involves the incorporation of histones, of which at least a fraction comes from a newly synthesized pool and not from recycled (evicted) histones. Interestingly, the presence of ubiquitinated H2A at sites of NER also requires CAF-1 (Zhu et al, 2009), suggesting that the CAF-1 dependent incorporation of H3.1 and H4 might allow the subsequent deposition of modified H2A and H2B.

Taken together, these findings show a role for CAF-1 in the later steps of the NER pathway. Since the complex is not required for the removal of DNA lesions, CAF-1 is thought to play a role in the restoration of chromatin organization after DNA repair. This places CAF-1 in the last step of the "access-repair-restore" model that was put forward to explain how DNA repair can take place in the context of chromatin organization (Figure 28) (Green & Almouzni, 2002; Smerdon, 1991). To date, CAF-1 is the only factor that has been convincingly implicated in this step. CAF-1 has also been implicated in the repair of single strand breaks and double strand breaks (Kim & Haber, 2009; Lewis et al, 2005; Linger & Tyler, 2005; Nabatiyan et al, 2006; Okano et al, 2003; Polo et al, 2006), suggesting that its function in the restoration of chromatin organization is not restricted to NER, but is a general requirement upon DNA repair in a chromatin context.

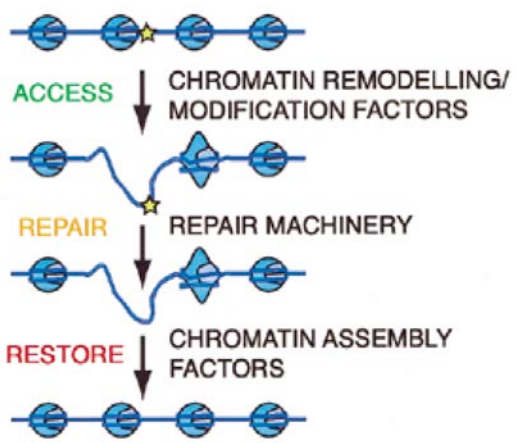

Figure 28: The Access-Repair-Restore model. The organization of DNA into chromatin requires that chromatin remodeling and modifying factors render the DNA lesion accessible in order for DNA repair to occur. Subsequently, chromatin organization should be restored to the initial status. The Access-Repair-Restore model was initially proposed in the context of NER, but probably also applies to other types of DNA damage. Image from Green & Almouzni, 2002.

It must be noted that the role of CAF-1 during DNA repair has been studied exclusively in proliferating cells. Yet, quiescent cells, which constitute the main fraction of cells in an adult human body, are exposed to genotoxic stresses like proliferating cells and also need to preserve both DNA integrity and chromatin organization to maintain cellular identity. In addition, quiescent cells cannot rely on any known cell cycle

checkpoint to survey genome and chromatin integrity. This raises the intriguing question of how non-proliferating cells, which express very low levels of p150 and p60 (Polo et al, 2004), deal with chromatin assembly coupled to DNA repair. In addition, the expression of the histone variant H3.1, which specifically associates with CAF-1 (Tagami et al, 2004), is also cell cycle-dependent and low during quiescence (Polo & Almouzni, 2006). Thus, whether chromatin assembly coupled to DNA repair can occur efficiently in quiescent cells, and which factors are involved, remains an open issue. This question brings me to my second main PhD project, in which I have compared the chromatin dynamics coupled to DNA repair in proliferative and quiescent cells.

II. RESULTS

Key Questions and Approaches: an overview

During my PhD, my interest has been focused on chromatin-related mechanisms underlying cell proliferation and tumorigenesis. For this, I particularly focused my attention on two chromatin-related factors, discussed in detail in the introduction: CAF-1 and HP1.

Our laboratory has previously shown that the expression of CAF-1 p150 and p60 is dependent on cell proliferation and constitutes a proliferation marker of clinical value in breast cancer (Polo et al, 2004; Quivy et al, 2008). However, in cell line models, the chromatin assembly capacity of CAF-1 is not essential for cell proliferation (Polo et al, 2006). Intriguingly, only the interaction of CAF-1 p150 with the HP1 proteins is required for DNA replication and thus for cell survival (Quivy et al, 2008; Quivy et al, 2004). This tight connection between CAF-1 and HP1 proteins in proliferative cells suggested a potential role for HP1 proteins in tumorigenesis. The involvement of HP1 proteins in crucial cellular processes, such as gene silencing and chromosome segregation (see introduction), and their interaction with the tumor suppressor protein Rb (Nielsen et al, 2001b; Williams & Grafi, 2000), are in line with this hypothesis. In a first study, we have therefore addressed how the three human HP1 isoforms relate to cell proliferation and tumorigenesis. For this, we have used tumoral and primary human cell lines, siRNA approaches and a selection of tumoral human patient samples, which were chosen and analyzed in collaboration with the Institut Curie hospital and the department of biostatistics. These data gave rise to publication #1: DE KONING Leanne, SAVIGNONI Alexia, BOUMENDIL Charlène, REHMAN Haniya, ASSELAIN Bernard, SASTRE-GARAU Xavier, ALMOUZNI Geneviève, *Heterochromatin Protein 1 alpha: a hallmark of cell proliferation relevant in clinical oncology*, EMBO Molecular Medecine, Vol 1 (issue 3), p 178-191, 2009.

Using the same series of breast cancer patient samples, we have analyzed, in addition to HP1, the expression of other factors involved in chromatin organization. We focused on the histone chaperones CAF-1 p60, CAF-1 p150, Asf1a and Asf1b. Since CAF-1 p60 is a known proliferation marker in breast cancer (Polo et al, 2004), we aimed to

determine if CAF-1 p150 behaves similarly. In addition, unpublished data from the laboratory, concerning Asf1a and b, suggested that the two isoforms might not fulfill identical functions. Therefore, we analyzed how the two Asf1 isoforms are regulated in relation to cell proliferation and tumorigenesis and compared their expression to the expression of CAF-1 and HP1. This work gave rise to manuscript #2 that is currently in preparation: CORPET Armelle, DE KONING Leanne, TOEDLING Joern, SERVANT Nicolas, SAVIGNONI Alexia, BOUMENDIL Charlène, BARILLOT Emmanuel, ASSELAIN Bernard, SASTRE-GARAU Xavier and ALMOUZNI Geneviève, *Distinct functions for the two Asf1 isoforms in relation to proliferation*, in prep.

Next, I have addressed in more detail the role of CAF-1 in chromatin dynamics coupled to DNA repair. The efficiency by which cells are able to repair DNA lesions largely determines the clinical response to anti-cancer treatments such as chemotherapy and radiotherapy, both in terms of tumor reduction and toxicity to healthy cells (Helleday et al, 2008). A differential capacity between proliferative tumoral cells and quiescent healthy cells in term of their capacity to repair DNA and to restore chromatin organization could therefore affect cancer treatment. Until now, the role of CAF-1 in chromatin dynamics coupled to DNA repair has been studied exclusively in proliferative cells. This raises the question of how quiescent cells, which express very low levels of CAF-1 p150 and p60 (Polo et al, 2004), deal with chromatin assembly coupled to DNA repair. To address these issues, chromatin dynamics coupled to DNA repair in quiescent human cells has been studied. As a model for DNA damage, I applied UV irradiation, as the role of CAF-1 during UV repair in proliferative cells is particularly well studied (see introduction). This work gave rise to manuscript #3 that is currently in preparation: Leanne DE KONING, Sophie E. POLO, Danièle ROCHE and Geneviève ALMOUZNI, *Quiescent human cells show distinct chromatin restoration kinetics upon UV damage*, in prep.

Finally, I will present preliminary but intriguing observations concerning a potential role of CAF-1 p60 in the initiation of the tumorigenesis process. Indeed, CAF-1 p60 is not essential for cell survival in proliferative cells (Polo et al, 2006; Quivy et al, 2008), where its expression levels are constantly high (Polo et al, 2004). However, rapid neosynthesis of CAF-1 p60 occurs upon exit from the quiescent state (Polo et al, 2004).

Using siRNA approaches, we addressed if this upregulation is crucial for cell cycle entry and thus initiation of cell proliferation and tumorigenesis.

Each of these four projects, all closely related to cell proliferation and tumorigenesis, and the obtained results, will be presented and summarized in the following chapters.

Publication #1:

Heterochromatin Protein 1 alpha: a hallmark of cell proliferation relevant in clinical oncology

DE KONING Leanne, SAVIGNONI Alexia, BOUMENDIL Charlène, REHMAN Haniya, ASSELAIN Bernard, SASTRE-GARAU Xavier, ALMOUZNI Geneviève

EMBO Molecular Medecine, Vol 1 (issue 3), p 178-191, 2009

In this manuscript, we address how the three human isoforms of HP1 relate to cell proliferation and tumorigenesis. As discussed in the introduction, the three HP1 isoforms are highly similar in sequence and in protein structure. Their functional differences are poorly understood and experimental findings concerning one isoform are often generalized and considered as applicable to all three isoforms. Yet, the three proteins show different nuclear distribution patterns. While the nuclear staining of HP1α is enriched at constitutive pericentric heterochromatin, HP1γ shows less preferential binding to these regions and would be more often associated with facultative heterochromatin and gene silencing (Gilbert et al, 2003; Minc et al, 1999; Nielsen et al, 2001a). Furthermore, the three isoforms can interact with different binding partners (Kwon & Workman, 2008; Quivy et al, 2004). Finally, distinct posttranslational modifications have been identified on individual HP1 isoforms (Lomberk et al, 2006a; Minc et al, 1999).

The three isoforms are encoded by different genes, under control of different promoters, and a differential regulation of expression may further diversify their

functions. However, little is known about how the HP1 proteins are regulated. We addressed this issue in relation to cell proliferation and tumorigenesis, with a major focus on breast cancer. As discussed in the introduction, the clinical and genetic heterogeneity of breast cancer suggests that alterations in chromatin organization may play an important role in this type of cancer. In addition, the Institut Curie is specialized in breast cancer treatment and patient sample collections. However, the heterogenic nature of breast cancers renders its study more complex and, therefore, appropriate cellular models and adequate selections of patient samples are required. We used a selection of primary and tumoral (mammary) cell lines and tissue samples, when possible derived from the same patient, to study the expression patterns of HP1 isoforms. Our major results are summarized below. More detailed information, concerning methodology or obtained results, can be found in the corresponding publication (De Koning et al., Heterochromatin Protein 1 alpha: a hallmark of cell proliferation relevant in clinical oncology, EMBO Mol Med, Vol 1 (issue 3), p 178-191, 2009).

Results

1. Expression of HP1α, but not β or γ, is dependent on cell proliferation

First, we analyzed how the three HP1 isoforms are regulated during the cell cycle and within quiescence. Using both primary cells, which can be arrested in quiescence by serum starvation, and mammary carcinoma cells, which can be arrested by anti-estrogens, we show that the protein and mRNA levels of HP1α, but not HP1β or γ, are downregulated in quiescent cells. In contrast to CAF-1, HP1α expression is induced before the onset of DNA replication upon release from quiescence. In continuously proliferating cells, HP1α protein and mRNA levels do not show important variations among the different phases of the cell cycle. Thus, the expression of HP1α, but not HP1β or γ, is dependent on cell proliferation status, being specifically downregulated in quiescent cells.

2. *HP1α is overexpressed in human cancer cell lines*

Next, we addressed the expression levels in human cancer. As a first approach, we used tumoral and a healthy mammary cell lines, which have been derived from the same patient (Hackett et al, 1977). We studied both the total protein pool and the

chromatin-bound fraction of the three HP1 isoforms. We show that HP1α is overexpressed and highly chromatin-bound in the tumor cells compared to the healthy cells, while HP1β or γ show little differences. In addition, colocalization studies by immunofluorescence show an increased concentration of HP1α in foci in the tumor cells. These foci colocalize at least partially with centromeric regions and are not cell cycle dependent. Thus, a large fraction of the overexpressed HP1α seems to be recruited to pericentric heterochromatin.

3. HP1α downregulation results in mitotic defects

To study the specific function of HP1α related to tumor cell proliferation, we developed a siRNA approach to specifically downregulate each HP1 isoform. Our results show that downregulation of HP1α, but not HP1β or γ, results in mitotic defects in cancer cells. In primary cells, which have a normal cell cycle and checkpoint activity, HP1α depletion leads to a slightly delayed chromosome segregation. These observations suggest a specific function for HP1α in facilitating mitosis, which could be of particular importance in a cancer context.

4. HP1α is overexpressed in human cancer samples

To address whether our cell line models reflect the *in vivo* situation, we analyzed the expression of the three HP1 isoforms in human cancer samples. Using available transcriptome data as a first approach, we concluded that HP1α mRNA is overexpressed in multiple tumor samples compared to healthy tissue, while HP1β and γ show little differences. Next, we studied HP1α protein levels by immunohistochemistry on tumoral and corresponding healthy frozen tissues. Again, HP1α, but not HP1β or γ, was overexpressed in the tumoral tissues compared to the healthy tissues. Finally, we addressed whether the expression of HP1α was related to disease outcome. For this, we analyzed HP1α mRNA levels in an annotated selection of early stage breast cancers for which a patient follow-up of > 10 years was available. We show that the expression levels of HP1α are predictive for the overall survival of the patients, the formation of metastasis and the progression of the disease. Thus, HP1α is a prognostic factor in early breast cancers. In addition, multivariate analyses, which compare the combined value of individual prognostic factors, show that HP1α better predicts overall survival in this

series of patient samples than classical factors like tumor grade and size, age, mitotic index, etc. Although these conclusions remain to be confirmed in a second, independent set of patient samples (ongoing), they underline the strength of HP1α as a prognostic marker. These observations have given rise to the deposition of a patent, entitled "HP1alpha as a prognostic marker in human cancer" (appendix 2).

We thus conclude that HP1α, but not HP1β or γ, is expressed in a proliferation dependent manner and overexpressed in human cancer. Increased HP1α expression might confer a growth advantage to tumor cells, probably linked to the chromatin organization of pericentromeric regions and the passage of mitosis, and is indicative of bad prognosis.

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Molecular Medicine

Heterochromatin protein 1α: a hallmark of cell proliferation relevant to clinical oncology

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Mammalian cells contain three closely related heterochromatin protein 1 (HP1) isoforms, HP1α, β and γ, which, by analogy to their unique counterpart in *Schizosaccharomyces pombe*, have been implicated in gene silencing, genome stability and chromosome segregation. However, the individual importance of each isoform during normal cell cycle and disease has remained an unresolved issue. Here, we reveal that HP1α shows a proliferation-dependent regulation, which neither HP1β nor γ display. During transient cell cycle exit, the HP1α mRNA and protein levels diminish. Transient depletion of HP1α, but not HP1β or γ, in tumoural and primary human cells leads to defects in chromosome segregation. Notably, analysis of an annotated collection of samples derived from carcinomas reveals an overexpression of HP1α mRNA and protein, which correlates with clinical data and disease outcome. Our results unveil a specific expression pattern for the HP1α isoform, suggesting a unique function related to cell division and tumour growth. The overexpression of HP1α constitutes a new example of a potential epigenetic contribution to tumourigenesis that is of clinical interest for cancer prognosis.

INTRODUCTION

Cancer has been considered for a long time as a genetic disease induced mainly by hereditary or spontaneous mutations in DNA sequences (Hanahan & Weinberg, 2000; Weinberg, 1996). However, changes affecting chromatin organization have recently been implicated as well in tumourigenesis (Jones & Baylin, 2007), and intense work has been dedicated to understand how these processes relate to each other. Much effort has been put into the characterization of alterations in DNA methylation and different histone modifications (Esteller, 2007). Drugs designed to target these modifications have now been started to be used with some success in cancer treatment (Mulero-Navarro & Esteller, 2008). A present challenge is to find how, beyond DNA and histones, the higher order nuclear organization of chromatin (Misteli, 2007), which is often affected in cancer cells, participates in tumourigenesis. Breast cancer is a particularly interesting model in this respect. Given its clinical and genetic heterogeneity, it cannot be explained as a genetic disease. Thus, to consider if breast cancer cells show particular chromatin alterations that could promote tumourigenesis to proceed is particularly relevant.

In this study, we focus on a key component of constitutive heterochromatin regions, heterochromatin protein 1 (HP1) (Kwon & Workman, 2008; Maison & Almouzni, 2004), highly conserved from *Schizosaccharomyces pombe* to humans.

The unique HP1 homologue in *S. pombe*, Swi6, has been implicated in chromatin compaction and gene silencing (Kwon & Workman, 2008), through its interaction with histone H3 methylated on lysine 9 (H3K9me) (Bannister et al, 2001; Ekwall et al, 1996). In addition, Swi6 is involved in mitotic segregation (Ekwall et al, 1995), by promoting sister chromatid cohesion (Bernard et al, 2001) and through the establishment of the centromeric histone H3 variant CENP-A^{Cnp1} (Folco et al, 2008).

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Mammalian cells present three HP1 isoforms, HP1α, β and γ, that, by analogy to their fission yeast homologue, have been collectively implicated in gene silencing (Kwon & Workman, 2008). They can all interact with a trimethylated H3K9 (H3K9me3) peptide *in vitro* (Lachner et al, 2001) and targeting of either HP1α or β to a transgene array results in a local chromatin compaction (Verschure et al, 2005). HP1 isoforms also accumulate at pericentric heterochromatin (Maison & Almouzni, 2004). However, understanding the exact role of mammalian HP1 proteins in chromosome segregation is still at an early stage. Indeed, HP1 proteins interact with components of the centromere and the kinetochore complex (Ainsztein et al, 1998; Obuse et al, 2004; Wheatley et al, 2001) and downregulation (Auth et al, 2006; Obuse et al, 2004) or mislocalization of HP1 isoforms due to either the absence of H3K9me3 (Guenatri et al, 2004) or treatment with the histone deacetylase inhibitor (HDACi) Trichostatin-A (TSA) (Taddei et al, 2001), result in mitotic defects. Intriguingly, a recent report indicates that in contrast to Swi6 in *S. pombe*, the correct localization of HP1 is not required for the recruitment of cohesins to centromeric regions (Koch et al, 2008). Yet, the HP1α isoform seems to help in protecting cohesins from degradation by recruiting the Shugoshin protein (Yamagishi et al, 2008).

There are several indications that the three mammalian HP1 isoforms, HP1α, β and γ, may not fulfil identical functions. First, they show differences in their nuclear pattern of distribution. The overall nuclear staining of HP1α marks strongly the pericentric heterochromatin, whereas HP1γ shows less specificity for these regions (Gilbert et al, 2003; Minc et al, 1999; Nielsen et al, 2001a). Furthermore, despite their high similarity in structure and function, the three isoforms are not always present together and can interact with different binding partners (Kwon & Workman, 2008; Quivy et al, 2004). Finally, distinct post-translational modifications on individual HP1 isoforms (Lomberk et al, 2006; Minc et al, 1999) may further diversify their functions.

A first possible link between HP1 proteins and tumourigenesis was put forward through the observation that HP1 interacts with the tumour suppressor Retinoblastoma protein (Rb) (Nielsen et al, 2001b; Williams & Grafi, 2000) and participates in the Rb-dependent silencing of cell cycle genes such as Cyclin E (Nielsen et al, 2001b). Similarly, HP1 interacts with the transcriptional co-repressor KAP-1 (Ryan et al, 1999), which is involved in the regulation of the E2F1 (Wang et al, 2007) and p53 (Wang et al, 2005) proteins. Furthermore, HP1α and γ have been found in complex with Chromatin assembly factor 1 (CAF-1) (Murzina et al, 1999; Quivy et al, 2004), of which the intermediate subunit p60 is a validated proliferation marker in breast cancer (Polo et al, 2004). These arguments prompted us to consider how the different HP1 isoforms are regulated in relation to cell proliferation and tumourigenesis. Interestingly, the promoter region of HP1α contains potential target sites for the E2F proteins (Oberley et al, 2003; Weinmann et al, 2002) and myc transcription factors (Kim et al, 2008; Li et al, 2003). Moreover, all the three HP1 isoforms are downregulated in differentiated blood lymphocytes compared to their undifferentiated precursors (Baxter et al, 2004; Gilbert et al, 2003; Istomina et al, 2003; Ritou et al, 2007). Whether this downregulation is a general response to cell cycle exit or a specific feature of blood cell differentiation has not been addressed. Downregulation of HP1α has also been linked to the higher invasive potential of breast cancer cells (Kirschmann et al, 2000; Norwood et al, 2006), but again it is unclear to which aspect of the metastasis process this downregulation relates. Thus, the specific and/or common regulation patterns of the three HP1 isoforms in relation to cell proliferation, quiescence and cancer remain elusive.

To clarify these issues, we decided to carry out a comprehensive study of the behaviour of the distinct HP1 isoforms during cell proliferation, cell cycle exit and tumourigenesis, using both human cell line models and a collection of human tumour-derived tissue samples. We demonstrate that HP1α shows the unique property of displaying a proliferation-dependent expression pattern. Upon transient cell cycle exit, the expression of HP1α, but not β or γ, is reduced. Breast cancer cell lines show overexpression of HP1α, but not β or γ, compared to non-tumoural mammary cells derived from the same patient. Remarkably, HP1α is overexpressed in pancreas, uterus, ovary, prostate and breast carcinomas, as well as in uterine leiomyoma, compared to corresponding non-tumoural tissues. Furthermore, HP1α expression levels in breast carcinomas with a long-term patient follow-up show a significant correlation with disease progression and occurrence of metastasis. Our results demonstrate that HP1α levels are clearly associated with cell proliferation, which is relevant to tumourigenicity and useful for prognosis assessment in breast cancer.

RESULTS

HP1α expression depends on cell proliferation

Recent studies have described a downregulation of HP1 proteins in differentiated blood cells compared to undifferentiated blood cells (Baxter et al, 2004; Gilbert et al, 2003; Istomina et al, 2003; Ritou et al, 2007). This downregulation could be a common response to cell cycle exit, or a specific consequence of (blood) cell differentiation. To address this issue, we examined whether transient cell cycle exit, which is not accompanied by a differentiation process, similarly results in HP1 downregulation. Using two different human primary fibroblast cell lines (WI38 and BJ), in which quiescence is induced by serum starvation, we observed lower protein levels of HP1α, but not β or γ, in quiescent cells compared to proliferating cells (Fig 1A). MCF7 breast carcinoma cells, which are arrested in quiescence by anti-estrogen treatment (Carroll et al, 2000), show a similar downregulation of HP1α, but not β or γ. Thus, using two different means to induce quiescence, we find a specific downregulation of the HP1α isoform, the extent of which correlates with the duration of time the cells have spent in quiescence (not shown). As a control, we verified the downregulation of CAF-1 p60 in all quiescent cells (Polo et al, 2004) (Fig 1A) and assessed the synchronization efficiency by flow cytometry (Fig 1B). Similar to CAF-1 p150 and p60, HP1α

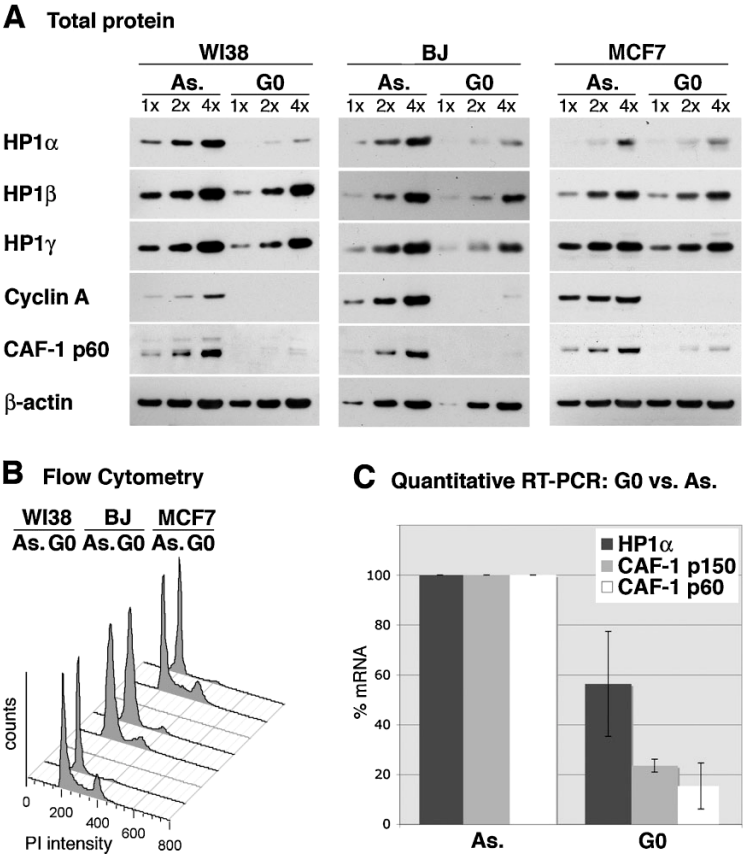

Figure 1. The expression of HP1α, but not β or γ, is downregulated in quiescence.

A. Total protein levels of HP1α, β and γ are detected by Western blot in asynchronously proliferating (As.) and quiescent (G0) WI38 lung fibroblasts, MCF7 breast cancer cells and BJ primary foreskin fibroblasts. Fibroblasts are arrested in quiescence by serum starvation and MCF7 cells by anti-estrogen treatment. Increasing amounts (x) of total cell extracts are loaded and β-actin serves as a loading control. CAF-1 p60 (Polo et al, 2004) and Cyclin A are used as markers for cell proliferation.

B. Flow cytometry analysis of the cell cycle distribution of the cells shown in A.

C. HP1α mRNA levels in proliferating (As.) and quiescent (G0) BJ foreskin fibroblasts, as determined by quantitative RT-PCR. Levels are normalized to the reference gene ribosomal protein Po-like protein (RPLPO) (de Cremoux et al, 2004) and levels in proliferating cells are set to 100%. CAF-1 p60 and CAF-1 p150 levels are shown for comparison. The error bar represents data from three independent experiments.

downregulation in transient quiescence relates in part to transcriptional regulation, since quiescent BJ cells also show decreased HP1α mRNA levels when compared to asynchronously proliferating cells (determined by quantitative RT-PCR, Fig 1C). Upon exit from the quiescent state, HP1α protein levels gradually increase between 16 and 24 h after release (Fig S1A in Supporting Information), which corresponds to the moment of cell cycle entry (determined by flow cytometry, Fig S1B in Supporting Information). The accumulation of HP1α occurs earlier than the one of CAF-1 p60 (Fig S1A in Supporting Information), mostly observed at the onset of DNA replication.

The observed downregulation of HP1α in quiescence could either be specific for the quiescent state or reflect an expression restricted to a specific stage of the cell cycle. We therefore analysed HP1α levels during the cell cycle in synchronized HeLa cells. In contrast to the cell cycle marker Cyclin A, we did not observe significant variation in HP1α levels in the synchronized cell populations (Fig S2A, B in Supporting Information). Similarly, human primary fibroblasts, which display a normal cell cycle regulation, show essentially similar levels of HP1α protein (Fig S2C in Supporting Information) and mRNA in the synchronized cell population (Fig 2D, E). In this respect, HP1α behaves similarly to CAF-1 p60 and p150, which are also ubiquitously expressed during the cell cycle but downregulated in quiescence (Fig 1 and S2 in Supporting Information; (Polo et al, 2004)). In conclusion, HP1α expression levels are high at all stages of the cell cycle in proliferative cells, and HP1α downregulation is specific to the quiescent state.

HP1α is overexpressed in breast cancer cells

The proliferation-dependent expression of HP1α suggests a possible differential expression between tumoural and non-tumoural cells, as found for proliferation markers including CAF-1 p60 (Polo et al, 2004). To examine this issue, we used mammary cells derived from the same patient, either tumoural (Hs578T) or non-tumoural (Hs578Bst) (Hackett et al, 1977), to be relevant in our comparison. Besides the total cell extract, we analysed levels of HP1 proteins both in the soluble fraction and in the fraction bound to chromatin (Fig 2A). Indeed, HP1 proteins distribute into different nuclear fractions, distinguished by their capacity to resist extraction with high salt concentrations or with Triton X-100 detergent. The salt- or detergent-resistant pool of HP1 is considered as the active, chromatin-bound pool of HP1 and represents less than 10% of the total HP1 in human and rodent cells, as determined by Western blot quantification (Taddei et al, 2001) or Fluorescence recovery

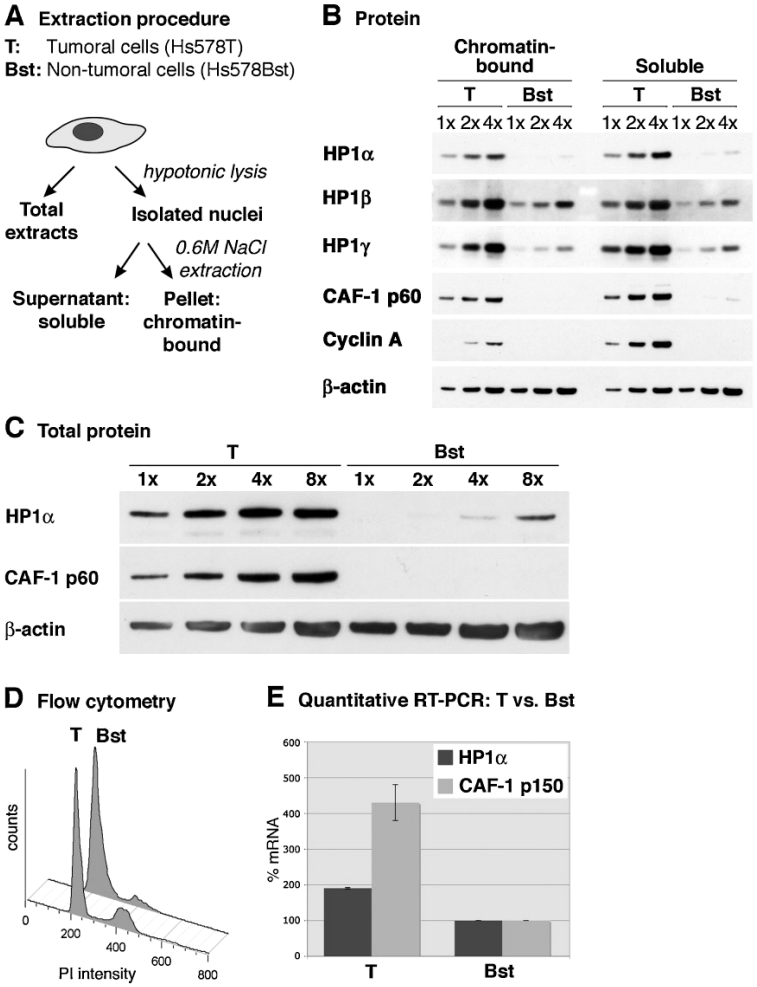

Figure 2. HP1α is overexpressed in breast cancer cells and associated with chromatin.

A. Scheme of the experimental procedure applied to obtain total, soluble and chromatin-bound cell extracts as used in B.

B. HP1α, β and γ protein levels are analysed by Western blotting in soluble and chromatin-bound nuclear extracts from the breast cancer cell line Hs578T (T) and the non-tumoural mammary cell line Hs578Bst (Bst), which are derived from the same patient (Hackett et al, 1977). Cyclin A and CAF-1 p60 are shown for comparison. Increasing amounts (x) of cell extracts are loaded; β-actin serves as a loading control.

C. Relative quantification of total HP1α protein levels in tumoural (T) and non-tumoural (Bst) mammary cells, compared to the amounts of CAF-1 p60 (Polo et al, 2004). Increasing amounts (x) of total cell extracts are loaded; β-actin serves as a loading control.

D. Flow cytometry analysis of tumoural (T) and non-tumoural (Bst) mammary cells in order to assess polyploidy. Tumoural (T) and non-tumoural (Bst) cells contain 25 and 13% of cells in S-phase, respectively.

E. Relative HP1α mRNA levels in tumoural (T) and non-tumoural (Bst) mammary cells, as determined by quantitative RT-PCR. Levels are normalized to the reference gene ribosomal protein Po-like protein (RPLPO) (de Cremoux et al, 2004) and non-tumoural cells are set to 100%. CAF-1 p150 is shown for comparison. Error bars represent data from two independent experiments.

after photobleaching (FRAP) (Cheutin et al, 2003; Dialynas et al, 2007; Festenstein et al, 2003).

Both in the chromatin-bound and in the soluble nuclear fractions, we observed higher HP1α levels in tumoural Hs578T cells than in non-tumoural Hs578Bst cells, whereas HP1β or γ display little differences in expression compared to the loading control β-actin (Fig 2B). Loading of identical amounts of cells also show tumoural overexpression of HP1α, specifically (Fig S3 in Supporting Information). Semi-quantitative Western blot on total cell extracts shows that tumoural Hs578T cells contain approximately eight times more HP1α protein than non-tumoural Hs578Bst cells, compared to the loading control (Fig 2C). We could exclude the possibility that HP1α expression levels reflect the DNA content of the cell, since the tumoural Hs578T cells display only a very moderate aneuploidy in flow cytometry (Fig 2D). The overexpression of HP1α mRNA, detected by quantitative RT-PCR (Fig 2E), further indicates a regulation that involves, at least in part, transcription.

Our analysis of chromatin-bound and soluble fractions suggests that overexpressed HP1α in tumoural cells is partially chromatin-bound and thus possibly important for chromatin organization. This seems to be consistent with its nuclear localization, which is granular and diffuse in the non-tumoural mammary cells but clearly localized into discrete spots in a large fraction of the breast cancer cells (Fig S4A in Supporting Information). These spots largely localize to centromeric regions, detected by the CREST autoimmune serum (Fig S4A in Supporting Information). Yet, the different forms of localization of HP1α were not accompanied by an altered nuclear distribution of H3K9me3 (Fig S4B in Supporting Information), and the different patterns of HP1α staining do not seem to be associated with specific stages of the cell cycle, as detected by staining for cell cycle markers (Fig S4C in Supporting Information). In conclusion, our cell line model shows an overexpression of HP1α, but not HP1β or γ, in tumoural mammary cells compared to non-tumoural mammary cells.

A large fraction of HP1α in breast cancer cells is chromatin-bound and localizes to centromeric regions.

HP1α downregulation results in mitotic defects

The proliferation-dependent expression of HP1α, which is not observed for HP1β or γ, points to a unique function of this isoform and suggests that high amounts of this protein could confer an advantage for cell growth. Interestingly, down-regulation of CAF-1 p150 results in a pronounced S-phase arrest in a manner that depends on its interaction with HP1 proteins (Quivy et al, 2008; Quivy et al, 2004). We thus tested whether high expression levels of HP1α, β or γ are required for human cancer cell proliferation. By transfecting HeLa cells with siRNA against HP1α, β or γ, we obtained a specific downregulation of each of the HP1 isoforms (Fig 3A). In contrast to downregulation of its binding partner CAF-1 p150 (Quivy et al, 2008), downregulation of the HP1 isoforms did not result in any obvious effect on cell proliferation, as assessed by flow cytometry (Fig 3B). We obtained similar results for HP1α downregulation in the mammary carcinoma cell line Hs578T (data not shown). However, in agreement with previous reports (Auth et al, 2006; Obuse et al, 2004), we did observe an increased fraction of mitotic profiles displaying lagging chromosomes, misalignments and chromosome bridges (Fig 3C) after downregulation of HP1α. We quantified these as the fraction of aberrant mitoses (Fig 3D). Interestingly, under our experimental conditions, downregulation of HP1β or γ did not give rise to increased mitotic defects, suggesting that only the HP1α isoform is critical for faithful mitosis. Furthermore, defects in mitosis were supported by observation of a three-fold increase in micronuclei formation after downregulation of HP1α, but not HP1β or γ (Fig 3E, F).

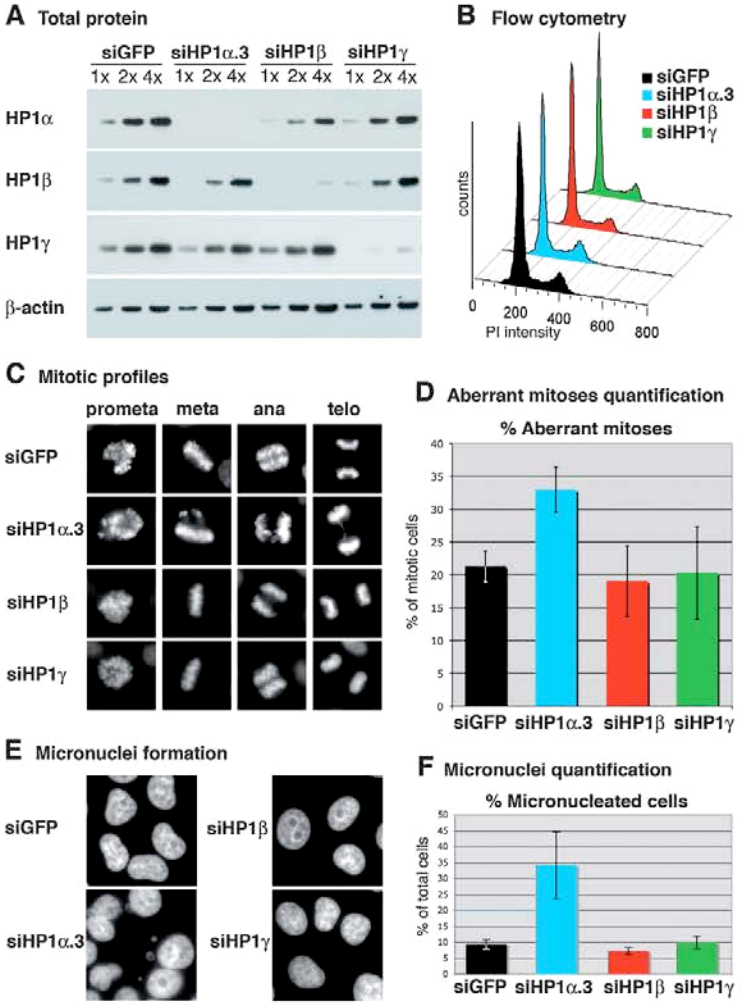

Figure 3. Downregulation of HP1α, but not HP1β or γ, leads to mitotic defects in HeLa cells.

A. Western blot analysis of total cell extracts from HeLa cells 72 hours after transfection with siRNA sequences targeting HP1α (named siHP1α.3), HP1β, HP1γ or control siRNA (siGFP). Increasing amounts (x) of total cell extracts are loaded and β-actin is used as a loading control.

B. Flow cytometry analysis of the cell cycle distribution of cells shown in A.

C. Typical mitotic figures after transfection with siRNA sequences targeting HP1α, HP1β, HP1γ or mock siRNA (siGFP).

D. Percentage of aberrant mitotic figures (lagging chromosomes, misalignments, chromosome bridges) in asynchronous HeLa cells 72 h after transfection with siRNA sequences targeting HP1α, HP1β, HP1γ or mock siRNA (siGFP). The error bars represent data from three independent, blind counted, experiments.

E. DAPI staining reveals micronuclei formation 72 h after transfection with an siRNA sequence targeting HP1α.

F. Quantification of the percentage of micronucleated cells 72 h after transfection with siRNA sequences targeting HP1α, HP1β, HP1γ or control siRNA (siGFP). Error bars represent data from three independent experiments of which two were blind counted.

Since HeLa cells already show a high fraction of deficient mitoses (~20%) and of micronucleated cells (~10%), we used primary fibroblasts, proficient for cell cycle control and checkpoint activity for further analysis. Transient transfection with two different siRNAs against HP1α resulted in a specific downregulation of this isoform (Fig S5A in Supporting Information). Again, we did not detect any significant effect on global cell cycle distribution by flow cytometry (Fig S5B in Supporting Information), but we observed a small but reproducible increase in the percentage of prometaphases and a decreased percentage of metaphases by microscopy (Fig S5C in Supporting Information). Interestingly, this effect mimics observations upon mutation of the *Drososophila* homologue of HP1α (Kellum & Alberts, 1995) or inactivation of the centromeric histone H3 variant CENPA in chicken cells (Regnier et al, 2005). Using live cell imaging, we measured a small but statistically significant ($p = 4.9 \times 10^{-7}$) increase in the duration of mitosis after downregulation of HP1α compared to control (upper graph Fig S5D in Supporting Information). Interestingly, the delay again mostly affected the steps preceding actual chromosome segregation (Fig S5D in Supporting Information, lower graph). In conclusion, our observations in both tumoural and primary cells suggest a role for HP1α in early mitosis, possibly contributing to the correct alignment or the stable attachment of chromosomes at the metaphase plate.

HP1α overexpression in human cancer samples

The proliferation-dependent expression of HP1α and its overexpression in breast cancer cell lines prompted us to study HP1α expression in the physiological context of human cancer. First, we analysed data from published transcriptome studies performed in different tissue types (Andersson et al, 2007; Pyeon et al, 2007; Quade et al, 2004; Ramaswamy et al, 2003; Richardson et al, 2006; Yu et al, 2004), using the Oncomine database (Rhodes et al, 2004). These data showed that HP1α is significantly and consistently overexpressed in several types of malignancies (Fig S6A in Supporting Information), while HP1γ and especially HP1β can be found either up- or downregulated when carrying out similar analyses (not shown). It is remarkable that in leukaemia, only HP1α, but not HP1β or γ, shows an important overexpression that correlates with the time for the disease to relapse (Kirschner-Schwabe et al, 2006) and with the expression of its binding partner CAF-1 p150 and the proliferation marker Ki67 (Fig S6B in Supporting Information).

Our results in cultured cells systematically showed that the difference in HP1α protein levels was more pronounced than the corresponding mRNA levels (Figs 1 and 2), possibly reflecting a post-translational regulation. This encouraged us to analyse the HP1α protein levels in frozen tumoural and non-tumoural human tissue sections by immunohistochemistry. We systematically observed an intense HP1α staining in tumoural cell nuclei in pancreas, uterus, ovary, prostate and breast malignancies (Fig 4A). In the corresponding non-tumoural tissues, HP1α levels were mostly below the limit of detection. This differential expression is specific to HP1α, since HP1β and γ show nuclear staining both in tumoural and non-tumoural tissues (Fig 4B). The nuclei showing intense HP1α immunostaining correspond to carcinoma cells, which also stain positive for the epithelial cytokeratin marker KL-1 (Fig 4C). In addition, a polyclonal antibody against HP1α, which provides a more intense staining than the monoclonal antibody used in Fig 4, also shows a clear overexpression in carcinoma cells with some staining in non-tumoural tissue (Fig S7A in Supporting Information). Staining for H3K9me3, however, did not show a clear difference between non-tumoural and tumoural tissues (Fig S7B in Supporting Information), suggesting that either the HP1α detection is more sensitive or the overexpression occurs independently from H3K9me3.

To determine the importance of HP1α overexpression for tumour growth and disease outcome, we selected cryopreserved breast carcinoma specimens that were collected in 1995. We focused on node negative and metastasis-free invasive carcinoma of size (median 18 mm; range 6–50 mm) that permitted primary conservative tumourectomy. These cases would benefit from new prognostic markers for the indication of adjuvant chemotherapy. Patient's and tumour characteristics are provided in Table SI in Supporting Information. The median follow-up is 146 months (range 30–161). At 10 years, overall survival, the distant recurrence and the disease progression rates were 90 [83–97], 87 [80–95] and 70% [61–81%], respectively. We measured HP1α mRNA expression levels by quantitative RT-PCR in 86 samples and normalized the expression levels to that of the reference gene ribosomal protein P0-like protein (RPLPO) (de Cremoux et al, 2004). We found that high levels of HP1α tend to associate with increased age of the patient ($p = 0.0014$) and larger, highly mitotic, grade III tumours (non-significant) (Fig 5A). In univariate analysis, HP1α continuous expression was significantly associated with an increased risk of disease progression: a one-unit increase of log 2(HP1α) increased the risk of disease progression by 3% (relative risk (RR) = 1.03 (1.00–1.05), $p = 0.041$). We determined a cut-off value of 10, which divided patients into two groups (74.4% with HP1α levels ≤10 and 25.6% with HP1α levels >10) and was significantly associated with disease progression ($p = 0.0113$; RR = 2.47 (1.20–5.09)). At 10 years, 75% [64–87] of the patients with low HP1α levels compared to 57% [39–83] of the patients with high HP1α levels had not shown disease progression. Moreover, this cut-off is also significantly associated with overall survival ($p = 0.0134$; RR = 3.76 (1.03–13.7)) and the occurrence of distant recurrences ($p = 0.011$; RR = 3.74 (1.26–11.2)) (Fig 5B).

In multivariate analyses, adjusted for known prognostic factors (*i.e.* patient's age, mitotic index, tumour grade, tumour size, hormone receptor status and Ki67 levels), only HP1α expression is an independent prognostic factor for overall survival ($p = 0.0431$): high (>10) HP1α levels are associated with an increased risk of death (RR = 3.76 (1.03-13.7)). Similarly, adjusted for the same parameters, only high HP1α expression ($p = 0.0077$; RR = 3.02 (1.38–6.63)) and high tumour grade ($p = 0.0170$; RR for grade III = 4.53 (1.51–13.6)) are pejorative for disease progression.

In conclusion, our immunohistochemistry analyses demonstrate that HP1α, but not β or γ, is overexpressed in multiple types of human cancer cells. Furthermore, quantitative RT-PCR analysis carried out for HP1α showed significant correlation

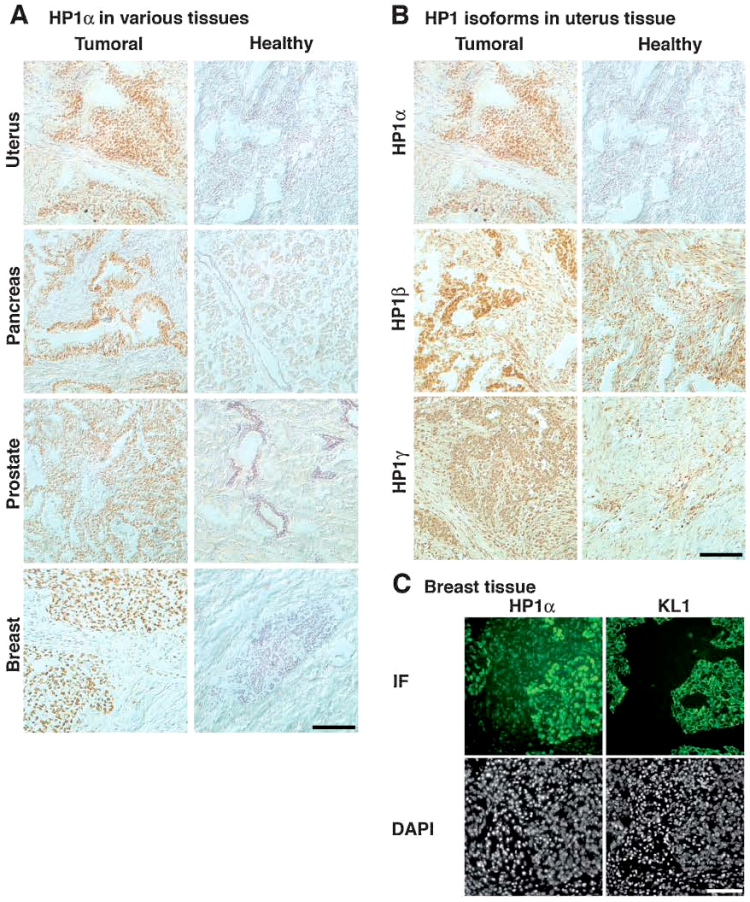

Figure 4. HP1α protein is overexpressed in multiple types of human cancer.

A. Frozen human tissue arrays containing sections from tumoural and healthy origin are stained for HP1α by immunohistochemistry and counterstained with hematoxylin.

B. Frozen uterus tissue sections were stained for HP1α, β and γ by immunohistochemistry and counterstained with hematoxylin. Tissue arrays were processed identically for the three antibodies.

C. Two adjacent sections from a frozen medullary breast carcinoma were stained for HP1α and epithelial pan-cytokeratin marker KL-1, by IF. DNA is revealed with DAPI. Areas of HP1α overexpression correspond to regions of epithelial tumour cells. Scale bars: 100 μm.

with clinico-pathological data and disease outcome. In our series of 86 small breast tumours, high HP1α expression remained the only independent prognostic factor for overall survival after adjustment for classical prognostic markers, such as the mitotic index, tumour grade, tumour size, hormone receptor status or Ki67. We are currently extending our analysis to a second, independent data set to test our cut-off value of 10 and confirm the highly prognostic value of HP1α compared to other markers. Taken together, our data demonstrate that HP1α constitutes a new chromatin-related marker of cell proliferation and tumourigenesis of clinical relevance for prognosis of breast cancer and potentially other types of cancer.

DISCUSSION

A unique regulation of the HP1α isoform

The HP1 family of proteins were identified more than 20 years ago (James & Elgin, 1986), but the common or divergent functions of the three isoforms remain largely unknown. Here, we show that in human cells, the HP1α isoform has unique properties, not shared by HP1β and γ. The protein is expressed in a proliferation-dependent manner, being downregulated during transient cell cycle exit. In line with these findings, cultured cancer cells overexpress HP1α compared to non-tumoural cells. Importantly, this is validated in patient samples. Our data thus demonstrate a unique regulation of HP1α, which is not paralleled by HP1β or γ. The region in which the HP1α gene is located is not often affected by genomic aberrations in breast cancer (Progenetix CGH database (Baudis & Cleary, 2001)). Genomic alterations are thus unlikely to cause HP1α overexpression, which might rather reflect an increased activation by E2F (Oberley et al, 2003; Weinmann et al, 2002) and/or myc transcription factors (Kim et al, 2008; Li et al, 2003).

An interesting question now is whether HP1α overexpression in tumour cells is accompanied by and related to alterations in other heterochromatic marks. We did not observe a difference in nuclear distribution or in staining intensity of H3K9me3 in breast cancer cells and in tumoural tissue samples. However, other factors can promote HP1 recruitment and maintenance in (pericentric) heterochromatin regions, among which is the H3K9-methyltransferase Suv39h1 (Stewart et al, 2005), an RNA component (Maison et al, 2002) and the largest subunit of CAF-1

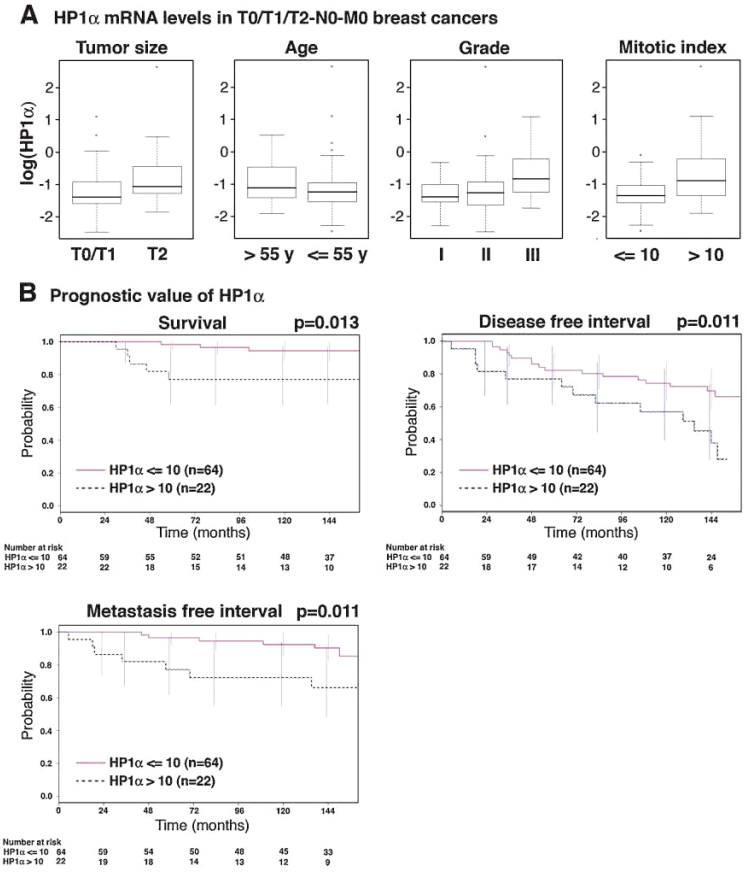

Figure 5. HP1α expression levels have a prognostic value in breast cancer patients.

A. Box plots representing logarithmic HP1α mRNA expression levels, according to the indicated clinico-pathological factors, in 86 breast cancer samples with a >10 year patient follow-up. Boxes represent the 25–75th percentile; brackets: range; black line: median; black dots: outliers.

B. Univariate Kaplan–Meier curves of the survival, the occurrence of metastasis and the disease-free interval (interval before the occurrence of local recurrence, regional lymph node recurrence, controlateral breast cancer or metastasis) in patients expressing high (>10) or low (≤10) levels of HP1α. The number of patients at risk at each time point is indicated below the graphs.

(p150) (Quivy et al, 2004, 2008). Post-translational modifications of HP1α itself (Lomberk et al, 2006) might also affect its stabilization and recruitment, as has been shown recently for HP1β in the context of DNA damage (Ayoub et al, 2008). Furthermore, it remains unclear how the increased levels of HP1α relate to loss of the heterochromatin mark H4K20me3 in cancer cells (Fraga et al, 2005) or the reported changes in DNA methylation (reviewed in (Esteller, 2007)). Future work should also explore the distinct or common repressive roles of HP1α and the polycomb group proteins. Indeed, HP1α interacts with the Suz12 subunit of the polycomb repressive complex 2 (PRC2) (Cao & Zhang, 2004; Yamamoto et al, 2004), in which all three subunits (Suz12, EZH2 and EED) are downregulated in quiescence (Bracken et al, 2003; Muller et al, 2001) and overexpressed in human cancer (reviewed in (Simon & Lange, 2008)). Considering how these different repressive marks function in cooperation will be necessary to obtain a more complete picture of the aberrant transcriptional repression mechanisms in cancer cells.

Potential functions of tumoural HP1α overexpression

The unique proliferation-dependent regulation of HP1α suggests the existence of a specific function that would distinguish it from HP1β and HP1γ. Interestingly, the three isoforms distribute differentially within the nucleus: HP1α is most specific for pericentric heterochromatin, whereas HP1β and especially HP1γ show a more diffuse distribution (Gilbert et al, 2003; Minc et al, 1999; Nielsen et al, 2001a). Hence, high HP1α levels in cancer cells might reflect an altered organization of certain heterochromatic regions, which could become more compact and/or more abundant. The increased amount of chromatin-bound HP1α and its different nuclear distributions (Fig 2 and Fig S4 in Supporting Information) in breast cancer cells compared to non-tumoural cells is in agreement with this hypothesis. It is noteworthy that the distinct nuclear distribution of HP1α in breast cancer cells (discrete spots) and non-tumoural cells (granular and diffuse) mimics the localization that has been observed in activated versus resting lymphocytes (Baxter et al, 2004; Grigoryev et al, 2004; Ritou et al, 2007). Yet, this is not solely a consequence of cell cycle status, since we did not observe a different HP1α localization between quiescent and proliferating primary fibroblasts (data not shown).

Besides a structural role in pericentric heterochromatin, the overexpression of HP1α in tumours could also relate to a function in regulating the euchromatic gene expression. Indeed, in *Drosophila*, HP1 is involved in the regulation of cell cycle genes

(De Lucia et al, 2005) and could even actively promote gene expression by stimulating H3K36 demethylation (Lin et al, 2008). Human HP1 plays a role in silencing Cyclin E by the tumour suppressor Rb (Nielsen et al, 2001b), and interacts with the co-repressor protein KAP1, the genomic targets of which include genes involved in crucial cellular pathways (O'Geen et al, 2007). However, there is currently no evidence in mammalian cells that regulation of gene expression would be a unique property of HP1α, not shared by HP1β and γ. Furthermore, if HP1α has a dominant regulatory role in gene expression, a profound impact on cell proliferation and survival would be expected upon its downregulation, which is not in line with our observations so far.

Although we do not formally exclude a more subtle role for HP1α in gene expression, which would deserve a specific study, our results rather favour a mitosis-related advantage of tumoural HP1α overexpression. Indeed, despite the fact that phosphorylation of H3S10 by Aurora B induces the release of HP1 proteins from pericentric chromatin during mitosis (Fischle et al, 2005; Hirota et al, 2005), at least a fraction of HP1α, specifically, remains tightly associated throughout mitosis (Guenatri et al, 2004; Hayakawa et al, 2003; Minc et al, 2001; Schmiedeberg et al, 2004). Hence, HP1α might play a unique role in mitosis. Indeed, we observe a partial colocalization of HP1α with (peri-) centromeric regions in breast cancer cells, suggesting that these heterochromatic regions might constitute the main target sequences of overexpressed HP1α. Furthermore, we show that downregulation of HP1α, but not HP1β or γ, results in mitotic defects, both in HeLa cells and in primary human fibroblasts. Interestingly, in primary fibroblasts, the phenotype includes a prolonged prometaphase, which could reflect a problem in the alignment of chromosomes at the metaphase plate. HP1α was recently shown to be necessary for the recruitment of the shugoshin protein (Yamagishi et al, 2008), which protects centromeric cohesins from degradation and thus prevents premature chromosome segregation. Deficient cohesion can lead to accumulation of cells in prometaphase (Watrin et al, 2006). Hence, a partial depletion of HP1α would affect cohesin protection and result in a prolonged prometaphase in primary cells due to checkpoint activity. In transformed cells, in which the mitotic checkpoint is often less efficient (Weaver & Cleveland, 2005), an increased fraction of aberrant mitoses and micronucleated cells is observed (Fig 3). Thus, elevated levels of HP1α would be more crucial for faithful mitosis in cancer cells when compared to healthy cells, and a positive selection for cancer cells overexpressing HP1α is more likely to occur when the mitotic checkpoint becomes deficient, in order to facilitate the passage through mitosis.

Importance of HP1α in cancer

To evaluate the clinical importance of HP1α expression, we analysed HP1α protein and mRNA levels in human cancer by immunohistochemistry and quantitative RT-PCR. Staining of cryopreserved human tissue samples showed a significant overexpression of HP1α, but not HP1β or γ, in tumoural cells. Thus, HP1α constitutes a potential marker for the diagnosis of multiple types of cancer. In contrast to other proliferation markers, such as Ki67, HP1α stains all tumoural cells and is thus highly suitable for the determination of the exact localization and extent of the tumour.

In order to quantify the HP1α expression and determine its prognostic value, we measured HP1α mRNA levels in 86 small breast tumours with >10 years follow-up. Our data reveal that high HP1α expression correlates with decreased survival and increased occurrence of metastasis over time. Furthermore, multivariate analyses demonstrate that HP1α levels predict disease outcome better than standard prognostic markers. Thus, HP1α constitutes a marker of prognostic value, in breast cancer and potentially in other types of cancer.

Previously, an increased expression of HP1α had been correlated with a decreased invasive potential among several breast cancer cell lines (Kirschmann et al, 2000; Norwood et al, 2006), possibly by silencing of pro-invasive genes. This observation might reflect the inverse correlation that has been suggested between proliferation and invasion (Berglund & Landberg, 2006). Indeed, metastasis requires the acquisition of invasive potential and the adaptation to a new environment, which are often incompatible with high proliferation rates. Thus, a temporal slowdown of tumour proliferation, accompanied by downregulation of HP1α, might permit the expression of pro-invasive genes and the occurrence of metastasis. Yet, the outgrowth of metastases requires cell proliferation and our data suggest that this process is dominant for final patient outcome, since high HP1α expression correlated with earlier diagnosis of metastasis.

Recently, inhibitors of HDAC have been used successfully in cancer treatment (Dokmanovic et al, 2007; Mulero-Navarro & Esteller, 2008). However, their precise mode of action remains elusive. It is interesting that, in proliferating cell lines, a major effect of HDACi is to increase the mobility (Cheutin et al, 2003; Dialynas et al, 2007) and dispersion (Bartova et al, 2005; Dialynas et al, 2007; Taddei et al, 2001) of HP1 proteins. Although the effect of HDACi treatment has not been assessed *in vivo* on post-therapeautic tissue samples, HP1α might be one of the main targets contributing to the anti-tumour effects of these drugs (Taddei et al, 2005). Without affecting the expression levels of HP1α, HDACi might delocalize the overexpressed HP1α *in vivo* and abrogate the contribution of HP1α overexpression to cancer cell growth and/or cell division.

In conclusion, HP1α expression levels reflect cell proliferation and are negatively correlated with disease outcome in early breast cancer. Our results favour a role of HP1α in facilitating mitosis, which might be more crucial in cancer cells. We demonstrate a potential clinical application of HP1α as a marker for cancer prognosis. As a consequence, HP1α should now be taken into account in fundamental cancer research to obtain a comprehensive picture of how heterochromatin domains are affected in cancer cells and contribute to tumourigenesis.

MATERIALS AND METHODS

Cell culture and synchronization

Wi38 primary lung fibroblasts (ATCC) and BJ primary foreskin fibroblasts (ATCC) were cultured in MEMα medium (GIBCO), MCF7 breast

cancer cells (gift from O. Delattre, Paris) and HeLa cervical carcinoma cells (gift from M. Bomens, Paris) in DMEM (GIBCO), Hs578T breast cancer cells (gift from O. Delattre, Paris) in RPMI medium (GIBCO) containing 10 mM insulin (Sigma) and Hs578Bst healthy mammary cells (ATCC) in DMEM containing 30 ng/ml epidermal growth factor (TEBU). The media were supplemented with 10% foetal calf serum (Eurobio) and 10 mg/ml penicillin and streptomycin (GIBCO).

For synchronization in quiescence, primary cells were grown in serum-free medium for at least 72 h and MCF7 cells for 48 h in a medium containing 10 nM of the anti-estrogen ICI182780 (Fischer Bioblock Scientific) (Carroll et al, 2000). HeLa cells were synchronized in the different stages of the cell cycle as described by Polo et al (2004). BJ cells were synchronized similarly, except that they were blocked for 14 h, released for 10 h, blocked again for 14 h and released for 3, 6 or 12 h for S-phase, G2 and G1, respectively. For mitotic BJ cells, 10 ng/ml nocodozole was added 4 h after the second release from thymidine and cells were harvested 10 h later. Synchronization was checked by flow cytometry as explained by Polo et al (2004), and the data were analysed using FlowJo (Tree Star Inc.).

Transfections and siRNAs

HeLa cells, plated in a medium without antibiotics, were transfected with 30 nM siRNA using Oligofectamine reagent (Invitrogen) and Optimem 1 medium (Gibco). BJ primary cells were transfected at passage 27 (8 passages after reception from ATCC) using nucleofection (Amaxa), according to manufacturer's instructions. Briefly, 4×10^5 cells were transfected with 0.8 nmol siRNA (Dharmacon) or 1 μg of plasmid DNA in 100 μl Nucleofector solution R (Amaxa), using the nucleofection program X-001. Cells were subsequently plated at a density of 7,000 cells/cm^2 to permit cell proliferation without reaching confluency within 4 days. siRNA sequences: siGFP: AAGCUGGAGUACAACUACAAC; siHP1α_1: CCUGAGAAAAACUUGGAUUTT (Obuse et al, 2004); siHP1α_3: GGGAGAAGTCAGAAAGTAA; siHP1β: AGGAATATGTGGTGGAAAA; siHP1γ: AGGTCTTGATCCTGAAAGA

Cell extracts

For *total cell extracts*, after a wash in phosphate buffered saline (PBS), we scraped cells from plates in 1× Laemmli buffer (60 mM Tris-Hcl, pH 6.8; 10% glycerol; 2% sodium dodecyl sulphate (SDS); 1% 2-mercaptoethanol and 0.002% bromophenol blue) and boiled these extracts 10 min before storage at −20°C.

Chromatin-bound cell extracts were made according to the method of Martini et al (1998). We determined protein concentration using Bio-Rad protein assay solution.

Antibodies

The following primary antibodies were used in the present study: Mouse monoclonal anti-HP1α 2HP-1H5 from Euromedex and rabbit polyclonal anti-HP1α H2164 from Sigma (directed against amino acids 67–119 and 177–191, respectively) for immunofluorescence (IF) and immunohistochemistry, mouse monoclonal anti-HP1α 2HP-2G9 for Western blot (Euromedex), mouse monoclonal anti-HP1β 1MOD1A9 (Euromedex), mouse monoclonal anti-HP1γ 2MOD-1G6-AS (Euromedex), anti-CREST human serum (gift from A. Ladurner), rabbit polyclonal anti-CAF-1 p60, mouse monoclonal anti-β-actin AC-15 (Sigma), mouse monoclonal anti-KL1 antibody (Dako), rabbit polyclonal anti-Cyclin A (Santa Cruz), mouse monoclonal anti-H3S10P (Abcam ab14955) and rabbit polyclonal anti-Cdt1 (Santa Cruz 28262).

Secondary antibodies: Fluorescein isothiocyanate (FITC) or Texas Red-coupled antibodies (IF) or horseradish peroxidase-coupled antibodies (Western Blotting) were from Interchim.

Western blotting

After 30 min treatment with 25 U benzonase nuclease (Novagen), cell extracts were loaded on 4–12% gradient gels (Invitrogen), using a 1 × MES migration buffer (Invitrogen), followed by Western blotting procedures as described by Martini et al (1998).

Immunofluorescence (IF)

After fixation in 2% paraformaldehyde, cells are permeabilized in 0.2% Triton X-100 in PBS. IF detection is carried out as in (Martini et al, 1998). Coverslips are mounted in Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector laboratories).

For IF on tissue samples, 8 μm cryosections made from frozen mammary tissues (Curie Institute, Paris, France) were fixed on glass slides in 3% paraformaldehyde and immunostained as above.

Immunohistochemistry

We used 8 μm cryosections from frozen mammary tissues (Curie Institute, Paris, France) or frozen tissue arrays (FMC401, Biomax). Sections, fixed on glass slides in 3% paraformaldehyde and permeabilized in PBS containing 0.5% Triton for 4 min, were incubated for 5 min in 3% H_2O_2 (Prolabo) for peroxidase inhibition and blocked in PBS containing 1% BSA and 5% non-fat milk. Incubation with primary antibody diluted in blocking solution was followed by revelation with horseradish peroxidase-coupled secondary antibody (DakoCytomation) and diaminobenzidine (DakoCytomation) before counterstaining with hematoxyline (Merck). Slides were dehydrated in increasing ethanol concentrations and toluene before mounting in Entellan mounting medium (Merck).

Live cell imaging

For live cell imaging, BJ cells were transfected twice with siRNA, with a 72 h interval. A plasmid coding for H2B-cherry was introduced in the second transfection, and cells were plated on glass bottom dishes (Mattek). Movies were made using the BioStation system (Nikon). Images were acquired every 20 min, during a period of 24 h, starting 48 h after the second transfection. Movies were analysed using Image J.

RNA extraction, quantitative RT-PCR and primers

We used the mRNeasy mini kit (Qiagen) for total RNA extraction from cell lines or frozen patient samples and produced cDNA using Superscript II reverse transcriptase (Invitrogen) with 1 μg RNA and 3 μg of random primers (Invitrogen) per reaction. We used the Lightcycler 2.0 System (Roche) and the Lightcycler FastStart DNA Master SYBR Green I reaction kit (Roche) for quantitative RT-PCR. For the patient samples, we used the 96-well plate Step One Plus system (Applied Biosystems) and the SYBR Green PCR Master mix (Applied Biosystems). We measured duplicates and carried out

The paper explained

PROBLEM:
Breast cancer is a clinically and genetically diverse disease. How alterations in chromatin organization contribute to its development should therefore be considered. Here, we focus on the three mammalian isoforms of heterochromatin protein 1 (HP1α, β and γ), key components of compact heterochromatin regions, in relation to cell proliferation and breast cancer.

RESULTS:
We reveal that HP1α shows a proliferation-dependent regulation, which neither HP1β nor γ display. During transient cell cycle exit, HP1α mRNA and protein levels diminish and depletion of HP1α leads to defects in chromosome segregation. Importantly, the levels of HP1α mRNA and protein are elevated in breast carcinomas and this upregulation correlates with clinical data and disease outcome. Altogether, we propose that HP1α has a role in mitosis and that this role provides a selective growth advantage to cancer cells.

IMPACT:
Our results suggest a unique function of the HP1α isoform, related to cell division and tumour growth. HP1α overexpression in breast cancer patient samples correlates with disease outcome and should be considered as a new epigenetic marker for prognosis assessment.

three subsequent cDNA dilutions to assess the primer efficiency. We designed primer pairs in order to overlap an intron, so as to distinguish cDNA amplification from putative genomic contamination. Primers: RPLPO forward: GGCGACCTGGAAGTCCAACT; RPLPO reverse: CCATCAGCACCACAGCCTTC; HP1α forward: GATCATTGGG-GCAACAGATT; HP1α reverse: TGCAAGAACCAGGTCAGCTT; CAF-1 p150 forward: CAGCAGTACCAGTCCCTTCC; CAF-1 p150 reverse: TCTTTGCAGTCTGAGCTTGTTC; CAF-1 p60 forward: CGGACACTCCACCAAGTTCT; CAF-1 p60 reverse: CCAGGCGTCTCTGACTGAAT. We normalized the quantity of HP1α mRNA according to the human acidic ribosomal phosphoprotein PO (RPLPO) (de Cremoux et al, 2004) by applying $x = 100/(E(\text{Cp RPLPO} - \text{Cp HP1}\alpha))$, where E is the mean efficiency of primer pairs and where x reflects the quantity of HP1α mRNA relative to the quantity of RPLPO mRNA in a given sample.

Breast cancer patient samples and statistics

This study includes 92 breast cancer samples, selected from the Institut Curie Biological Resources Center for treatment with primary conservative tumourectomy (median tumour size: 18 mm (6–50 mm)). Patients were diagnosed in 1995 and found to be lymph node negative (N0) and metastasis free (M0). Patients were informed of research purposes and did not express opposition. Patient's and tumour characteristics are provided in Table SI in Supporting Information). RNA were extracted from cryopreserved tissue and analysed as described above. RNA of 86 samples were of sufficient quality for further analysis.

Differences between groups were analysed by χ^2 or Fisher exact tests for categorical variables and Kruskal–Wallis for continuous variables. Recurrence-free and alive patients were censored at the date of their last known contact. Survival data were defined as the time from diagnosis of breast cancer until the occurrence of disease progression, defined as local recurrence in the treated breast, regional recurrence in lymph node-bearing areas, controlateral breast cancer or distant recurrences. Determination of a cut-off value prognostic for the disease free interval (DFI) was computed using a Cox proportional risks model. A Wald test was used to evaluate the prognostic value of this variable on each event. The overall survival (OS), metastasis free interval and DFI rates were estimated by the Kaplan–Meier method, and groups were compared using a log-rank test. Multivariate analysis was carried out to assess the relative influence of prognostic factors on OS and DFI, using the Cox stepwise forward procedure (Cox 1972). Significance level was 0.05. Analyses were performed using the R software 2.5.0 version.

Author contributions

LDK set up and performed the main experiments of this project and wrote the article, under supervision of GA. GA designed the concept and carried out the follow-up of the project both experimentally and in the writing. AS performed all statistical analyses concerning the breast cancer patient samples, under the supervision of BA. CB performed the Quantitative RT-PCRs on breast cancer patient samples and HR contributed to life cell imaging and siRNA depletion experiments. XS-G selected and provided the breast cancer patient samples along with their specific medical annotations.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

For more information

Oncomine transcriptome database:
http://www.oncomine.org/main/index.jsp

Progenetix CGH database:
http://www.progenetix.net/progenetix/index.html

References

Ainsztein AM, Kandels-Lewis SE, Mackay AM, Earnshaw WC (1998) INCENP centromere and spindle targeting: identification of essential conserved motifs and involvement of heterochromatin protein HP1. J Cell Biol 143: 1763–1774

Andersson A, Ritz C, Lindgren D, Eden P, Lassen C, Heldrup J, Olofsson T, Rade J, Fontes M, Porwit-Macdonald A, *et al* (2007) Microarray-based classification of a consecutive series of 121 childhood acute leukemias: prediction of leukemic and genetic subtype as well as of minimal residual disease status. Leukemia 21: 1198–1203

Auth T, Kunkel E, Grummt F (2006) Interaction between HP1alpha and replication proteins in mammalian cells. Exp Cell Res 28: 28

Ayoub N, Jeyasekharan AD, Bernal JA, Venkitaraman AR (2008) HP1-beta mobilization promotes chromatin changes that initiate the DNA damage response. Nature 453: 682–686

Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC, Kouzarides T (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. Nature 410: 120–124

Bartova E, Pachernik J, Harnicarova A, Kovarik A, Kovarikova M, Hofmanova J, Skalnikova M, Kozubek M, Kozubek S (2005) Nuclear levels and patterns of histone H3 modification and HP1 proteins after inhibition of histone deacetylases. J Cell Sci 118: 5035–5046

Baudis M, Cleary ML (2001) Progenetix.net: an online repository for molecular cytogenetic aberration data. Bioinformatics 17: 1228–1229

Baxter J, Sauer S, Peters A, John R, Williams R, Caparros ML, Arney K, Otte A, Jenuwein T, Merkenschlager M, *et al* (2004) Histone hypomethylation is an indicator of epigenetic plasticity in quiescent lymphocytes. EMBO J 23: 4462–4472

Berglund P, Landberg G (2006) Cyclin e overexpression reduces infiltrative growth in breast cancer: yet another link between proliferation control and tumor invasion. Cell Cycle 5: 606–609

Bernard P, Maure JF, Partridge JF, Genier S, Javerzat JP, Allshire RC (2001) Requirement of heterochromatin for cohesion at centromeres. Science 294: 2539–2542

Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K (2003) EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J 22: 5323–5335

Cao R, Zhang Y (2004) SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol Cell 15: 57–67

Carroll JS, Prall OW, Musgrove EA, Sutherland RL (2000) A pure estrogen antagonist inhibits cyclin E-Cdk2 activity in MCF-7 breast cancer cells and induces accumulation of p130-E2F4 complexes characteristic of quiescence. J Biol Chem 275: 38221–38229

Cheutin T, McNairn AJ, Jenuwein T, Gilbert DM, Singh PB, Misteli T (2003) Maintenance of stable heterochromatin domains by dynamic HP1 binding. Science 299: 721–725

de Cremoux P, Bieche I, Tran-Perennou C, Vignaud S, Boudou E, Asselain B, Lidereau R, Magdelenat H, Becette V, Sigal-Zafrani B, *et al* (2004) Inter-laboratory quality control for hormone-dependent gene expression in human breast tumors using real-time reverse transcription-polymerase chain reaction. Endocr Relat Cancer 11: 489–495

De Lucia F, Ni JQ, Vaillant C, Sun FL (2005) HP1 modulates the transcription of cell-cycle regulators in Drosophila melanogaster. Nucleic Acids Res 33: 2852–2858

Dialynas GK, Terjung S, Brown JP, Aucott RL, Baron-Luhr B, Singh PB, Georgatos SD (2007) Plasticity of HP1 proteins in mammalian cells. J Cell Sci 120: 3415–3424

Dokmanovic M, Clarke C, Marks PA (2007) Histone deacetylase inhibitors: overview and perspectives. Mol Cancer Res 5: 981–989

Ekwall K, Javerzat JP, Lorentz A, Schmidt H, Cranston G, Allshire R (1995) The chromodomain protein Swi6: a key component at fission yeast centromeres. Science 269: 1429–1431

Ekwall K, Nimmo ER, Javerzat JP, Borgstrom B, Egel R, Cranston G, Allshire R (1996) Mutations in the fission yeast silencing factors clr4+ and rik1+ disrupt the localisation of the chromo domain protein Swi6p and impair centromere function. J Cell Sci 109: 2637–2648

Esteller M (2007) Epigenetic gene silencing in cancer: the DNA hypermethylome. Hum Mol Genet 16: R50–59

Festenstein R, Pagakis SN, Hiragami K, Lyon D, Verreault A, Sekkali B, Kioussis D (2003) Modulation of heterochromatin protein 1 dynamics in primary Mammalian cells. Science 299: 719–721

Fischle W, Tseng BS, Dormann HL, Ueberheide BM, Garcia BA, Shabanowitz J, Hunt DF, Funabiki H, Allis CD (2005) Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. Nature 438: 1116–1122

Folco HD, Pidoux AL, Urano T, Allshire RC (2008) Heterochromatin and RNAi are required to establish CENP-A chromatin at centromeres. Science 319: 94–97

Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, *et al* (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat Genet 37: 391–400

Gilbert N, Boyle S, Sutherland H, de Las Heras J, Allan J, Jenuwein T, Bickmore WA (2003) Formation of facultative heterochromatin in the absence of HP1. EMBO J 22: 5540–5550

Grigoryev SA, Nikitina T, Pehrson JR, Singh PB, Woodcock CL (2004) Dynamic relocation of epigenetic chromatin markers reveals an active role of constitutive heterochromatin in the transition from proliferation to quiescence. J Cell Sci 117: 6153–6162

Guenatri M, Bailly D, Maison C, Almouzni G (2004) Mouse centric and pericentric satellite repeats form distinct functional heterochromatin. J Cell Biol 166: 493–505

Hackett AJ, Smith HS, Springer EL, Owens RB, Nelson-Rees WA, Riggs JL, Gardner MB (1977) Two syngeneic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs578T) and the diploid myoepithelial (Hs578Bst) cell lines. J Natl Cancer Inst 58: 1795–1806

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100: 57–70

Hayakawa T, Haraguchi T, Masumoto H, Hiraoka Y (2003) Cell cycle behavior of human HP1 subtypes: distinct molecular domains of HP1 are required for their centromeric localization during interphase and metaphase. J Cell Sci 116: 3327–3338

Hirota T, Lipp JJ, Toh BH, Peters JM (2005) Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin. Nature 438: 1176–1180

Istomina NE, Shushanov SS, Springhetti EM, Karpov VL, Krasheninnikov IA, Stevens K, Zaret KS, Singh PB, Grigoryev SA (2003) Insulation of the chicken beta-globin chromosomal domain from a chromatin-condensing protein, MENT. Mol Cell Biol 23: 6455–6468

James TC, Elgin SC (1986) Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene. Mol Cell Biol 6: 3862–3872

Jones PA, Baylin SB (2007) The epigenomics of cancer. Cell 128: 683-692
Kellum R, Alberts BM (1995) Heterochromatin protein 1 is required for correct chromosome segregation in Drosophila embryos. J Cell Sci 108: 1419-1431
Kim J, Lee JH, Iyer VR (2008) Global identification of Myc target genes reveals its direct role in mitochondrial biogenesis and its E-box usage in vivo. PLoS ONE 3: e1798
Kirschmann DA, Lininger RA, Gardner LM, Seftor EA, Odero VA, Ainsztein AM, Earnshaw WC, Wallrath LL, Hendrix MJ (2000) Down-regulation of HP1Hsalpha expression is associated with the metastatic phenotype in breast cancer. Cancer Res 60: 3359-3363
Kirschner-Schwabe R, Lottaz C, Todling J, Rhein P, Karawajew L, Eckert C, von Stackelberg A, Ungethum U, Kostka D, Kulozik AE, *et al* (2006) Expression of late cell cycle genes and an increased proliferative capacity characterize very early relapse of childhood acute lymphoblastic leukemia. Clin Cancer Res 12: 4553-4561
Koch B, Kueng S, Ruckenbauer C, Wendt KS, Peters JM (2008) The Suv39h-HP1 histone methylation pathway is dispensable for enrichment and protection of cohesin at centromeres in mammalian cells. Chromosoma 117: 199-210
Kwon SH, Workman JL (2008) The heterochromatin protein 1 (HP1) family: put away a bias toward HP1. Mol Cells 26: 217-227
Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. Nature 410: 116-120
Li Z, Van Calcar S, Qu C, Cavenee WK, Zhang MQ, Ren B (2003) A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. Proc Natl Acad Sci USA 100: 8164-8169
Lin CH, Li B, Swanson S, Zhang Y, Florens L, Washburn MP, Abmayr SM, Workman JL (2008) Heterochromatin protein 1a stimulates histone H3 lysine 36 demethylation by the Drosophila KDM4A demethylase. Mol Cell 32: 696-706
Lomberk G, Bensi D, Fernandez-Zapico ME, Urrutia R (2006) Evidence for the existence of an HP1-mediated subcode within the histone code. Nat Cell Biol 8: 407-415
Maison C, Almouzni G (2004) HP1 and the dynamics of heterochromatin maintenance. Nat Rev Mol Cell Biol 5: 296-304
Maison C, Bailly D, Peters AH, Quivy JP, Roche D, Taddei A, Lachner M, Jenuwein T, Almouzni G (2002) Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. Nat Genet 30: 329-334
Martini E, Roche DM, Marheineke K, Verreault A, Almouzni G (1998) Recruitment of phosphorylated chromatin assembly factor 1 to chromatin after UV irradiation of human cells. J Cell Biol 143: 563-575
Minc E, Allory Y, Courvalin JC, Buendia B (2001) Immunolocalization of HP1 proteins in metaphasic mammalian chromosomes. Methods Cell Sci 23: 171-174
Minc E, Allory Y, Worman HJ, Courvalin JC, Buendia B (1999) Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. Chromosoma 108: 220-234
Misteli T (2007) Beyond the sequence: cellular organization of genome function. Cell 128: 787-800
Mulero-Navarro S, Esteller M (2008) Epigenetic biomarkers for human cancer: the time is now. Crit Rev Oncol Hematol 68: 1-11
Muller H, Bracken AP, Vernell R, Moroni MC, Christians F, Grassilli E, Prosperini E, Vigo E, Oliner JD, Helin K (2001) E2Fs regulate the expression of genes involved in differentiation, development, proliferation, and apoptosis. Genes Dev 15: 267-285
Murzina N, Verreault A, Laue E, Stillman B (1999) Heterochromatin dynamics in mouse cells: interaction between chromatin assembly factor 1 and HP1 proteins. Mol Cell 4: 529-540
Nielsen AL, Oulad-Abdelghani M, Ortiz JA, Remboutsika E, Chambon P, Losson R (2001a) Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins. Mol Cell 7: 729-739
Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D, Firestein R, Cleary M, Jenuwein T, Herrera RE, *et al* (2001b) Rb targets histone H3 methylation and HP1 to promoters. Nature 412: 561-565
Norwood LE, Moss TJ, Margaryan NV, Cook SL, Wright L, Seftor EA, Hendrix MJ, Kirschmann DA, Wallrath LL (2006) A requirement for dimerization of HP1Hsalpha in suppression of breast cancer invasion. J Biol Chem 28: 28
O'Geen H, Squazzo SL, Iyengar S, Blahnik K, Rinn JL, Chang HY, Green R, Farnham PJ (2007) Genome-wide analysis of KAP1 binding suggests autoregulation of KRAB-ZNFs. PLoS Genet 3: e89
Oberley MJ, Inman DR, Farnham PJ (2003) E2F6 negatively regulates BRCA1 in human cancer cells without methylation of histone H3 on lysine 9. J Biol Chem 278: 42466-42476
Obuse C, Iwasaki O, Kiyomitsu T, Goshima G, Toyoda Y, Yanagida M (2004) A conserved Mis12 centromere complex is linked to heterochromatic HP1 and outer kinetochore protein Zwint-1. Nat Cell Biol 6: 1135-1141
Polo SE, Theocharis SE, Klijanienko J, Savignoni A, Asselain B, Vielh P, Almouzni G (2004) Chromatin assembly factor-1, a marker of clinical value to distinguish quiescent from proliferating cells. Cancer Res 64: 2371-2381
Pyeon D, Newton MA, Lambert PF, den Boon JA, Sengupta S, Marsit CJ, Woodworth CD, Connor JP, Haugen TH, Smith EM, *et al* (2007) Fundamental differences in cell cycle deregulation in human papillomavirus-positive and human papillomavirus-negative head/neck and cervical cancers. Cancer Res 67: 4605-4619
Quade BJ, Wang TY, Sornberger K, Dal Cin P, Mutter GL, Morton CC (2004) Molecular pathogenesis of uterine smooth muscle tumors from transcriptional profiling. Genes Chromosomes Cancer 40: 97-108
Quivy JP, Gerard A, Cook AJ, Roche D, Almouzni G (2008) The HP1-p150/CAF-1 interaction is required for pericentric heterochromatin replication and S-phase progression in mouse cells. Nat Struct Mol Biol 15: 972-979
Quivy JP, Roche D, Kirschner D, Tagami H, Nakatani Y, Almouzni G (2004) A CAF-1 dependent pool of HP1 during heterochromatin duplication. EMBO J 23: 3516-3526
Ramaswamy S, Ross KN, Lander ES, Golub TR (2003) A molecular signature of metastasis in primary solid tumors. Nat Genet 33: 49-54
Regnier V, Vagnarelli P, Fukagawa T, Zerjal T, Burns E, Trouche D, Earnshaw W, Brown W (2005) CENP-A is required for accurate chromosome segregation and sustained kinetochore association of BubR1. Mol Cell Biol 25: 3967-3981
Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A, Chinnaiyan AM (2004) ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia 6: 1-6
Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, Liao X, Iglehart JD, Livingston DM, Ganesan S (2006) X chromosomal abnormalities in basal-like human breast cancer. Cancer Cell 9: 121-132
Ritou E, Bai M, Georgatos SD (2007) Variant-specific patterns and humoral regulation of HP1 proteins in human cells and tissues. J Cell Sci 120: 3425-3435
Ryan RF, Schultz DC, Ayyanathan K, Singh PB, Friedman JR, Fredericks WJ, Rauscher FJ 3rd, (1999) KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. Mol Cell Biol 19: 4366-4378
Schmiedeberg L, Weisshart K, Diekmann S, Meyer Zu Hoerste G, Hemmerich P (2004) High- and low-mobility populations of HP1 in heterochromatin of mammalian cells. Mol Biol Cell 15: 2819-2833
Simon JA, Lange CA (2008) Roles of the EZH2 histone methyltransferase in cancer epigenetics. Mutat Res 647: 21-29
Stewart MD, Li J, Wong J (2005) Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment. Mol Cell Biol 25: 2525-2538
Taddei A, Maison C, Roche D, Almouzni G (2001) Reversible disruption of pericentric heterochromatin and centromere function by inhibiting deacetylases. Nat Cell Biol 3: 114-120

Taddei A, Roche D, Bickmore WA, Almouzni G (2005) The effects of histone deacetylase inhibitors on heterochromatin: implications for anticancer therapy? EMBO Rep 6: 520-524

Verschure PJ, van der Kraan I, de Leeuw W, van der Vlag J, Carpenter AE, Belmont AS, van Driel R (2005) In vivo HP1 targeting causes large-scale chromatin condensation and enhanced histone lysine methylation. Mol Cell Biol 25: 4552-4564

Wang C, Ivanov A, Chen L, Fredericks WJ, Seto E, Rauscher FJ 3rd, Chen J, Cress WD, Ryan RF, Schultz DC, *et al* (2005) MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. EMBO J 24: 3279-3290

Wang C, Rauscher FJ 3rd, Cress WD, Chen J (2007) Regulation of E2F1 function by the nuclear corepressor KAP1. J Biol Chem 282: 29902-29909

Watrin E, Schleiffer A, Tanaka K, Eisenhaber F, Nasmyth K, Peters JM (2006) Human Scc4 is required for cohesin binding to chromatin, sister-chromatid cohesion, and mitotic progression. Curr Biol 16: 863-874

Weaver BA, Cleveland DW (2005) Decoding the links between mitosis, cancer, and chemotherapy: the mitotic checkpoint, adaptation, and cell death. Cancer Cell 8: 7-12

Weinberg RA (1996) How cancer arises. Sci Am 275: 62-70

Weinmann AS, Yan PS, Oberley MJ, Huang TH, Farnham PJ (2002) Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis. Genes Dev 16: 235-244

Wheatley SP, Carvalho A, Vagnarelli P, Earnshaw WC (2001) INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis. Curr Biol 11: 886-890

Williams L, Grafi G (2000) The retinoblastoma protein - a bridge to heterochromatin. Trends Plant Sci 5: 239-240

Yamagishi Y, Sakuno T, Shimura M, Watanabe Y (2008) Heterochromatin links to centromeric protection by recruiting shugoshin. Nature 455: 251-255

Yamamoto K, Sonoda M, Inokuchi J, Shirasawa S, Sasazuki T (2004) Polycomb group suppressor of zeste 12 links heterochromatin protein 1alpha and enhancer of zeste 2. J Biol Chem 279: 401-406

Yu YP, Landsittel D, Jing L, Nelson J, Ren B, Liu L, McDonald C, Thomas R, Dhir R, Finkelstein S, *et al* (2004) Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. J Clin Oncol 22: 2790-2799

SUPPLEMENTARY DATA

Heterochromatin Protein 1 alpha: a hallmark of cell proliferation relevant in clinical oncology

DE KONING Leanne, SAVIGNONI Alexia, BOUMENDIL Charlène, REHMAN Haniya, ASSELAIN Bernard, SASTRE-GARAU Xavier, ALMOUZNI Geneviève

Supplementary figure legends:

Supplementary figure S1: HP1α levels progressively increase after exit from quiescence. **A.** BJ primary fibroblasts are arrested in quiescence (G0) by serum starvation and released by adding back serum for the indicated time period. Total protein levels are analyzed by Western blot. CAF-1 p60 (Polo et al, 2004) and Cyclin A are used as markers for cell proliferation. β-actin is used as a loading control. ***B***. Flow cytometry analysis of the cell cycle distribution of the cells shown in *A*.

Supplementary figure S2: HP1α expression does not visibly vary during the cell cycle. ***A***. HeLa cervical carcinoma cells are synchronized in the cell cycle by release from double thymidine block (G1, G1/S, S, G2) and by nocodazole (M). Total protein extracts are analyzed for HP1α expression levels using Western Blot. β-actin is used as a loading control and Cyclin A as a marker for G2/S phases. CAF-1 p60 is shown for comparison. ***B***. Flow cytometry cell cycle analysis of the synchronized cells used in *A*. ***C***. BJ foreskin primary fibroblasts are synchronized in the cell cycle by release from double thymidine block (G1, G1/S, S, G2) and by nocodazole (M). HP1α protein levels are analyzed in total cell extracts by Western Blot. β-actin is used as a loading control and Cyclin A as a marker for G2/S phases. CAF-1 p60 is shown for comparison. ***D***. Flow cytometry analysis of the cell cycle distribution of the synchronized cells shown in *C*. ***E***. HP1α mRNA levels in synchronized BJ cells, as determined by quantitative RT-PCR. Levels are normalized to the reference gene ribosomal protein P0-like protein (RPLPO) (de Cremoux et al, 2004) and levels in asynchronously proliferating cells are set to 100%. CAF-1 p60 and CAF-1 p150 levels are shown for comparison (Polo et al, 2004). Error bars represent data from two independent experiments.

Supplementary figure S3: Levels of HP1 proteins in soluble and chromatin-bound nuclear fractions. ***A.*** Scheme depicting the extraction procedure to obtain soluble and chromatin-bound nuclear fractions of the breast cancer cell line Hs578T (T) and the healthy mammary cell line Hs578Bst (Bst), which are derived from the same patient (Hackett et al, 1977). ***B.*** HP1α, β and γ protein levels are analyzed by Western Blot in soluble and chromatin-bound nuclear extracts from the cancer cells Hs578T (T) and the healthy cells Hs578Bst (Bst). Loading was corrected in a manner to deposit an identical amount of cells ($2x10^5$ and $4x10^5$ cells for each extract). We revealed several classical loading controls. While the fractionation of α-tubulin and the presence of β-actin differ slightly between tumoral and healthy cells, GAPDH reflects best the identical loading under these experimental conditions. Cyclin A and CAF-1 p60 are shown for comparison.

Supplementary figure S4: Nuclear distribution of HP1α, but not H3K9me3, is altered in breast cancer cells compared to healthy mammary cells. ***A***. Immunofluorescence staining of HP1α in the breast cancer cell line Hs578T (T) and in the non-tumoral mammary cell line Hs578Bst (Bst). Costaining for centromeres, using CREST patient serum, reveals a partial although incomplete colocalization with the HP1α spots in Hs578T breast cancer cells. DNA is stained with DAPI. Indicated below the microscopy images are the mean percentages of cells showing clearly distinguishable HP1α spots in the breast cancer cell line Hs578T (T) and in the healthy mammary cell line Hs578Bst (Bst). Standard error, based on two independent experiments, is indicated. **B.** Nuclear distribution of the H3K9me3 histone modification, compared to HP1α in the tumoral (T) and non-tumoral (Bst) mammary cells. DNA is revealed with DAPI. **C.** Costaining of HP1α with Cdt1 (indicative for G1 phase), Cyclin A (indicative for S and G2 phase), or histone H3 phosphorylated on S10 (H3S10P; indicative for G2 and M phase), does not reveal a correlation between cell cycle status and spotted HP1α patterns. Scale bars: 10 μm.

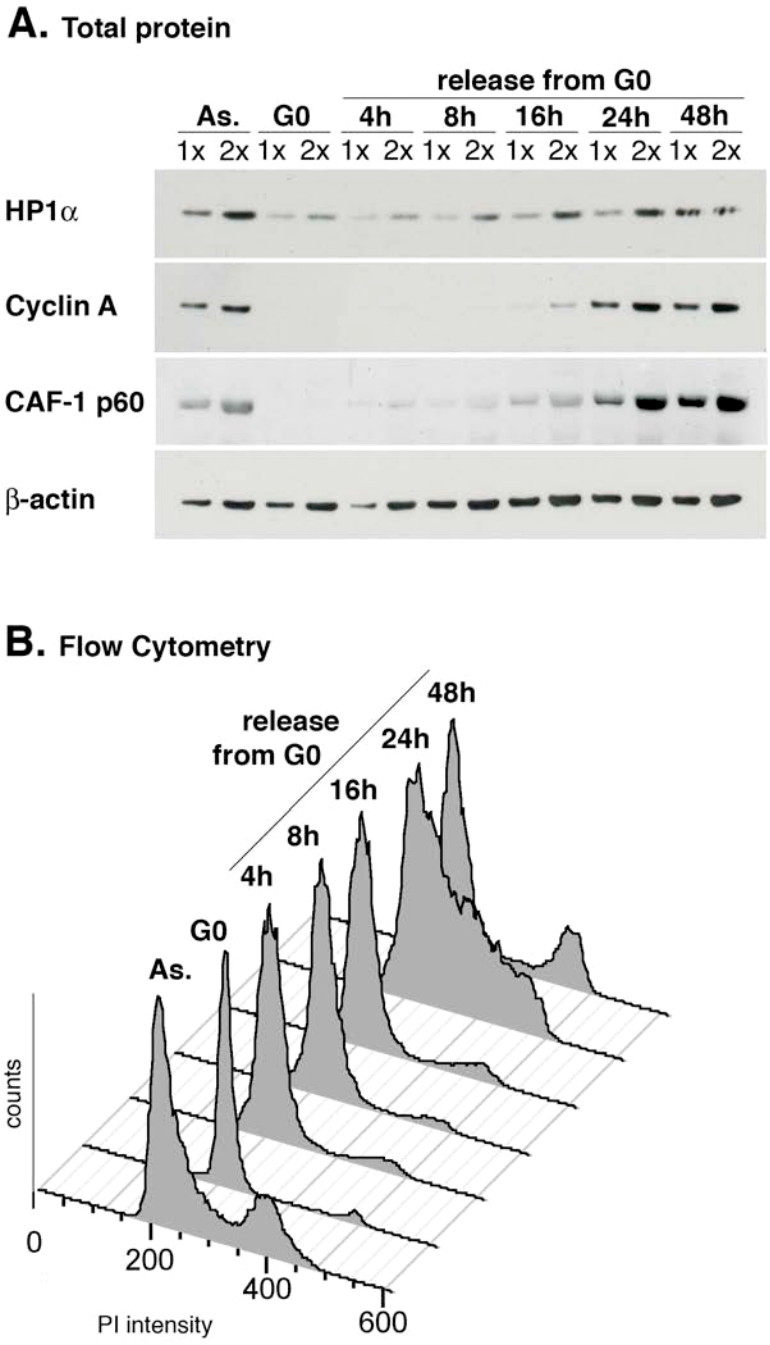
Supplementary figure S5: Downregulation of HP1α in primary cells affects (pro)metaphase. ***A.*** BJ primary fibroblasts are transfected by nucleofection with control siRNA (siGFP) or two different siRNA sequences targeting HP1α (α.1 or α.3). Total protein levels are analyzed by Western blot. HP1β and HP1γ are revealed to show the specificity of the siRNA for the HP1α isoform. ***B.*** Flow cytometry analysis of the cell cycle distribution of cells shown in *A*, 72 hours after transfection with control siRNA (siGFP) or two different

siRNA sequences targeting HP1α (siHP1α.1/3). ***C.*** Profiles of mitotic cells are analyzed by immunofluorescence 72h after transfection. Enrichment in mitotic cells is achieved by a 6 hours release from a 12h thymidine block. Depicted are typical mitotic profiles (upper panel) and the presence of each mitotic phase as a percentage of the total number of mitotic cells (graph). Error bars represent standard errors, based on three independent experiments (n>100 for each) of which two were blind counted. **D.** Time of mitosis after two subsequent transfections with control siRNA (siGFP) or HP1α siRNA (siHP1α.3), as determined by live cell imaging. DNA is visualized by expression of H2B-Cherry (red). Images were acquired every 20 minutes for a period of 24h, starting 48h after the second transfection. The time of mitosis (upper graph) was defined as the time between the condensation of DNA and the establishment of two distinct cells. The average number of images depicting mitotic cells in (pro-)metaphase, before the onset if chromosome segregation, was also determined (lower graph). Means of three independent experiments are depicted, representing a total number of 130 and 87 mitotic cells for siGFP and siHP1α, respectively. P-values were determined with a test of Mann-Whitney Wilcoxon. Mean H2B-cherry transfection efficiencies were 37% and 39% for siGFP and siHP1α, respectively.

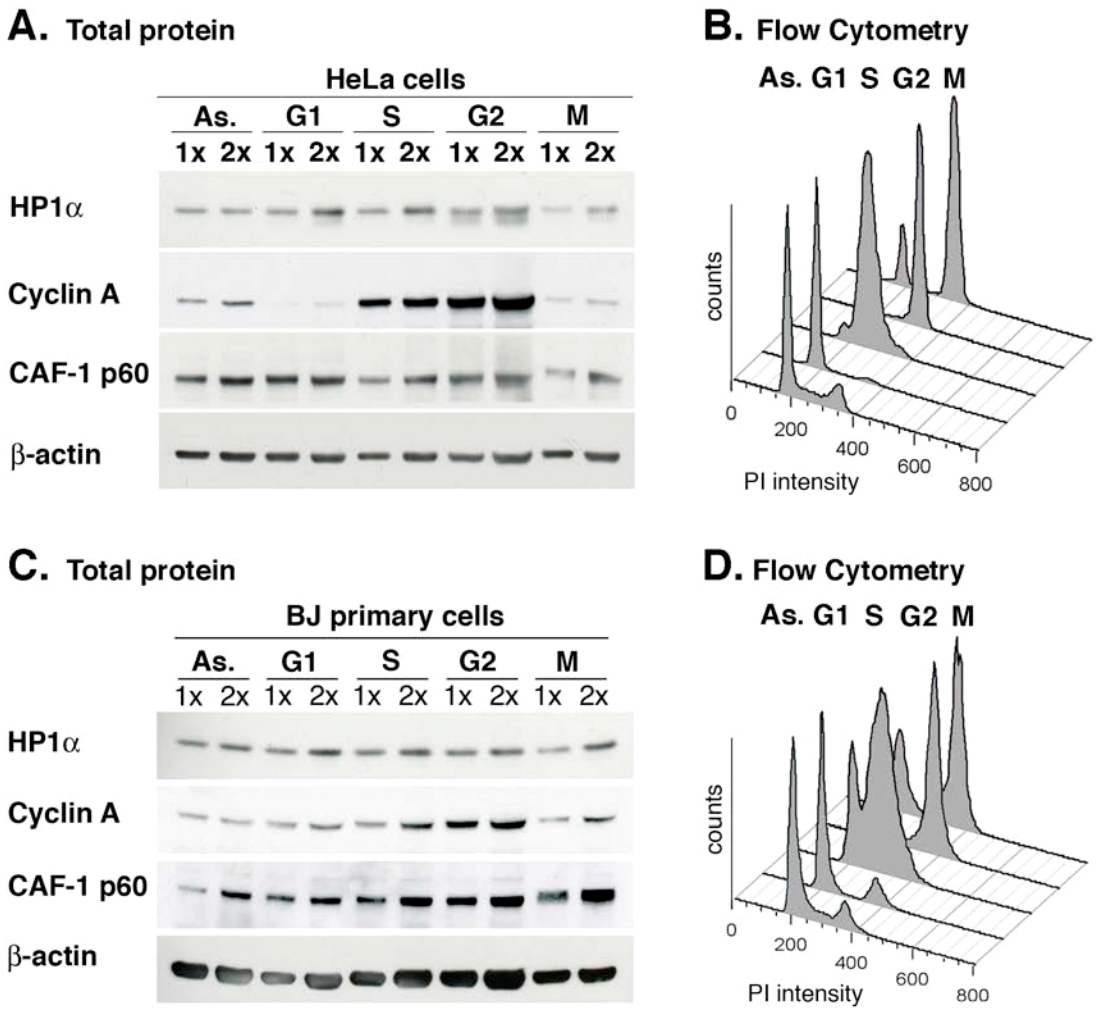
Supplementary figure S6: HP1α mRNA is overexpressed in multiple types of cancer and correlates with clinical and molecular data. ***A.*** Boxplot representation of microarray expression profiles of HP1α in different types of cancer (red/green) compared to normal tissue (blue). Boxes represent the 25th-75th percentile; brackets: range; black line: median; black dots: outliers; n: sample number. p-values are based on Student's T-test. Results are analyzed and plotted using ONCOMINE (Rhodes et al, 2004), and exploit data from transcriptome studies on different tumor types (Andersson et al, 2007; Pyeon et al, 2007; Quade et al, 2004; Ramaswamy et al, 2003; Richardson et al, 2006; Yu et al, 2004). ***B.*** *Left panel:* Correlation of HP1α expression levels with the time to relapse in childhood acute lymphoblastic leukemia (Kirschner-Schwabe et al, 2006). Relapse occurs very early (within 18 months after initial diagnosis), early (>18 months after initial diagnosis but <6 months after cessation of frontline treatment) or late (>6 months after cessation of frontline treatment). Boxplot representation as in *A. Right panels:* Scatter plot representation of the positive correlation of HP1α expression levels with CAF-1 p150 and the proliferation marker Ki67 expression levels in childhood acute lymphoblastic leukemia (Kirschner-Schwabe et al,

2006). Red line: line of best fit; R: Pearson's correlation coefficient. Results are analyzed and plotted using ONCOMINE (Rhodes et al, 2004).

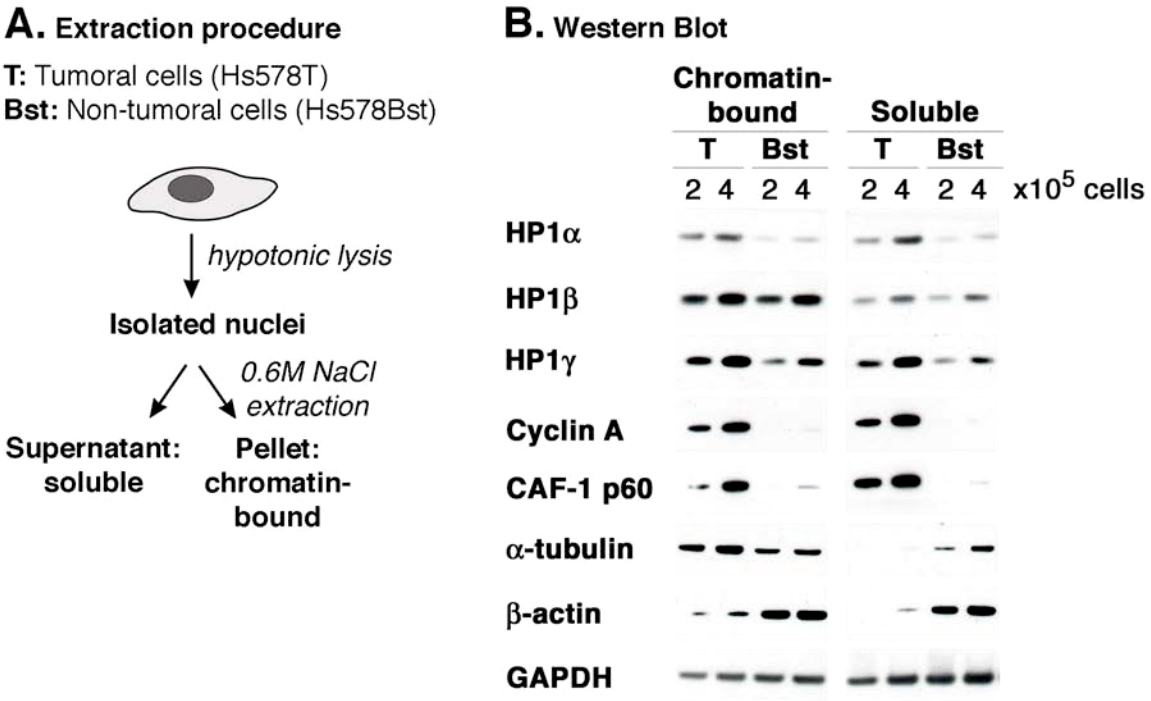
Supplementary figure S7: Overexpression of HP1α in human tumor samples is detected with a polyclonal antibody and is not accompanied by altered H3K9me3 staining. A. Immunohistochemistry staining, using a polyclonal HP1α antibody, of a tissue array containing frozen human tissue sections from tumoral and healthy origin. Tissues were counterstained with hematoxylin. **B.** A frozen human tissue array (same batch as in A.) is stained for H3K9me3 by immunohistochemistry and counterstained with hematoxylin. Scale bars: 50 μm.



Supplementary figure S1



Supplementary figure S2



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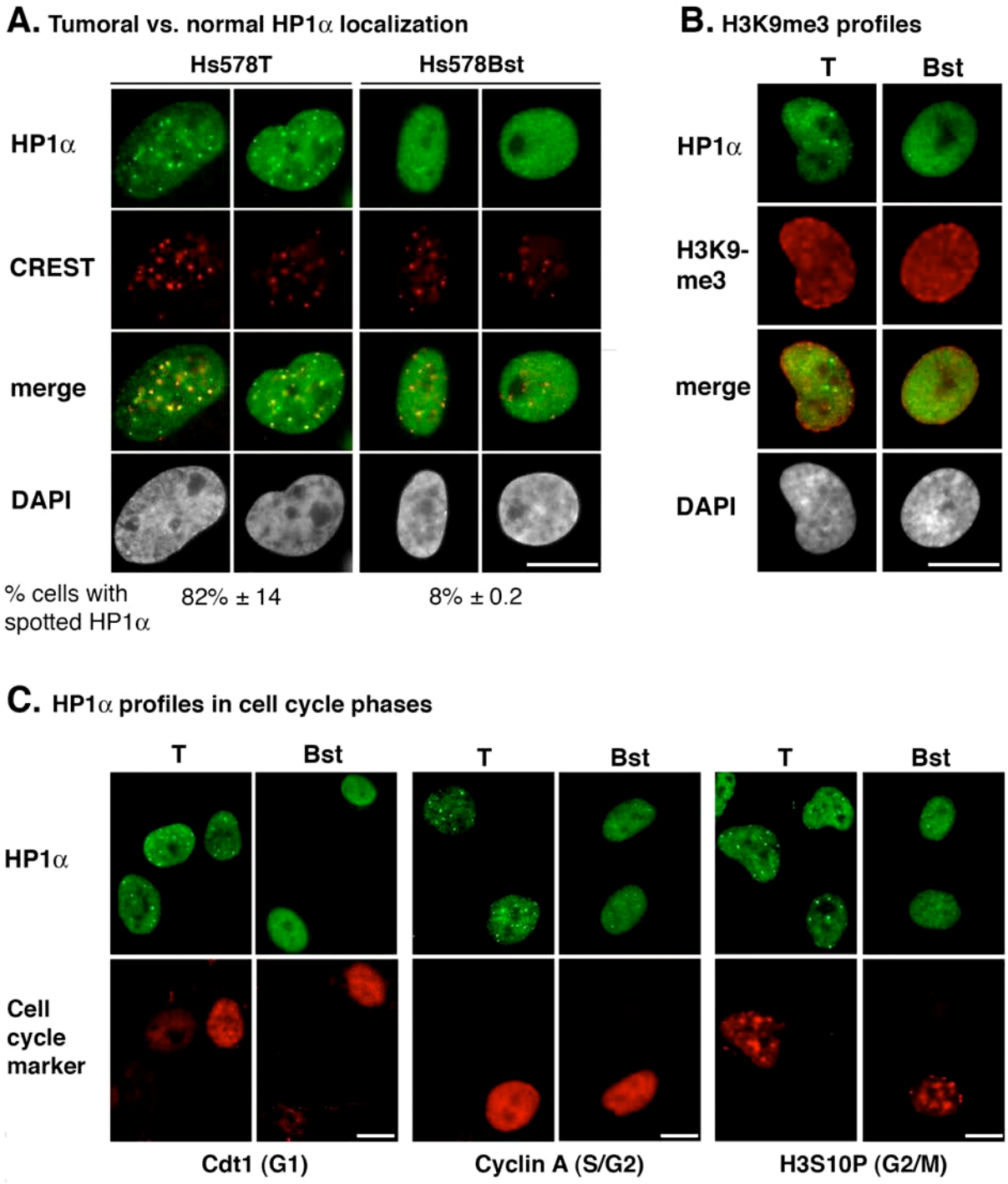

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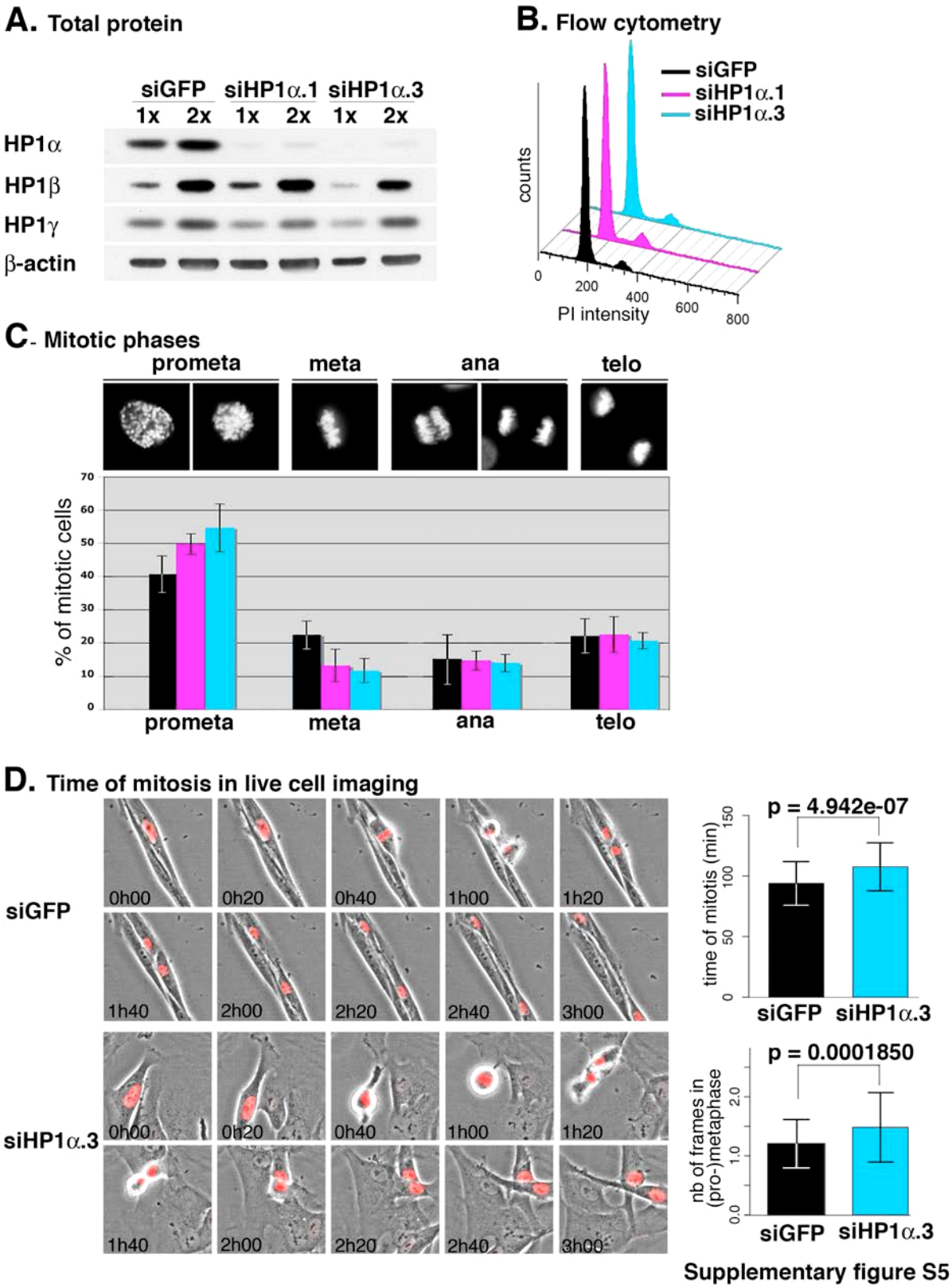

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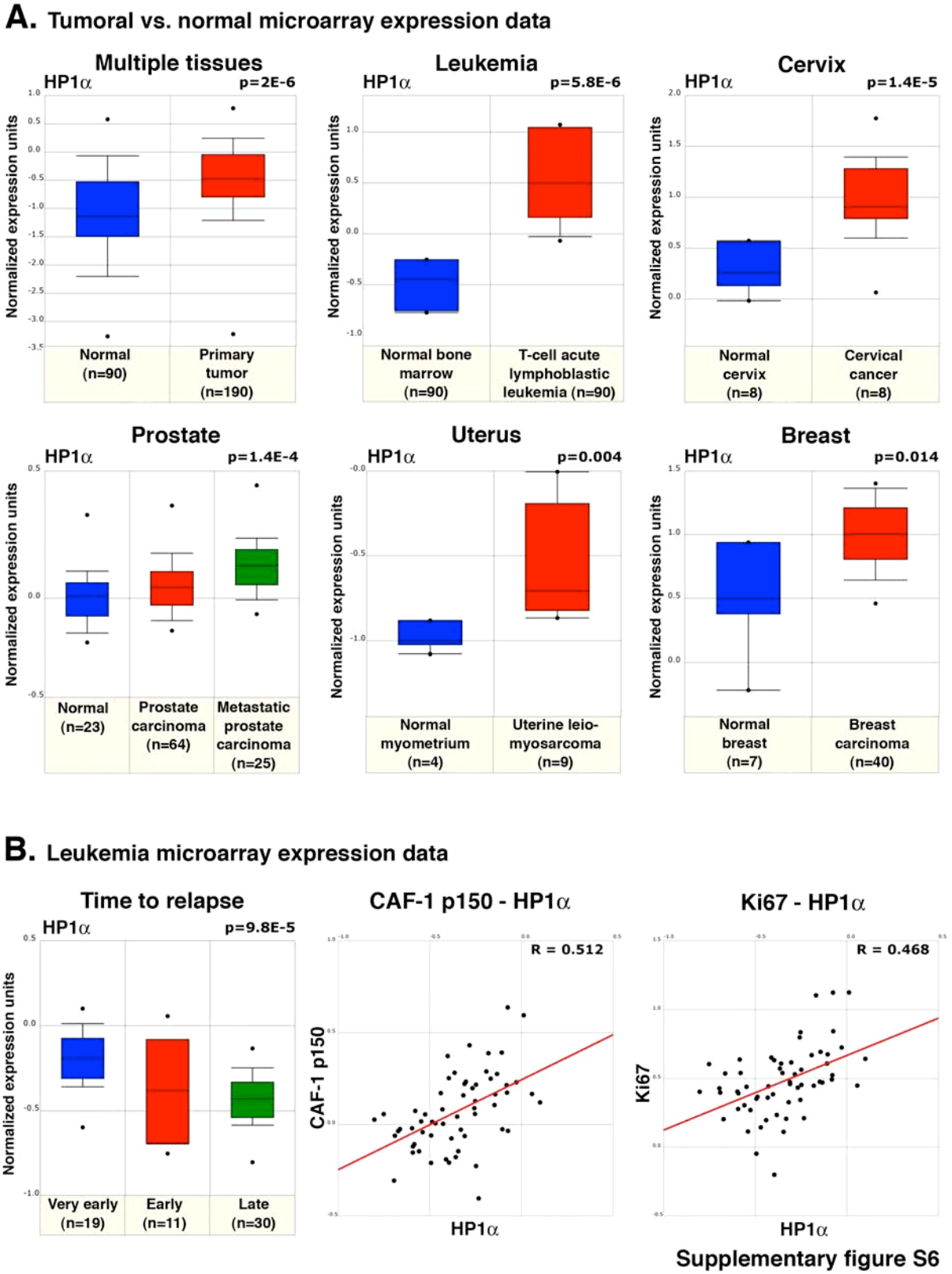

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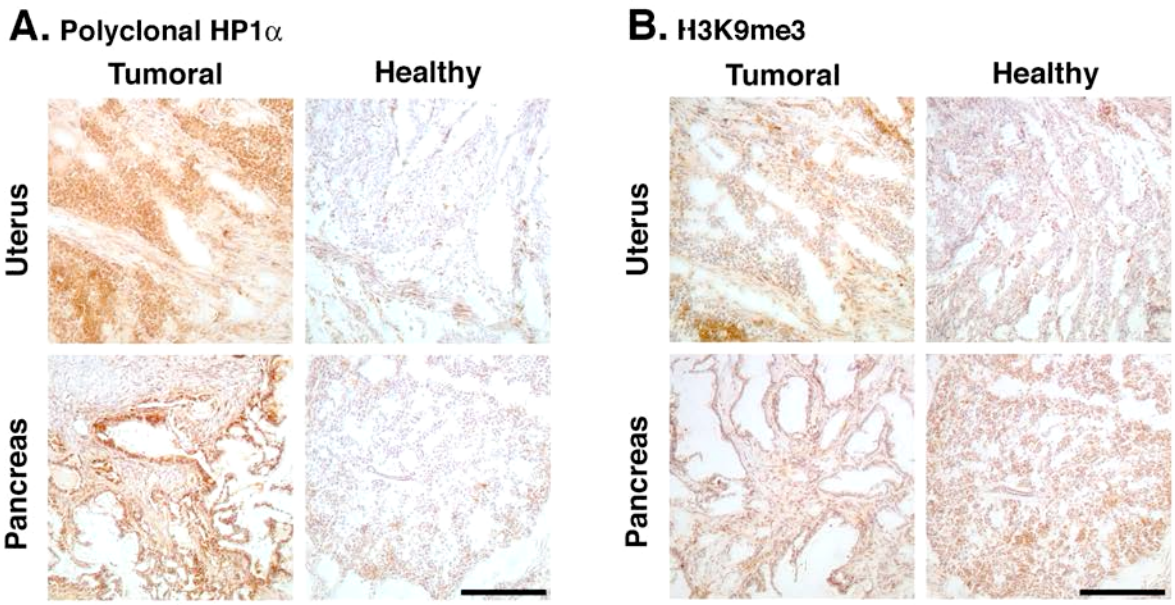

Supplementary figure S7

Table I: Description of the patient samples		
Age	median: 53 (range: 26-70)	
Menopaused	yes	54%
	no	46%
Histological type	ductal	88%
	lobular	9%
	papillary	1%
	tubular	1%
Size classification	T0/T1	62%
	T2	38%
Tumor size (mm)	median: 18 (range: 6-50)	
Grade EE	I	33%
	II	42%
	III	25%
Estrogen receptor	positive	86%
	negative	14%
Progesteron receptor	positive	69%
	negative	31%
Mitotic index	median: 8 (range: 0-105)	
Ki67	<= 15	52%
	15 - 40	24%
	> 40	24%
Adjuvant chemotherapy	no	93%
	yes	7%

Manuscript #2 (in preparation):

Distinct functions for the two Asf1 isoforms in relation to proliferation

CORPET Armelle, DE KONING Leanne, TOEDLING Joern, SERVANT Nicolas, SAVIGNONI Alexia, BOUMENDIL Charlène, BARILLOT Emmanuel, ASSELAIN Bernard, SASTRE-GARAU Xavier and ALMOUZNI Geneviève.

The importance of HP1α in breast cancer prognosis (publication #1) has led us to study other factors involved in chromatin organization that are related to cell proliferation and/or the proliferation marker CAF-1 p60 (Polo et al, 2004). In this manuscript, our main focus concerns the histone chaperone Anti-silencing function 1 (Asf1). As discussed in the introduction, Asf1 can serve as a histone donor for the histone chaperones CAF-1 and HIRA. Together with CAF-1, Asf1 can promote chromatin assembly coupled to DNA synthesis *in vitro* (Mello et al, 2002). In line with a role in DNA replication, depletion of Asf1 leads to S-Phase defects in various organisms (Mousson et al, 2007).

Human cells contain two Asf1 isoforms, Asf1a and Asf1b. They both interact with the Minichromosome Maintenance (MCM) proteins 2-7, which constitute the putative helicase involved in DNA unwinding ahead of the replication fork (Groth et al, 2007a). Together, the two isoforms are thought to promote replication fork progression by assisting histone eviction and deposition (Groth et al, 2007a). In addition, upon replication stress, the two isoforms could buffer the transient accumulation of histones (Groth et al, 2005). However, these data have addressed the functions of the two isoforms together and, to date, the specific function of each isoform in relation to cell proliferation has not been studied.

Interestingly, while both Asf1a and b interact with CAF-1 (Mello et al, 2002), only Asf1a interacts with HIRA (Daganzo et al, 2003; Tagami et al, 2004; Zhang et al, 2005). Thus, Asf1b could specifically be involved in CAF-1 dependent chromatin assembly coupled to DNA synthesis. In addition, Asf1b protein levels are highest in human testis

and other human proliferative tissues such as thymus, small intestine or colon (Umehara & Horikoshi, 2003), while Asf1a shows a more ubiquitous expression profile. These data suggest that the two Asf1 isoforms might not be regulated similarly. Therefore, we have addressed how the two Asf1 isoforms relate to cell proliferation and tumorigenesis. For this, we have used a combination of cell line models and human patients samples. Armelle Corpet, with help from Joern Toedling, Nicolas Servant and Emmanuel Barillot, performed genome-wide transcriptome data analyses. I provided Armelle Corpet with expertise and material from different primary and tumoral cell line models. In addition, with technical help from Charlène Boumendil, I set up and performed all experiments on the breast cancer patient samples, provided by Xavier Sastre-Garau, and I initiated the interaction with Alexia Savignoni and Bernard Asselain for the statistical analysis. Therefore, I will here particularly focus on the results obtained in these cell line models and in the breast cancer patient samples.

Results

Asf1b is more related to cell proliferation than Asf1a

Using a siRNA approach specific for either Asf1a or Asf1b, followed by genome-wide transcriptome analysis, Armelle Corpet and Joern Toedling were able to demonstrate that depletion of Asf1b, but not Asf1a, mainly affects the expression of genes involved in cell proliferation. In line with this observation, we showed that human tumoral or primary cells that are arrested in quiescence show an important downregulation in the levels of Asf1b mRNA and protein compared to proliferative cells, while the levels of Asf1a show little difference. Finally, the depletion of Asf1b, but not Asf1a, gave rise to aberrant nuclear shapes and micronuclei formation. Thus, these observations indicate that the expression of Asf1b is highly associated with cell proliferation, which is not the case for Asf1a.

Asf1b is a proliferation marker for breast cancer prognosis

Given the differential expression of Asf1a and b in relation to cell proliferation, we considered their expression levels in the context of cancer cells. For this, we analyzed the mRNA levels of Asf1a and Asf1b in cryopreserved human breast cancer samples by quantitative RT-PCR. We used the annotated selection of early stage node negative and metastasis-free invasive breast carcinomas, in which we previously determined HP1α

expression (see publication #1). These patients, for whom the disease progression is hard to predict, would benefit from new prognostic markers for the indication of adjuvant chemotherapy. In addition to Asf1a and b, we analyzed the expression of CAF-1 p60 and p150. Indeed, we aimed to determine if CAF-1 p150 and p60 behave similarly and we wished to compare the prognostic value of the different factors. Since the patients have been followed for > 10 years, the prognostic value can be reliably assessed.

As shown in Figure 1A, we found that the expression levels of CAF-1 p60 and p150 are highly related, which is consistent with their similar expression profiles in cell line models (Polo et al, 2004). They predict disease outcome in a similar manner and, at least in this series of patient samples, p150 does not seem to have an additional prognostic value compared to p60 (not shown). Therefore, we here focus on p60. High CAF-1 p60 levels are indicative for reduced global patient survival (p-value=0.035) and a reduced metastasis-free interval (p-value=0.03). This is the first report showing that CAF-1 is significantly associated with disease outcome in breast cancer. Therefore, CAF-1 is a marker not only for the diagnosis (Polo et al, 2004), but also for the prognosis of breast cancer. However, it must be kept in mind that CAF-1, and other proliferation markers, are not necessarily prognostic factors in all types of breast cancers, as exemplified by the highly specific subgroup of young breast cancer patients in which we measured CAF-1 expression (see appendix #3: Marc A Bollet, Alexia Savignoni, Leanne De Koning, Carine Tran Perennou, Catherine Barbaroux, Armelle Degeorges, Brigitte Sigal-Zafrani, Geneviève Almouzni, Paul Cottu, Rémy Salmon, Alain Fourquet, Patricia de Cremoux, *Is tumour aromatase expression prognostic for local control in young breast cancer patients after breast-conserving treatment?*, Breast Cancer Research).

Concerning the expression of the two Asf1 isoforms, Asf1b expression is highly correlated to the expression of CAF-1 p60 (p-value=2.4 x 10^{-10}), while Asf1a correlates less well with p60 (p-value=2.5 x 10^{-3}) (Figure 1A). Interestingly, the two Asf1 isoforms show very limited correlation in their expression (p-value=0.017). These data again show that Asf1b seems more related to cell proliferation than Asf1a. In addition, Asf1b, CAF-1 and the proliferation marker Ki67 all significantly correlate with large tumor size, increased mitotic index and high tumor grade, while Asf1a does not (Figure 1B). In conclusion, the expression of Asf1b, but not Asf1a, is proliferation-dependent and

correlates with clinico-pathological data. Therefore, we assessed the prognostic value of Asf1b. As shown in figure 2, high Asf1b expression levels are predictive for decreased global survival (p-value=0.03).

The statistical analyses to compare the expression of these factors with the expression levels of HP1α are currently ongoing. In addition, we aim to compare the prognostic value of all factors, using multivariate statistical analysis, in order to assess which factor(s) can independently predict disease progression and global survival in the most accurate manner. For this, we will determine cut-off values for each of the genes, as has been done for HP1α, in order to compare the most prognostic threshold values in each case. The obtained results, and the applied cut-off values will be validated in a second series of patient samples (ongoing), which have been extracted in 1996 and are in all clinico-pathological aspects comparable to the samples from 1995 used here.

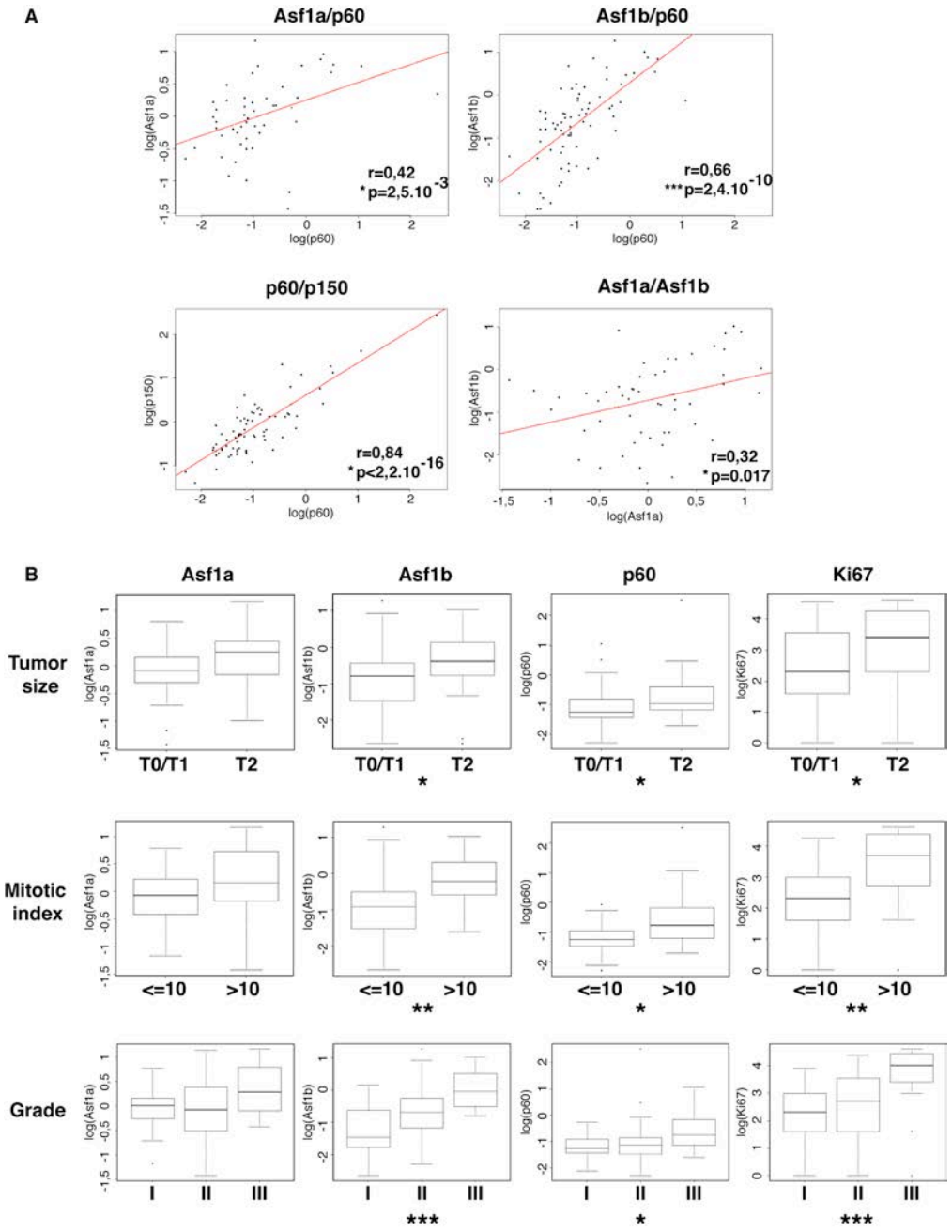

Figure 1: Expression of Asf1 and CAF-1 in early stage breast cancer samples. **A.** Correlations among the different factors analyzed, using Pearsons correlation coefficient. **B.** Boxplots representing the logarithmic expression of Asf1, CAF-1 and Ki67 according to tumor size, the mitotic index and the tumor grade. Boxes represent the 25–75th percentile; brackets: range; black line: median; black dots: outliers. Asterisks are indicative of significant correlations.

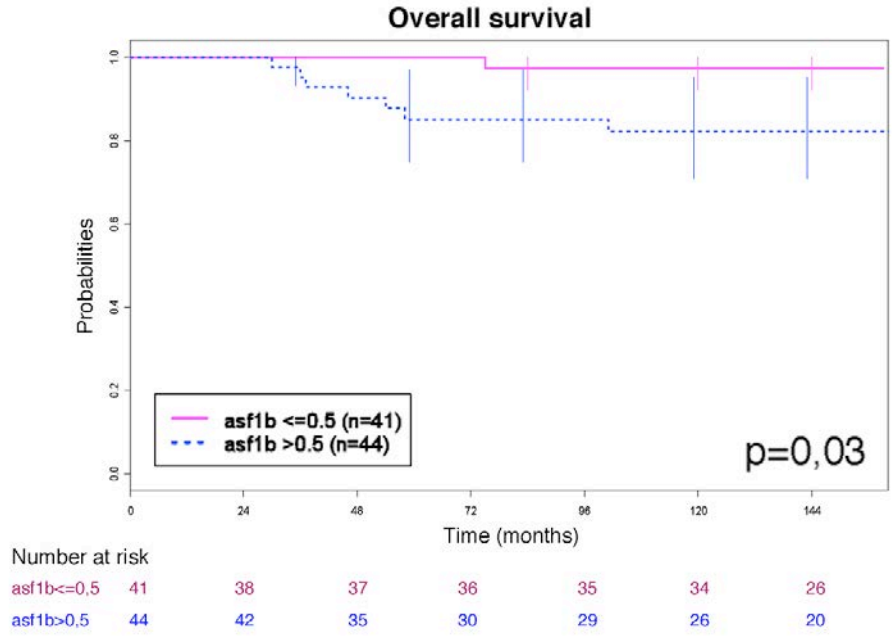

Figure 2: Univariate Kaplan–Meier survival curves in patients expressing high (>0.5) or low (<0.5) levels of Asf1b. The median expression level was used as the cut-off value. The number of patients at risk at each time point is indicated below the graphs.

Manuscript #3 (in preparation):

Quiescent human cells show distinct chromatin restoration kinetics upon UV damage

Leanne DE KONING, Sophie E. POLO, Danièle ROCHE and Geneviève ALMOUZNI

In this study, based on earlier investigations in the laboratory, we address how quiescent cells can deal with chromatin dynamics coupled to DNA repair. Indeed, quiescent cells constitute the major fraction of an adult organism and need to preserve DNA integrity during life span. Yet, the integrity of DNA is continuously challenged by endogenous and exogenous sources. In the context of cancer treatments, genotoxic radiations or drugs are administered, which do not only affect proliferative tumor cells but also healthy quiescent cells. A differential repair capacity between proliferating and quiescent cells can thus affect cancer treatment efficiency and toxicity.

To study chromatin dynamics coupled to DNA repair, we used UV lesions as a model system. As discussed in the introduction, UV irradiation induces two types of lesions: 6,4-PP lesions, which mainly occur in linker DNA, and CPD lesions, which can occur both in nucleosomal and linker DNA (Mitchell et al, 1990; Niggli & Cerutti, 1982; Suquet et al, 1995; Williams & Friedberg, 1979). The repair of UV lesions involves a step of chromatin restoration in which CAF-1 plays a major role (Gaillard et al, 1996; Moggs et al, 2000; Polo et al, 2006). However, CAF-1 is downregulated in quiescent cells (Polo et al, 2004), raising the intriguing question of how quiescent cells deal UV repair. More specifically, we focused on how the late steps of CPD and 6,4-PP repair, including chromatin restoration, can occur in UV-irradiated quiescent human cells. We will here summarize the main results. More detailed information can be found in the corresponding manuscript.

Results

1. UV irradiation does not induce CAF-1 expression in quiescent cells

Using different types of primary and tumoral cell lines, we confirm that CAF-1 p60 and p150 levels are indeed downregulated in quiescence. Importantly, UV irradiation does not result in an upregulation of CAF-1 levels.

2. Low CAF-1 levels are sufficient for a local recruitment in quiescence

To test if CAF-1 fulfills a function in UV repair in quiescent cells, like in proliferating cells, we analyzed its kinetics after UV irradiation. We demonstrate that the low amounts of CAF-1 p60 and p150 are sufficient for its local recruitment at UV damage sites, suggesting a role for CAF-1 in repair-coupled chromatin dynamics in quiescent cells. The recruitment of CAF-1 occurs with similar kinetics as in proliferating cells. However, its dissociation from chromatin is delayed, suggesting that the requirement for chromatin assembly activity is delayed or prolonged.

3. Late steps of CPD, but not 6,4-PP, repair is delayed in G0 cells

To understand why CAF-1 dissociation is delayed in quiescent cells, we studied the DNA repair kinetics in quiescent cells. While the repair of 6,4-PP lesions occurs with similar kinetics in proliferating and quiescent cells, the repair of CPD lesions, specifically, is delayed in quiescence. In addition, cells synchronized in G1 repair CPD lesions like asynchronous cells (Fig 4C), suggesting that the delayed CPD repair is specific for the quiescent state. Using markers for different steps of NER, we show that the repair of CPD lesions is specifically impaired in late steps of the NER pathway, which comprises concomitant DNA synthesis and chromatin reassembly.

Our findings evidence that the repair of CPD lesions, specifically, is impaired in quiescent cells. This impairment seems to occur in the late steps of the NER pathway, coinciding with DNA synthesis and chromatin reassembly. While a defect in DNA synthesis would equally affect the repair of both 6,4-PP and CPD lesions, the chromatin assembly requirement might be different for the two types of lesions. Indeed, while 6,4-PP lesions are mostly found in linker DNA, CPD lesions can occur both in linker and in nucleosomal DNA (Gale & Smerdon, 1990; Mitchell et al, 1990; Niggli & Cerutti, 1982; Suquet et al, 1995). One can readily imagine that that the repair in linker DNA might require less extensive chromatin dynamics than the repair in nucleosomal DNA. Thus, we hypothesize that quiescent cells show impaired repair of nucleosomal CPD lesions, specifically, for which the nucleosome assembly activity could be of particular

importance. We currently aim to test this hypothesis using *in vitro* repair and chromatin assembly assays.

Quiescent human cells show distinct chromatin restoration kinetics upon UV damage

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Running title: Chromatin response to UV damage in quiescent cells

Keywords: **quiescence, UV irradiation, Nucleotide Excision Repair, chromatin assembly.**

ABSTRACT

To preserve both genome integrity and chromatin organization is a major issue in quiescent cells. Indeed, these cells need to maintain cell function and identity over a long period. In proliferating cells, the complex Chromatin Assembly Factor 1 (CAF-1) is involved in the reassembly of chromatin coupled to the repair of DNA lesions. However, CAF-1 levels are massively downregulated in quiescence, raising the intriguing question of how quiescent cells deal with chromatin restoration coupled to DNA repair. Here, we use UV irradiation as a means to study this issue. UV irradiation induces mainly cyclobutane pyrimidine dimers (CPD), which can occur both in nucleosomal and linker DNA, and 6-4-photoproducts (6,4-PP), which are largely restricted to linker DNA. We show that, in quiescent cells, the limited amounts of CAF-1 are sufficient to induce a detectable recruitment to local UV damage sites, as observed in proliferating cells. Yet, CAF-1 dissociation is delayed in quiescent cells, in parallel with a delayed repair of cyclobutane pyrimidine dimers (CPD) lesions, specifically. Indeed, 6,4-PP lesions are repaired normally. Only late steps of CPD repair, including DNA synthesis and chromatin reassembly, are affected. Since impaired DNA synthesis is expected to affect both 6,4-PP and CPD repair, we put forward that the localization of CPD lesions within nucleosomes constitutes a major challenge in quiescent cells, both in terms of repair and chromatin restoration.

INTRODUCTION

Cellular quiescence, defined as reversible or irreversible cell cycle exit, is a distinct cell status (Coller, 2007), characterized by unique gene expression profiles (Coller et al, 2006; Coppock et al, 1993; Iyer et al, 1999; Schneider et al, 1988; Venezia et al, 2004; Williams & Penman, 1975) and a distinct chromatin organization (Ait-Si-Ali et al, 2004; Baxter et al, 2004; Grigoryev et al, 2004; Meshorer et al, 2006; Talasz et al, 2005; Zinner et al, 2006). It is the most prominent cell status encountered in tissues of adult organisms. Quiescent cells need to preserve DNA integrity and chromatin organization to ensure cell identity over a long period, in the absence of any known checkpoint to survey genome and chromatin integrity.

Genome integrity and chromatin organization are challenged when cells are exposed to DNA damaging agents. Ultra-Violet (UV) irradiation mainly induces covalent bonds between two adjacent pyrimidines, thus creating pyrimidine dimers. Structurally, two types of pyrimidine dimers can be distinguished: cyclobutane pyrimidine dimers (CPD) and 6,4-photoproducts (6,4-PP). Micrococcal nuclease digestion, followed by high pressure liquid chromatography (HPLC) or detection with specific antibodies, showed that CPDs are randomly distributed between nucleosomal core DNA and linker DNA in UV-irradiated confluent human skin fibroblasts (Mitchell et al, 1990; Niggli & Cerutti, 1982; Suquet et al, 1995; Williams & Friedberg, 1979). In contrast, identical methods showed that 6,4-PP lesions are approximately six fold more abundant in nuclease-sensitive linker DNA (Mitchell et al, 1990; Suquet et al, 1995). Thus, these two lesions could challenge chromatin organization in distinct manners.

Mammalian cells can repair UV lesions by a dedicated repair pathway, nucleotide excision repair (NER), which involves DNA unwinding and excision of a stretch of nucleotides comprising the dimer, followed by DNA resynthesis. How NER operates within a chromatin context and how chromatin organization is preserved is still a matter of debate. The "access-repair-restore" model proposes that a local disruption of chromatin organization occurs to make the lesion accessible for the repair machinery (Green & Almouzni, 2002; Groth et al, 2007b; Smerdon, 1991). Next, DNA repair and chromatin restoration occur, preferentially in a coordinated manner. While several factors are thought to provide access to the DNA lesion (Gong et al, 2005), only one factor has been convincingly implied in the repair-coupled chromatin restoration: the protein complex Chromatin Assembly Factor 1 (CAF-1).

CAF-1 is a highly conserved protein complex composed of three subunits: p150, p60 (Kaufman et al, 1995a) and p48 (Verreault et al, 1996) in human cells. The complex is involved in chromatin assembly coupled to DNA synthesis, through the direct interaction of p150 with the polymerase accessory factor PCNA (Shibahara & Stillman, 1999). Thus, CAF-1 operates not only at sites of DNA replication (Shibahara & Stillman, 1999; Stillman, 1986) but also in the context of DNA repair (Gaillard et al, 1996; Moggs et al, 2000; Polo et al, 2006). Indeed, in proliferating cells, CAF-1 is recruited to local UV damage sites *in vivo* (Green & Almouzni, 2003b; Martini et al, 1998). The complex specifically associates with the replicative histone variant H3.1 (Tagami et al, 2004) and mediates deposition of this histone variant at sites of DNA damage (Polo et al, 2006). Since CAF-1 is dispensable for a functional DNA repair pathway in both yeast and human cells (Game & Kaufman, 1999; Kim & Haber, 2009; Polo et al, 2006), the complex is thought to play a role in the restoration of chromatin structure after DNA repair (Green & Almouzni, 2002; Smerdon, 1991). Chromatin restoration by CAF-1 is believed to occur not only after NER, but also in the context of other repair pathways that involve DNA synthesis (Lewis et al, 2005; Moggs et al, 2000; Nabatiyan et al, 2006; Okano et al, 2003). Furthermore, the increased UV sensitivity of proliferating yeast strains lacking CAF-1 (Game & Kaufman, 1999; Kaufman et al, 1997) points to the importance of CAF-1 in the recovery from UV damage.

Till now, the role of CAF-1 in chromatin assembly upon DNA damage has been studied exclusively in proliferating cells. However, quiescent and proliferating cells can encounter similar genotoxic stresses and should repair and restore chromatin organization in a similar manner in order to ensure genome integrity. Yet, CAF-1 p150 and p60 are massively downregulated in quiescent cells (Polo et al, 2004), raising the intriguing question of how quiescent cells can deal with chromatin assembly coupled to DNA repair.

We here focus on how the late steps of CPD and 6,4-PP repair, including chromatin restoration, can occur in UV-irradiated quiescent human cells. We show that CAF-1 expression is not induced upon UV damage in quiescent cells and that the limited pool of CAF-1 is sufficient for a local recruitment at UV damage sites. However, we observe a delayed dissociation from chromatin of both CAF-1 and PCNA in quiescent cells. This is associated with a delayed repair of CPD lesions, but not of 6,4-PP lesions. Our results indicate that early NER factors dissociate normally and that only late CPD repair steps, coinciding with DNA synthesis and chromatin restoration, are delayed in quiescence. We hypothesize that the delayed CPD repair could reflect a difficulty in repairing nucleosomal lesions, of

which the additional chromatin restoration step might represent a limiting factor in quiescence.

RESULTS

UV irradiation does not induce CAF-1 expression in quiescent cells

We previously put forward a function of CAF-1 in re-establishment of chromatin structure coupled to repair of UV lesions in cycling cells (Polo et al, 2006). Whether a similar mechanism would occur in quiescent (G0) cells, which express low amounts of CAF-1 p150 and p60 (Polo et al, 2004), was an open question. To investigate this issue, we used MCF7 cells, which can be arrested in quiescence by anti-estrogen treatment (Carroll et al, 2000). We first confirmed that the two largest subunits of CAF-1 were indeed downregulated in quiescence while PCNA levels and several NER proteins are expressed at similar levels in asynchronous and quiescent cells (Fig S1A). Next, we investigated whether CAF-1 expression could be induced by UV irradiation. We followed the expression level of CAF-1 p150 and p60 upon global UV irradiation. We extended the analysis up to 24 h post irradiation, a time lapse that is sufficient to allow protein neosynthesis and completion of the major part of NER. Despite a visible induction of UV lesions in all cells (Fig. S1D), we could not detect any increase in CAF-1 protein levels upon UV irradiation, neither in quiescent nor in asynchronous cells (Fig. S1B, C). Similarly, WI38 normal diploid fibroblasts, which were arrested in G0 by serum starvation, do not display any upregulation of CAF-1 p150 and p60 upon global UV irradiation (Fig. S1E, F). Thus, quiescent cells dispose of very low levels of CAF-1, both in the absence and in the presence of UV damage.

Low levels of CAF-1 are sufficient for its local recruitment at UV damage sites

Considering that UV response in quiescent cells occurs in the presence of a limited pool of CAF-1, we hypothesized that CAF-1 might not be needed in response to UV insults. Alternatively, a low amount of CAF-1 could be sufficient to take part in UV response. In order to test this hypothesis, we examined CAF-1 recruitment to UV damaged chromatin in quiescent MCF7 cells. For this, we focused on the chromatin-bound fraction of CAF-1 subunits, which correspond to the fraction that is detergent-resistant (Martini et al, 1998). Using local UV irradiation, we observed that the chromatin-bound fraction of CAF-1 p60 and p150 localized to the sites of UV lesions (as detected by CPD) both in asynchronous and quiescent MCF7 cells (Fig 1). The local recruitment of CAF-1 is accompanied by PCNA recruitment. Since CAF-1 expression was not induced upon UV irradiation in quiescence, its local detection was not attributable to protein neosynthesis, as further confirmed by

cycloheximide treatment (Fig. 1). CAF-1 and PCNA recruitment to UV lesions was also detected in quiescent primary fibroblasts (Fig S2), in which quiescence is induced by serum starvation. These results show that the limited pool of CAF-1 in quiescent cells is sufficient to be mobilized upon DNA damage and can locally reach a threshold level that is necessary for immunofluorescence detection.

We hypothesized that the reduced availability of CAF-1 in quiescent cells might result in a delayed recruitment of a detectable amount of CAF-1 at UV damage sites. We therefore visualized and quantified the kinetics of CAF-1 and PCNA recruitment after local UV irradiation. Surprisingly, CAF-1 recruitment became visible both in asynchronous and in G0 cells at 5 minutes after irradiation (Fig 2A), suggesting that the low levels of CAF-1 in G0 cells are highly mobile and can accumulate to form a distinct signal as fast as in proliferating cells. The recruitment of CAF-1 and PCNA was maximal at 30 minutes post-irradiation, both in asynchronous and G0 cells, and then rapidly declined in asynchronous cells. Intriguingly, in G0 cells, CAF-1 and PCNA recruitment remains visible for as long as 24 hours, although the intensity of the signal diminishes over time (Fig 2B, C). Thus, CAF-1 p150 and p60 are recruited to local UV damage sites in quiescent cells with similar kinetics as in proliferating cells. However, the dissociation of CAF-1 and PCNA from the damage sites is delayed in quiescence, suggesting that the requirement for chromatin assembly activity is delayed or prolonged in these cells.

Repair of CPD, but not 6,4-PP lesions, is delayed in G0 cells

To investigate why the dissociation of CAF-1 and PCNA is delayed in quiescent cells, we analyzed the capacity of these cells to repair the two main DNA lesions induced by UV, CPDs and 6,4-PPs. While repair of 6,4-PP lesions takes place in a short time window after irradiation, the repair of CPD lesions is known to take longer (Costa et al, 2003). Indeed, 6,4-PP lesions were removed within two hours following local UV irradiation and the percentage of cells displaying 6,4-PP lesions was highly similar in asynchronous and G0 arrested MCF7 cells at all studied time points (Fig 3A). In contrast, CPD removal showed distinct kinetics in asynchronous and G0 cells (Fig 3B). Quantification indicated that >70% of the asynchronous cells were free of detectable CPD signal 24 hours after local UV, while >80% of quiescent cells still displayed CPD lesions at this time. Similarly, in primary BJ fibroblasts, 6,4-PP lesions were removed with identical kinetics in asynchronous and serum-starved G0 cells, while the removal of CPD lesions, and the dissociation of PCNA, is delayed in G0 (Fig S3).

The delayed repair is not a side effect of serum-starvation, since confluent BJ primary cells, grown in the presence of serum, also show delayed CPD repair (Fig S4).

Next, we investigated if quiescent cells show delayed CPD repair due to the absence of DNA replication, which could enhance its detection or lead to translesions synthesis. We synchronized MCF7 cells in G1 by a release from a nocodazole block (Fig 4A, B). G1 cells displayed CPD repair kinetics that were identical to asynchronous cells (Fig 4C), suggesting that the delayed CPD repair is specific for the quiescent state. In conclusion, the repair of CPD lesions, but not 6,4-PP lesions, is specifically impaired in quiescence and this is not due uniquely to the absence of cells undergoing replication.

Late steps of CPD repair are impaired in quiescence

To determine which step of the CPD repair pathway is inhibited in quiescent cells, we studied the dissociation kinetics of several factors involved in NER. First, we focused on an early NER factor, XPA, which is an essential component of the pre-incision complex. In contrast to PCNA, XPA dissociation was similar in asynchronous and G0 BJ primary fibroblasts (Fig 5A). This suggests that early CPD repair steps are able to occur normally in quiescent cells, while only later steps, possibly after incision of the damaged strand, are affected. Therefore, we analyzed the recruitment of the single strand DNA binding protein RPA, which is, like XPA, recruited before incision takes place but remains bound until gap filling is completed. Although the signal-to-background ration does not permit the detection of RPA at very late time points, the recruitment can be detected longer in G0 than in asynchronous cells (Fig 5B). These observations suggest that early steps of CPD repair occur normally and that only later steps are affected in quiescent cells.

To confirm that gap-filling is indeed delayed in quiescent cells, as the above mentioned observations suggest, we analyzed the ability of these cells to incorporate labeled nucleotides in NER patches, using a *semi-in vivo* DNA synthesis assay. For this, we extracted cells at different time points after UV irradiation and, providing all soluble factors critical for DNA synthesis, assessed the incorporation of labeled nucleotides by immunodetection (Fig 6A). Although the signal-to-background ration does not permit visualization at very late time points, we noted that the gap-filling capacity could indeed be observed later after local UV irradiation in G0 cells compared to asynchronous cells (Fig 6B). Since we only provide soluble factors for DNA synthesis, this observation not only indicates that quiescent cells retain open gaps longer after UV irradiation, but also that all chromatin-bound factors necessary for DNA synthesis are recruited and operational. Still, the actual DNA synthesis,

involving concurrent chromatin reassembly, is delayed in quiescent cells for CPD repair specifically. Since many factors involved in DNA synthesis and chromatin reassembly are downregulated in quiescence, such as ligase I (Vitolo et al, 2005), polymerase δ (Yang et al, 1992) and ε (Tuusa et al, 1995) and CAF-1 p150 and p60 (Polo et al, 2004), we hypothesized that high local doses of UV might saturate the DNA synthesis machinery. However, even at lower doses of local UV irradiation, the differential repair capacity between proliferating and quiescent cells remained visible (Fig S5), although CPD removal was faster at lower doses both in proliferating and quiescent cells. In addition, a further depletion of CAF-1 p60 by siRNA transfection in primary cells did not cause any additional delay of CPD repair in quiescent cells (Fig S6). In conclusion, quiescent cells show impaired removal of CPD, but not 6,4-PP. The defect occurs in late steps of CPD repair, including DNA synthesis and chromatin reassembly, in a manner that seems independent from UV dose or CAF-1 p60 levels.

DISCUSSION

A function for CAF-1 at UV damage sites in quiescence

In this manuscript, we addressed the issue of how low CAF-1 levels can contribute to chromatin restoration after repair of UV damage in quiescent cells. Surprisingly, we found that, like in proliferating cells, CAF-1 is recruited to UV damage in quiescent cells, while there is no detectable upregulation of CAF-1. Our observations suggest that low CAF-1 levels in quiescent cells are sufficient for its recruitment and its functional implication in chromatin restoration after UV repair. It is interesting to note that, while CPD signal intensity was similar in asynchronous and quiescent cells, we systematically observed brighter PCNA signal at local UV damage in quiescent cells than in proliferating cells. This may reflect a better accessibility for the PCNA antibody, specifically, or the accumulation of more PCNA proteins in quiescence, which could contribute to an improved local recruitment of the few available CAF-1 complexes.

An important question in the context of chromatin restoration is whether the histone variant H3.1, which is specifically deposited by CAF-1 in DNA synthesis-coupled chromatin assembly (Polo et al, 2006; Tagami et al, 2004), is sufficiently present in quiescence for a local incorporation at sites of UV damage. Indeed, histone H3.1 expression is restricted to proliferating cells (reviewed in Polo & Almouzni, 2006), but remaining levels may suffice for gap filling. In the absence of a specific antibody for the H3.1 variant, we can only assess the incorporation of exogenous, tagged H3.1 (Polo et al, 2006). Although overexpression of H3.1 in quiescent cells induced escape from quiescence in 15% (s.d. 4%) of the cells, we observed local incorporation of e-H3.1 at NER sites in 63% (s.d. 10%) of transfected cells (data not shown). This observation suggests that CAF-1, recruited at UV damage in quiescent cells, is able to deposit exogenous histone H3.1. A future challenge will be to detect if endogenous H3.1 is incorporated at UV damage sites by CAF-1.

Impaired CPD repair in quiescence: from detection to DNA synthesis

Intriguingly, CAF-1 and PCNA dissociation from local UV damage was delayed in quiescent cells, most likely due to the delay that we observe in the repair of CPD, but not 6,4-PP, lesions. We focused our efforts on specifying the steps of CPD repair that are delayed in quiescence.

CPD repair relies more on transcription-coupled detection and repair than 6,4-PP repair, of which the important DNA distorting nature makes it more amenable to global genome repair (reviewed in Costa et al, 2003). Our quiescent cells show a two-fold reduction in global transcription levels (based on an FluoroUridine incorporation assay; data not shown), which could impact on CPD detection. Yet, we observe recruitment of CAF-1 and PCNA in quiescence, two factors that can only be recruited if the endonucleases XPF and XPG are present at both sides of the lesion (Miura et al, 1996; Green & Almouzni, 2003b; Staresincic et al, 2009). Thus, CAF-1 and PCNA recruitment indicates that damage recognition and incision have taken place normally. In addition, when we used cells mutated for XPC (XP21RO), which are deficient for global genome repair and need to repair both 6,4-PP and CPD lesions by transcription-coupled repair, we did not observe a delayed repair of 6,4-PP lesions in quiescent cells (data not shown). We thus tend to conclude that delayed CPD repair in quiescence is not due to different mechanisms of detection between the two types of lesions. Finally, the early NER factor XPA dissociates with similar kinetics in asynchronous and quiescent cells. Only factors that remain bound during late steps, such as RPA and PCNA, show delayed dissociation. Taken together, our results suggest that the defect occurs in late NER steps, coinciding with DNA synthesis and chromatin restoration, and that the detection of CPD lesions takes place normally in quiescence.

It has been proposed that gap filling capacity is perturbed in quiescent cells due to low levels of factors involved in DNA synthesis (Matsumoto et al, 2007). However, our *semi-in vivo* BiodU incorporation assay, where we only provide critical soluble factors that are removed by the extraction procedure, demonstrates that quiescent cells are proficient for DNA synthesis and that all required chromatin-bound factors are recruited and functionally active. Thus, only low levels of one of the soluble factors that we provide in excess in our assay could be at the origin of a reduced DNA synthesis capacity *in vivo*. Low levels of dNTPs, which are present in lower quantities in quiescent cells and not upregulated upon DNA damage (Hakansson et al, 2006), would be a candidate. However, if DNA synthesis was impaired in quiescent cells, we would expect this to affect the repair of both CPD and 6,4-PP lesions, since gap filling is required in both cases. Yet, we do not observe any delay in 6,4-PP repair, either in MCF7 breast cancer cells or in BJ primary cells, suggesting that delayed CPD repair in quiescence cannot be due solely to inefficient DNA synthesis.

Impaired CPD repair in quiescence: a role for chromatin restoration?

Next, we sought to determine what could differ in late repair steps between CPD and 6,4-PP repair. It is striking that, while 6,4-PP lesions are mostly found in linker DNA between adjacent nucleosomes, CPD lesions can occur both in linker and in nucleosomal DNA (Gale & Smerdon, 1990; Mitchell et al, 1990; Niggli & Cerutti, 1982; Suquet et al, 1995). Although chromatin organization can be destabilized over a large region surrounding a lesion (Althaus, 1992), the DNA synthesis patches only cover about 30 nucleotides. When localized within nucleosomal core DNA, and more particularly within the 50 bases that are relatively refractory to repair and require nucleosomal sliding or removal (Arnold et al, 1987; Jensen & Smerdon, 1990), the nucleosome assembly activity could be of particular importance during repair. In contrast to surrounding chromatin, which can be restored by DNA synthesis-independent mechanisms, this short patch of newly synthesized DNA could rely uniquely on CAF-1 dependent H3.1 incorporation. The reduced levels of CAF-1 in quiescent cells (Fig S1) could therefore specifically affect repair within nucleosomal core DNA. Our hypothesis would imply a biphasic CPD repair kinetics: repair of CPD lesions localized in linker DNA would be similar in asynchronous and quiescent cells, while only nucleosomal CPD lesions would show delayed repair in quiescence. Although our quantifications rather display exponential repair curves, biphasic repair has been described for CPD repair (D'Errico et al, 2003) and for lesions induced by the UV-mimetic agent NA-AAF (Tang et al, 1989; van Oosterwijk et al, 1996). Further studies, including *in vitro* assays to evaluate the capacity of extracts from asynchronous and quiescent cells to repair CPD lesions in either naked DNA or chromatin templates, will permit to address these issues and test our hypothesis that quiescent cells show delayed repair of nucleosomal CPD lesions specifically.

Our findings could be of particular importance in the context of cancer treatment by genotoxic approaches such as radiation or certain drugs. Indeed, if the repair of other types of DNA damage is also delayed in quiescent cells, this implies that genotoxic treatments will differentially affect healthy (quiescent) and tumoral (proliferating) cells, which could impact on treatment efficiency and patient outcome. It will therefore be of particular interest to analyze the DNA and chromatin restoring capacity of quiescent vs. asynchronous cells in the context of other types of DNA damage that can occur in nucleosomal DNA.

MATERIALS AND METHODS

Cell culture, siRNA Transfection, Drugs

MCF7 breast carcinoma cells (gift from O. Delattre, Paris, France), WI38 diploid fibroblasts (LGC Promochem, Molsheim, France), and BJ primary fibroblasts (ATCC) and EJ30 bladder carcinoma cells (gift from T. Krude, Cambridge, U.K.) were grown in Petri dishes (Falcon Plastics, Cockeysville, MD) in the appropriate medium (GIBCO) complemented with 10% fetal calf serum (Eurobio) and 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO). MCF7 and EJ30 cells were grown in DMEM and WI38 and BJ cells were grown in MEM alpha medium. To arrest cells in G0, we used serum starvation for at least 72h in WI38 and BJ cells and a 48h treatment with 10 nM ICI 182780, an estrogen receptor antagonist (Fischer Bioblock Scientific) in MCF7 cells (Carroll et al, 2000; Polo et al, 2004). For protein synthesis inhibition, we treated for 30 min with 10 mM cycloheximide (Sigma Chemical). We induced mitotic arrest in MCF7 cells by a 19h treatment with 10ng/ml nocodazole (Sigma). To transfect BJ cells, we used Amaxa Cell Line Nucleofector Kit R (Lonza) according to manufacturer's instructions. siRNA target sequences: siGFP: AAGCUGGAGUACAACUACAAC; sip60: AAGCGUGUGGCUUUCAAUGUU.

UV irradiation

We used UV-C irradiation (254 nm) using a low-pressure mercury lamp at various fluences and set conditions using a VLX-3W dosimeter (Vilbert-Lourmat). We applied either global UV irradiation (10 J/m^2) or local irradiation (150 J/m^2, unless indicated differently) through a 3mm pore filter (Millipore) as described (Gerard et al, 2006b; Gérard et al, 2006; Green & Almouzni, 2003a; Mone et al, 2001).

Antibodies

We used primary antibodies for CAF-1 subunits: monoclonal anti-p150 (ab7655 Abcam); polyclonal anti-p150 characterized in our laboratory (Quivy et al, 2004); monoclonal anti-p60 that recognizes only phosphorylated p60 (ab8133 Abcam); polyclonal anti-p60 characterized in our laboratory (Green & Almouzni, 2003b) and polyclonal anti-p60 (gift from T. Krude, Cambridge, UK) that both recognize phosphorylated and unphosphorylated p60; anti-p48 (ab1766 Abcam). Other primary

antibodies were anti-HIRA (gift from P. Adams, Fox Chase center, PA), anti-Asf1a/b characterized in our laboratory (Mello et al, 2002), anti-PCNA (PC10 from Dako and FL-261 from Santa-Cruz), anti-XPC (gift from F. Hanaoka, Osaka, Japan), anti-XPA (12F5 Clinisciences), anti-CPD (KTM53 Kamiya Biomedicals), anti-6,4-photoproducts (gift from T. Matsunaga, Kanazawa, Japan), anti-H3 (ab7834, Abcam), anti-HA (3F10 Roche), anti-β-actin (AC15 Sigma Chemical), anti-γ-H2AX (JBW301 Upstate), anti-RPA p32 (9H8 Labvision). Secondary antibodies coupled to Fluorescein IsoThioCyanate, Texas red, Cyanin3 or horseradish peroxidase are from Jackson ImmunoResearch Laboratories, FITC-labeled streptavidin from ENZO Life Sciences and biotin-labeled anti-streptavidin from AbCys.

Immunofluorescence

We performed immunofluorescence on paraformaldehyde fixed cells, image capture and processing as described (Green & Almouzni, 2003b). We scored for percentages of positively stained cells by scoring at least 200 cells in each case. We excluded S-phase cells when scoring cells positive for local CAF-1, PCNA and RPA recruitment.

Cell extracts, Western Blot

We made cytosolic, nuclear, total and Triton treated cell extracts for Western Blotting as in (Martini et al, 1998).

Flow cytometry

Cells were fixed in ice-cold 70% ethanol before DNA staining with 50 mg/ml propidium iodide (Sigma Aldrich) in Phosphate Buffer Saline containing 0.5 mg/ml RNAse A (Amersham) and analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest Pro software (Becton Dickinson).

DNA synthesis assay

We permeabilized cells as for immunofluorescence (Green & Almouzni, 2003b) at several time points after local UV irradiation and incubated for 45min at 37°C in run-on buffer (40mM Hepes pH 7.8 ; 7mM MgCl2 ; 3mM ATP ; 0.5mM DTT ; 20mM creatin phosphate ; 25 μg/ml creatin kinase; 0.1mM of dATP, dCTP, dGTP; 40 μM of Bio-16-

dUTP; 300mM sucrose) and stopped the reaction by 3 washes with cold stop buffer (40mM Hepes pH 7.8 ; 7mM MgCl2 ; 3mM CaCl2 ; 0.5mM DTT). After, we revealed by immunofluoresence as in (Green & Almouzni, 2003b), using signal amplification for Bio-16-dUTP detection with FITC-labeled streptavidin, biotin-labeled anti-streptavidin and again FITC-labeled streptavidin.

REFERENCES

Ait-Si-Ali S, Guasconi V, Fritsch L, Yahi H, Sekhri R, Naguibneva I, Robin P, Cabon F, Polesskaya A, Harel-Bellan A (2004) A Suv39h-dependent mechanism for silencing S-phase genes in differentiating but not in cycling cells. *Embo J* **23**(3)**:** 605-615

Althaus FR (1992) Poly ADP-ribosylation: a histone shuttle mechanism in DNA excision repair. *J Cell Sci* **102 (Pt 4):** 663-670

Arnold GE, Dunker AK, Smerdon MJ (1987) Limited nucleosome migration can completely randomize DNA repair patches in intact human cells. *J Mol Biol* **196**(2)**:** 433-436

Baxter J, Sauer S, Peters A, John R, Williams R, Caparros ML, Arney K, Otte A, Jenuwein T, Merkenschlager M, Fisher AG (2004) Histone hypomethylation is an indicator of epigenetic plasticity in quiescent lymphocytes. *Embo J* **23**(22)**:** 4462-4472

Carroll JS, Prall OW, Musgrove EA, Sutherland RL (2000) A pure estrogen antagonist inhibits cyclin E-Cdk2 activity in MCF-7 breast cancer cells and induces accumulation of p130-E2F4 complexes characteristic of quiescence. *J Biol Chem* **275**(49)**:** 38221-38229

Coller HA (2007) What's taking so long? S-phase entry from quiescence versus proliferation. *Nat Rev Mol Cell Biol* **8**(8)**:** 667-670.

Coller HA, Sang L, Roberts JM (2006) A new description of cellular quiescence. *PLoS Biol* **4**(3)**:** e83. Epub 2006 Mar 2007.

Coppock DL, Kopman C, Scandalis S, Gilleran S (1993) Preferential gene expression in quiescent human lung fibroblasts. *Cell Growth Differ* **4**(6)**:** 483-493.

Costa RM, Chigancas V, Galhardo Rda S, Carvalho H, Menck CF (2003) The eukaryotic nucleotide excision repair pathway. *Biochimie* **85**(11)**:** 1083-1099

D'Errico M, Teson M, Calcagnile A, Proietti De Santis L, Nikaido O, Botta E, Zambruno G, Stefanini M, Dogliotti E (2003) Apoptosis and efficient repair of DNA damage protect human keratinocytes against UVB. *Cell Death Differ* **10**(6)**:** 754-756

Gaillard PH, Martini EM, Kaufman PD, Stillman B, Moustacchi E, Almouzni G (1996) Chromatin assembly coupled to DNA repair: a new role for chromatin assembly factor I. *Cell* **86**(6)**:** 887-896.

Gaillard PH, Roche D, Almouzni G (1999) Nucleotide excision repair coupled to chromatin assembly. *Methods Mol Biol* **119:** 231-243

Gale JM, Smerdon MJ (1990) UV induced (6-4) photoproducts are distributed differently than cyclobutane dimers in nucleosomes. *Photochem Photobiol* **51**(4)**:** 411-417

Game JC, Kaufman PD (1999) Role of Saccharomyces cerevisiae chromatin assembly factor-I in repair of ultraviolet radiation damage in vivo. *Genetics* **151**(2)**:** 485-497.

Gerard A, Polo SE, Roche D, Almouzni G (2006) Methods for studying chromatin assembly coupled to DNA repair. *Methods Enzymol* **409:** 358-374

Gong F, Kwon Y, Smerdon MJ (2005) Nucleotide excision repair in chromatin and the right of entry. *DNA Repair (Amst)* **4**(8)**:** 884-896

Green CM, Almouzni G (2002) When repair meets chromatin. First in series on chromatin dynamics. *EMBO Rep* **3**(1)**:** 28-33.

Green CM, Almouzni G (2003) Local action of the chromatin assembly factor CAF-1 at sites of nucleotide excision repair in vivo. *Embo J* **22**(19)**:** 5163-5174.

Grigoryev SA, Nikitina T, Pehrson JR, Singh PB, Woodcock CL (2004) Dynamic relocation of epigenetic chromatin markers reveals an active role of constitutive heterochromatin in the transition from proliferation to quiescence. *J Cell Sci* **117**(Pt 25)**:** 6153-6162

Groth A, Rocha W, Verreault A, Almouzni G (2007) Chromatin challenges during DNA replication and repair. *Cell* **128**(4)**:** 721-733.

Hakansson P, Hofer A, Thelander L (2006) Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem* **281**(12)**:** 7834-7841

Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JC, Trent JM, Staudt LM, Hudson J, Jr., Boguski MS, Lashkari D, Shalon D, Botstein D, Brown PO (1999) The transcriptional program in the response of human fibroblasts to serum. *Science* **283**(5398)**:** 83-87.

Jensen KA, Smerdon MJ (1990) DNA repair within nucleosome cores of UV-irradiated human cells. *Biochemistry* **29**(20)**:** 4773-4782

Kaufman PD, Kobayashi R, Kessler N, Stillman B (1995) The p150 and p60 subunits of chromatin assembly factor I: a molecular link between newly synthesized histones and DNA replication. *Cell* **81**(7)**:** 1105-1114.

Kaufman PD, Kobayashi R, Stillman B (1997) Ultraviolet radiation sensitivity and reduction of telomeric silencing in Saccharomyces cerevisiae cells lacking chromatin assembly factor-I. *Genes Dev* **11**(3)**:** 345-357.

Kim JA, Haber JE (2009) Chromatin assembly factors Asf1 and CAF-1 have overlapping roles in deactivating the DNA damage checkpoint when DNA repair is complete. *Proc Natl Acad Sci U S A* **106**(4)**:** 1151-1156

Lewis LK, Karthikeyan G, Cassiano J, Resnick MA (2005) Reduction of nucleosome assembly during new DNA synthesis impairs both major pathways of double-strand break repair. *Nucleic Acids Res* **33**(15)**:** 4928-4939. Print 2005.

Martini E, Roche DM, Marheineke K, Verreault A, Almouzni G (1998) Recruitment of phosphorylated chromatin assembly factor 1 to chromatin after UV irradiation of human cells. *J Cell Biol* **143**(3)**:** 563-575.

Matsumoto M, Yaginuma K, Igarashi A, Imura M, Hasegawa M, Iwabuchi K, Date T, Mori T, Ishizaki K, Yamashita K, Inobe M, Matsunaga T (2007) Perturbed gap-filling synthesis in nucleotide excision repair causes histone H2AX phosphorylation in human quiescent cells. *J Cell Sci* **120**(Pt 6)**:** 1104-1112

Mello JA, Sillje HH, Roche DM, Kirschner DB, Nigg EA, Almouzni G (2002) Human Asf1 and CAF-1 interact and synergize in a repair-coupled nucleosome assembly pathway. *EMBO Rep* **3**(4)**:** 329-334

Meshorer E, Yellajoshula D, George E, Scambler PJ, Brown DT, Misteli T (2006) Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. *Dev Cell* **10**(1)**:** 105-116.

Mitchell DL, Nguyen TD, Cleaver JE (1990) Nonrandom induction of pyrimidine-pyrimidone (6-4) photoproducts in ultraviolet-irradiated human chromatin. *J Biol Chem* **265**(10)**:** 5353-5356

Miura M, Nakamura S, Sasaki T, Takasaki Y, Shiomi T, Yamaizumi M (1996) Roles of XPG and XPF/ERCC1 endonucleases in UV-induced immunostaining of PCNA in fibroblasts. *Exp Cell Res* **226**(1)**:** 126-132

Moggs JG, Grandi P, Quivy JP, Jonsson ZO, Hubscher U, Becker PB, Almouzni G (2000) A CAF-1-PCNA-mediated chromatin assembly pathway triggered by sensing DNA damage. *Mol Cell Biol* **20**(4)**:** 1206-1218.

Mone MJ, Volker M, Nikaido O, Mullenders LH, van Zeeland AA, Verschure PJ, Manders EM, van Driel R (2001) Local UV-induced DNA damage in cell nuclei results in local transcription inhibition. *EMBO Rep* **2**(11)**:** 1013-1017

Nabatiyan A, Szuts D, Krude T (2006) Induction of CAF-1 expression in response to DNA strand breaks in quiescent human cells. *Mol Cell Biol* **26**(5)**:** 1839-1849.

Niggli HJ, Cerutti PA (1982) Nucleosomal distribution of thymine photodimers following far- and near-ultraviolet irradiation. *Biochem Biophys Res Commun* **105**(3)**:** 1215-1223

Okano S, Lan L, Caldecott KW, Mori T, Yasui A (2003) Spatial and temporal cellular responses to single-strand breaks in human cells. *Mol Cell Biol* **23**(11)**:** 3974-3981.

Polo SE, Almouzni G (2006) Chromatin assembly: a basic recipe with various flavours. *Curr Opin Genet Dev* **16**(2)**:** 104-111

Polo SE, Roche D, Almouzni G (2006) New histone incorporation marks sites of UV repair in human cells. *Cell* **127**(3)**:** 481-493.

Polo SE, Theocharis SE, Klijanienko J, Savignoni A, Asselain B, Vielh P, Almouzni G (2004) Chromatin assembly factor-1, a marker of clinical value to distinguish quiescent from proliferating cells. *Cancer Res* **64**(7)**:** 2371-2381

Quivy JP, Roche D, Kirschner D, Tagami H, Nakatani Y, Almouzni G (2004) A CAF-1 dependent pool of HP1 during heterochromatin duplication. *Embo J* **23**(17)**:** 3516-3526

Schneider C, King RM, Philipson L (1988) Genes specifically expressed at growth arrest of mammalian cells. *Cell* **54**(6)**:** 787-793.

Shibahara K, Stillman B (1999) Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. *Cell* **96**(4)**:** 575-585.

Smerdon MJ (1991) DNA repair and the role of chromatin structure. *Curr Opin Cell Biol* **3**(3)**:** 422-428.

Staresincic L, Fagbemi AF, Enzlin JH, Gourdin AM, Wijgers N, Dunand-Sauthier I, Giglia-Mari G, Clarkson SG, Vermeulen W, Scharer OD (2009) Coordination of dual incision and repair synthesis in human nucleotide excision repair. *Embo J*

Stillman B (1986) Chromatin assembly during SV40 DNA replication in vitro. *Cell* **45**(4)**:** 555-565.

Suquet C, Mitchell DL, Smerdon MJ (1995) Repair of UV-induced (6-4) photoproducts in nucleosome core DNA. *J Biol Chem* **270**(28)**:** 16507-16509

Tagami H, Ray-Gallet D, Almouzni G, Nakatani Y (2004a) Histone h3.1 and h3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell* **116**(1)**:** 51-61.

Tagami H, Ray-Gallet D, Almouzni G, Nakatani Y (2004b) Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell* **116**(1)**:** 51-61

Talasz H, Lindner HH, Sarg B, Helliger W (2005) Histone H4-lysine 20 monomethylation is increased in promoter and coding regions of active genes and correlates with hyperacetylation. *J Biol Chem* **280**(46)**:** 38814-38822. Epub 32005 Sep 38815.

Tang MS, Bohr VA, Zhang XS, Pierce J, Hanawalt PC (1989) Quantification of aminofluorene adduct formation and repair in defined DNA sequences in mammalian cells using the UVRABC nuclease. *J Biol Chem* **264**(24)**:** 14455-14462

Tuusa J, Uitto L, Syvaoja JE (1995) Human DNA polymerase epsilon is expressed during cell proliferation in a manner characteristic of replicative DNA polymerases. *Nucleic Acids Res* **23**(12)**:** 2178-2183

van Oosterwijk MF, Versteeg A, Filon R, van Zeeland AA, Mullenders LH (1996) The sensitivity of Cockayne's syndrome cells to DNA-damaging agents is not due to defective transcription-coupled repair of active genes. *Mol Cell Biol* **16**(8)**:** 4436-4444

Venezia TA, Merchant AA, Ramos CA, Whitehouse NL, Young AS, Shaw CA, Goodell MA (2004) Molecular signatures of proliferation and quiescence in hematopoietic stem cells. *PLoS Biol* **2**(10)**:** e301. Epub 2004 Sep 2028.

Verreault A, Kaufman PD, Kobayashi R, Stillman B (1996) Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. *Cell* **87**(1)**:** 95-104.

Vitolo B, Lidonnici MR, Montecucco C, Montecucco A (2005) A new monoclonal antibody against DNA ligase I is a suitable marker of cell proliferation in cultured cell and tissue section samples. *Eur J Histochem* **49**(4)**:** 349-354

Williams JG, Penman S (1975) The messenger RNA sequences in growing and resting mouse fibroblasts. *Cell* **6**(2)**:** 197-206.

Williams JI, Friedberg EC (1979) Deoxyribonucleic acid excision repair in chromatin after ultraviolet irradiation of human fibroblasts in culture. *Biochemistry* **18**(18)**:** 3965-3972

Yang CL, Chang LS, Zhang P, Hao H, Zhu L, Toomey NL, Lee MY (1992) Molecular cloning of the cDNA for the catalytic subunit of human DNA polymerase delta. *Nucleic Acids Res* **20**(4)**:** 735-745

Zinner R, Albiez H, Walter J, Peters AH, Cremer T, Cremer M (2006) Histone lysine methylation patterns in human cell types are arranged in distinct three-dimensional nuclear zones. *Histochem Cell Biol* **125**(1-2)**:** 3-19.

FIGURE LEGENDS

Figure 1: The limited pool of CAF-1 in quiescent cells is sufficient for a local recruitment to UV damage sites

Recruitment of CAF-1 p150 and p60 subunits and PCNA to UV damage sites analyzed by immunofluorescence in asynchronous and G0 arrested MCF7 cells at the indicated times after local UV irradiation. S-phase cells, displaying replication-dependent recruitment of CAF-1 p150, are excluded from the analysis in the asynchronous population and shown cells are therefore in G1 or G2. We permeabilized the cells with Triton prior to fixation to remove soluble nuclear components. NER sites are visualized by staining for CPD lesions. Cycloheximide (CHX) was added to inhibit protein neosynthesis in quiescent cells.

Figure 2: Kinetics of CAF-1 recruitment is similar but dissociation is delayed in quiescent vs. asynchronous cells

A. CAF-1 p150 recruitment, together with PCNA, to UV damage sites in asynchronous and G0 arrested MCF7 cells by immunofluorescence at the indicated times after local UV irradiation. S-phase cells, displaying replication-dependent recruitment of CAF-1 p150, are excluded from the analysis in the asynchronous population and shown cells are therefore in G1 or G2. We permeabilized the cells with Triton prior to fixation. ***B.*** Quantification of the percentage of cells with detectable CAF-1 p150 recruitment in G1/G2 cells and in G0 arrested MCF7 cells, at the indicated times after local UV irradiation. ***C.*** Quantification of the percentage of cells with detectable PCNA recruitment in G1/G2 cells and in G0 arrested MCF7 cells, at the indicated times after local UV irradiation. Error bars represent standard error from two independent experiments.

Figure 3: Quiescent cells efficiently repair 6,4-PP lesions but show delayed CPD repair

A. Kinetics of 6,4 photoproducts (6,4-PP) removal in asynchronous (As., *upper panel*) and G0 arrested (*lower panel*) MCF7 cells, visualized by immunofluorescence at different time points after local UV irradiation. Scale bar: 10μm. *Graph*: Quantification of the percentage of cells displaying detectable 6,4-PP signal in asynchronous (As.) and G0 arrested MCF7 cells at indicated times after local UV irradiation. ***B.*** Kinetics of

cyclobutane pyrimidine dimer (CPD) removal in asynchronous (As., *upper panel*) and G0 arrested (*lower panel*) MCF7 cells, visualized by immunofluorescence at different time points after local UV irradiation. Scale bar: 10μm. *Graph:* Quantification of the percentage of cells displaying detectable CPD signal in asynchronous (As.) and G0 arrested MCF7 cells at indicated times after local UV irradiation. Error bars represent standard error from three independent experiments.

Figure 4: Delayed CPD repair is specific for the quiescent state. ***A.*** Experimental scheme for analysis of the kinetics of CPD repair in G1 cells. Two hours after release from a nocodazole block, we exposed cells to local UV irradiation. Cell cycle progression is followed by flow cytometry. CPD repair of G1 cells is compared to asynchronous and G0 cells. ***B.*** Flow cytometry analysis of BJ primary cells that are asynchronously growing (As.), synchronized in G1 or arrested in G0. ***C.*** Quantification of the percentage of cells displaying detectable CPD signal in BJ primary cells that are asynchronously growing (As.), synchronized in G1 or arrested in G0, at different time points after local UV irradiation.

Figure 5: Only proteins involved in late NER steps show delayed dissociation in quiescent cells.

A. Visualization of XPA and PCNA recruitment to UV damage sites in asynchronous (As.) and G0 arrested MCF7 cells by immunofluorescence at the indicated times after local UV irradiation. We permeabilized the cells with Triton prior to fixation. Scale bar: 10μm. Quantification of the percentage of cells showing XPA (top graph) or PCNA (bottom graph) recruitment in asynchronous (As.) and G0 arrested MCF7 cells is shown at the right. S-phase cells, displaying replication-dependent recruitment of PCNA, are excluded for PCNA quantification. ***B.*** Recruitment of the 32kDa subunit of RPA, together with PCNA, to UV damage sites in asynchronous (As.) and G0 arrested MCF7 cells by immunofluorescence at the indicated times after local UV irradiation. Cells are permeabilized with Triton prior to fixation. Scale bar: 10μm. Quantification of the percentage of cells showing RPA recruitment in asynchronous (As.) and G0 arrested MCF7 cells is shown at the right. S-phase cells, displaying replication-dependent recruitment of RPA, are excluded.

Figure 6: DNA synthesis capacity at NER patches is delayed in quiescent cells

A. Experimental scheme of the BiodU incorporation procedure. Cells are exposed to local UV irradiation, permitted to recover for different time periods and extracted with Triton. Biotin-dUTP (BiodU), together with crucial soluble factors for DNA synthesis, are provided for 45min. BiodU incorporation at NER patches is detected by immunofluorescence. ***B.*** Visualization of BiodU incorporation at UV damage sites in asynchronous (As.) and G0 arrested MCF7 cells by immunofluorescence at the indicated times after local UV irradiation. Scale bar: 10μm.

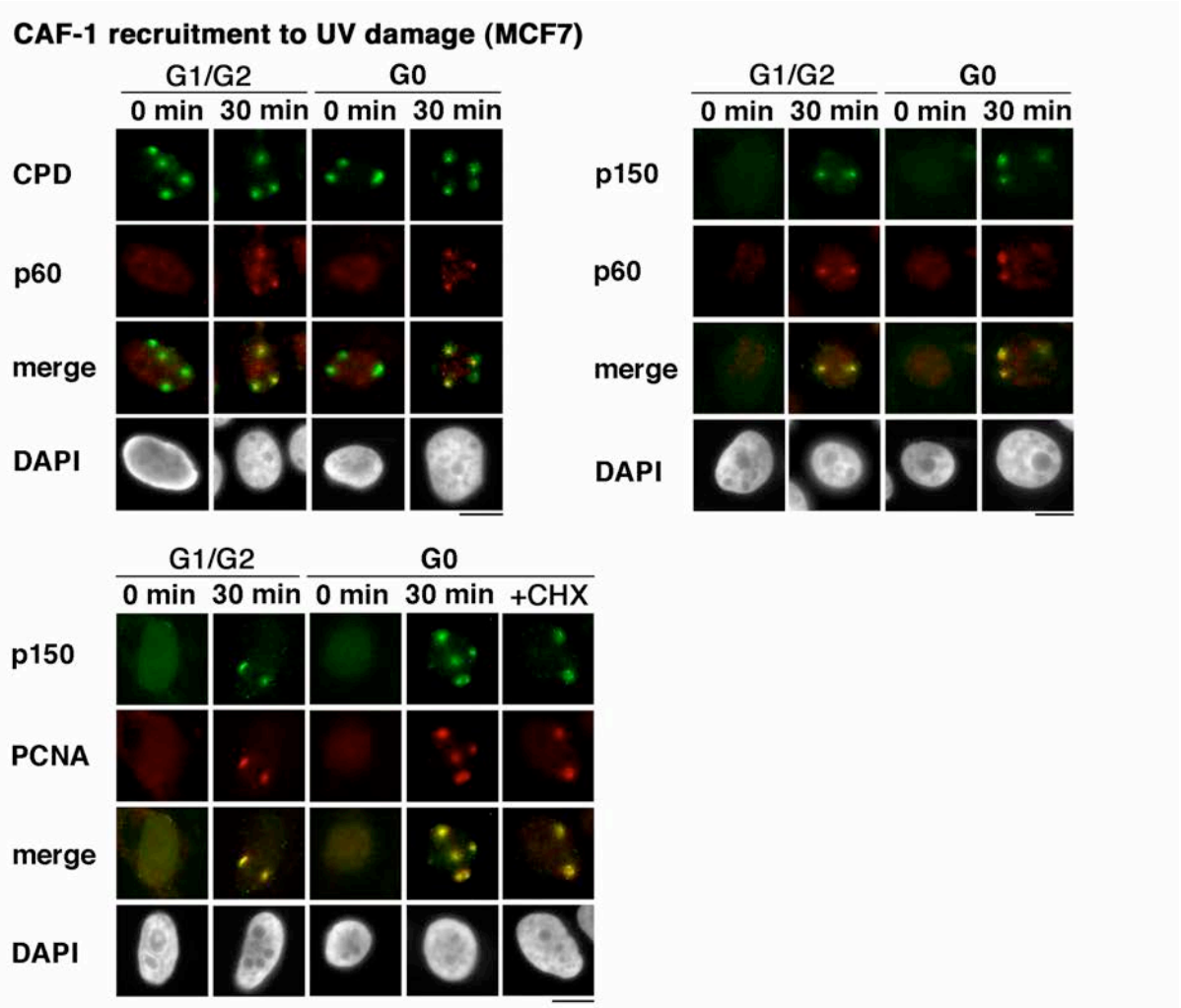
CAF-1 recruitment to UV damage (MCF7)
G1/G2
G0
0 min
30 min
0 min
30 min
CPD
p60
merge
DAPI
G1/G2
G0
0 min
30 min
0 min
30 min
p150
p60
merge
DAPI
G1/G2
G0
0 min
30 min
0 min
30 min
+CHX
p150
PCNA
merge
DAPI

Figure 1

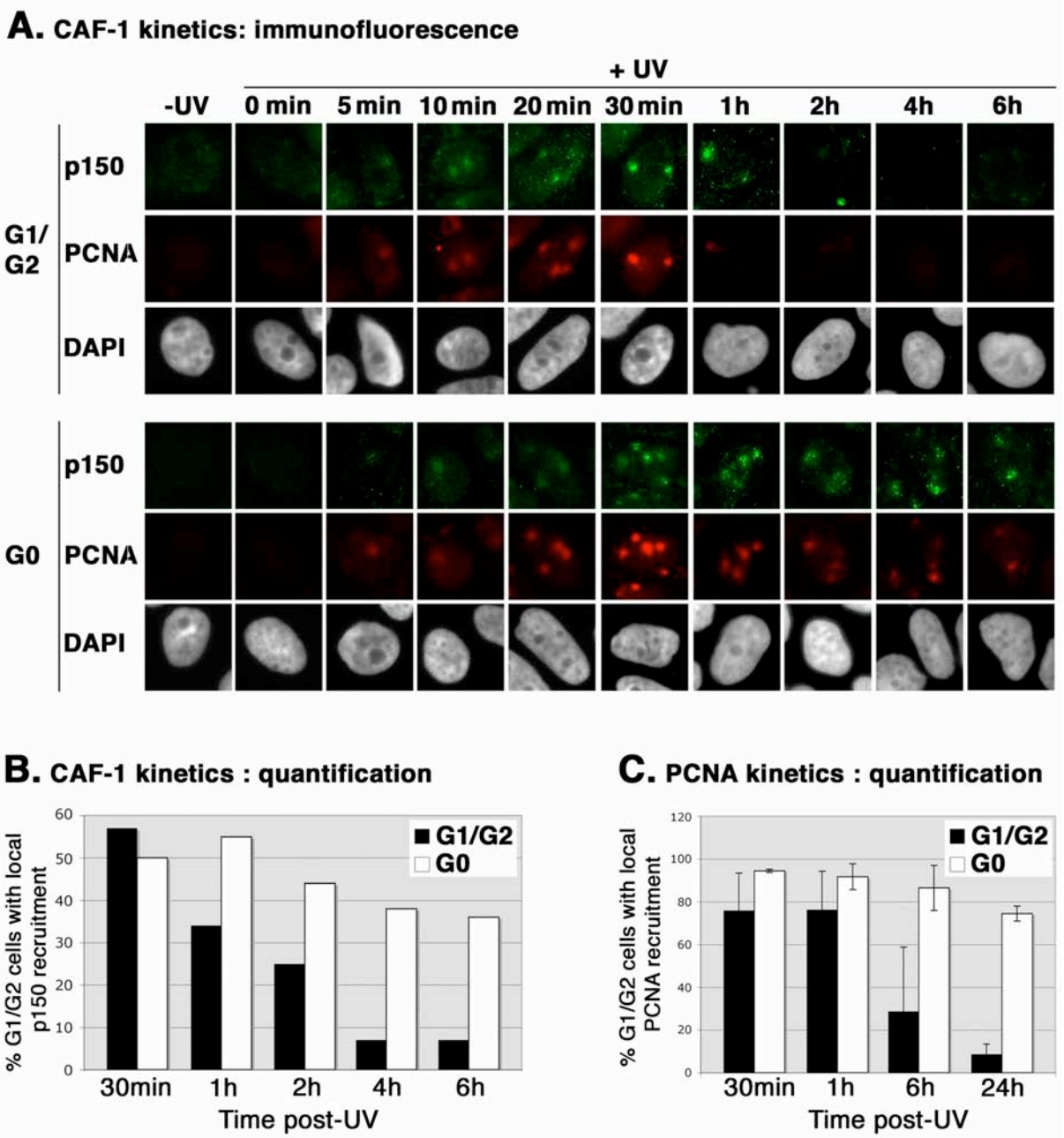
A. CAF-1 kinetics: immunofluorescence
+ UV
-UV
0 min
5 min
10 min
20 min
30 min
1h
2h
4h
6h
G1/ G2
p150
PCNA
DAPI
G0
p150
PCNA
DAPI
B. CAF-1 kinetics : quantification
% G1/G2 cells with local p150 recruitment
G1/G2
G0
30min
1h
2h
4h
6h
Time post-UV
C. PCNA kinetics : quantification
% G1/G2 cells with local PCNA recruitment
G1/G2
G0
30min
1h
6h
24h
Time post-UV

Figure 2

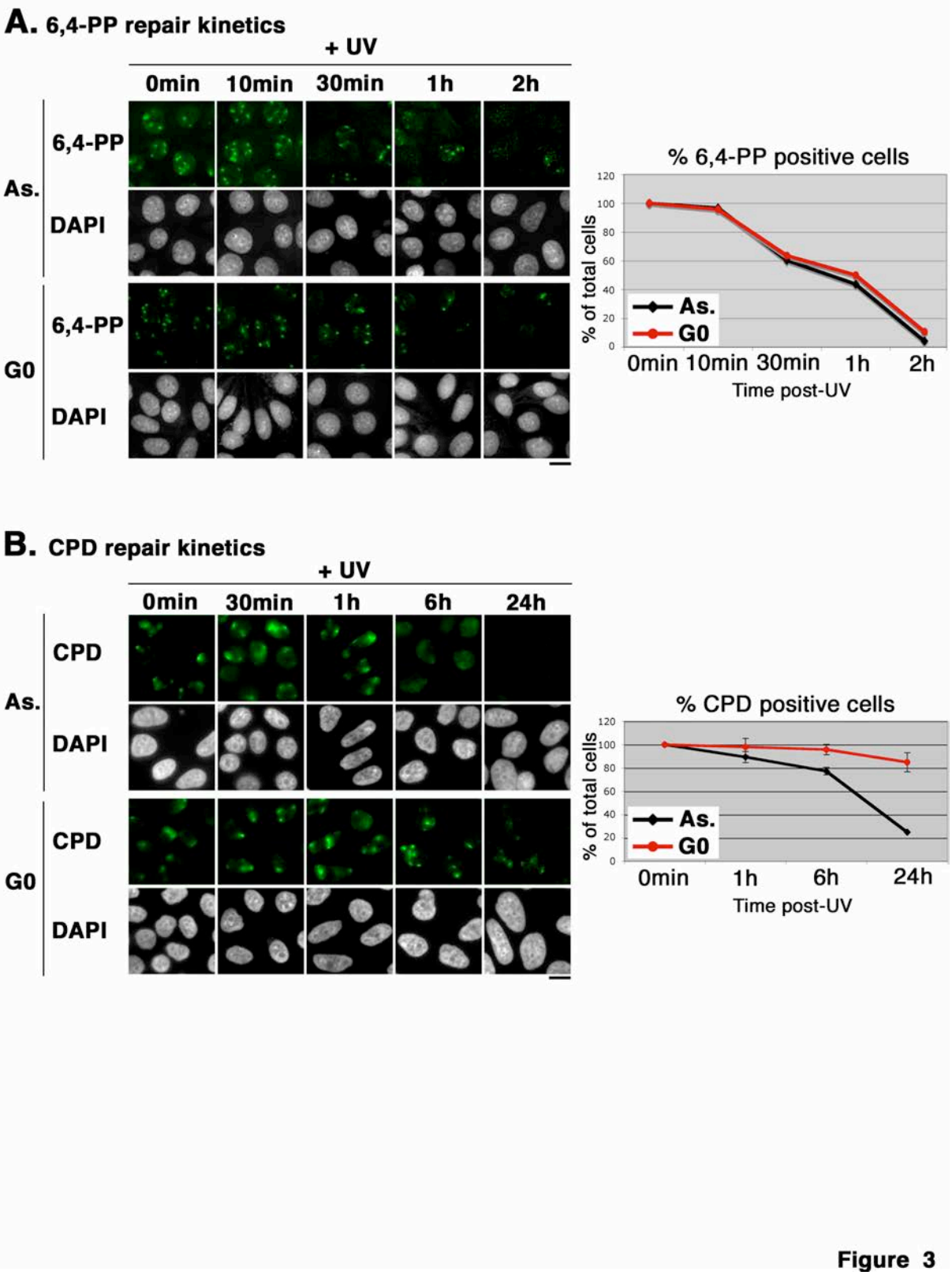
A. 6,4-PP repair kinetics
+ UV
0min
10min
30min
1h
2h
As.
6,4-PP
DAPI
G0
6,4-PP
DAPI
% 6,4-PP positive cells
% of total cells
120
100
80
60
40
20
0
As.
G0
0min 10min 30min 1h 2h
Time post-UV
B. CPD repair kinetics
+ UV
0min
30min
1h
6h
24h
As.
CPD
DAPI
G0
CPD
DAPI
% CPD positive cells
% of total cells
120
100
80
60
40
20
0
As.
G0
0min 1h 6h 24h
Time post-UV

Figure 3

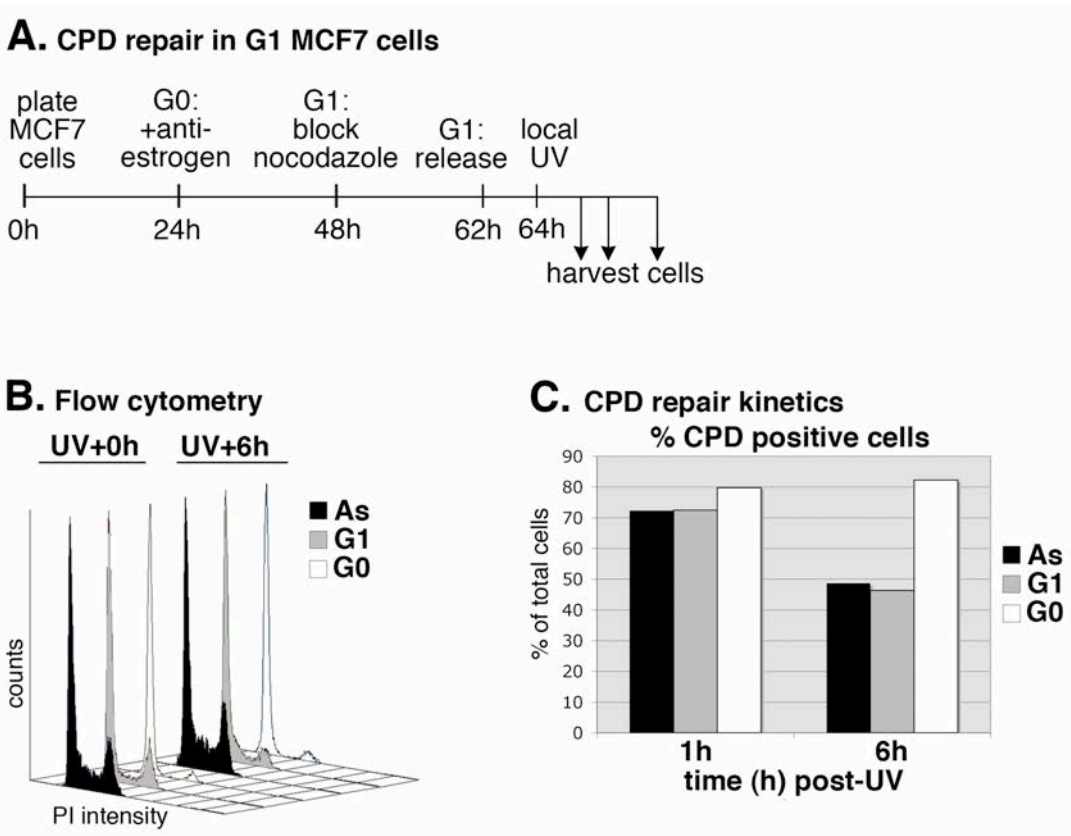
A. CPD repair in G1 MCF7 cells
plate MCF7 cells
G0: +anti-estrogen
G1: block nocodazole
G1: release
local UV
0h
24h
48h
62h
64h
harvest cells
B. Flow cytometry
UV+0h
UV+6h
As
G1
G0
counts
PI intensity
C. CPD repair kinetics
% CPD positive cells
% of total cells
90
80
70
60
50
40
30
20
10
0
1h
6h
time (h) post-UV
As
G1
G0

Figure 4

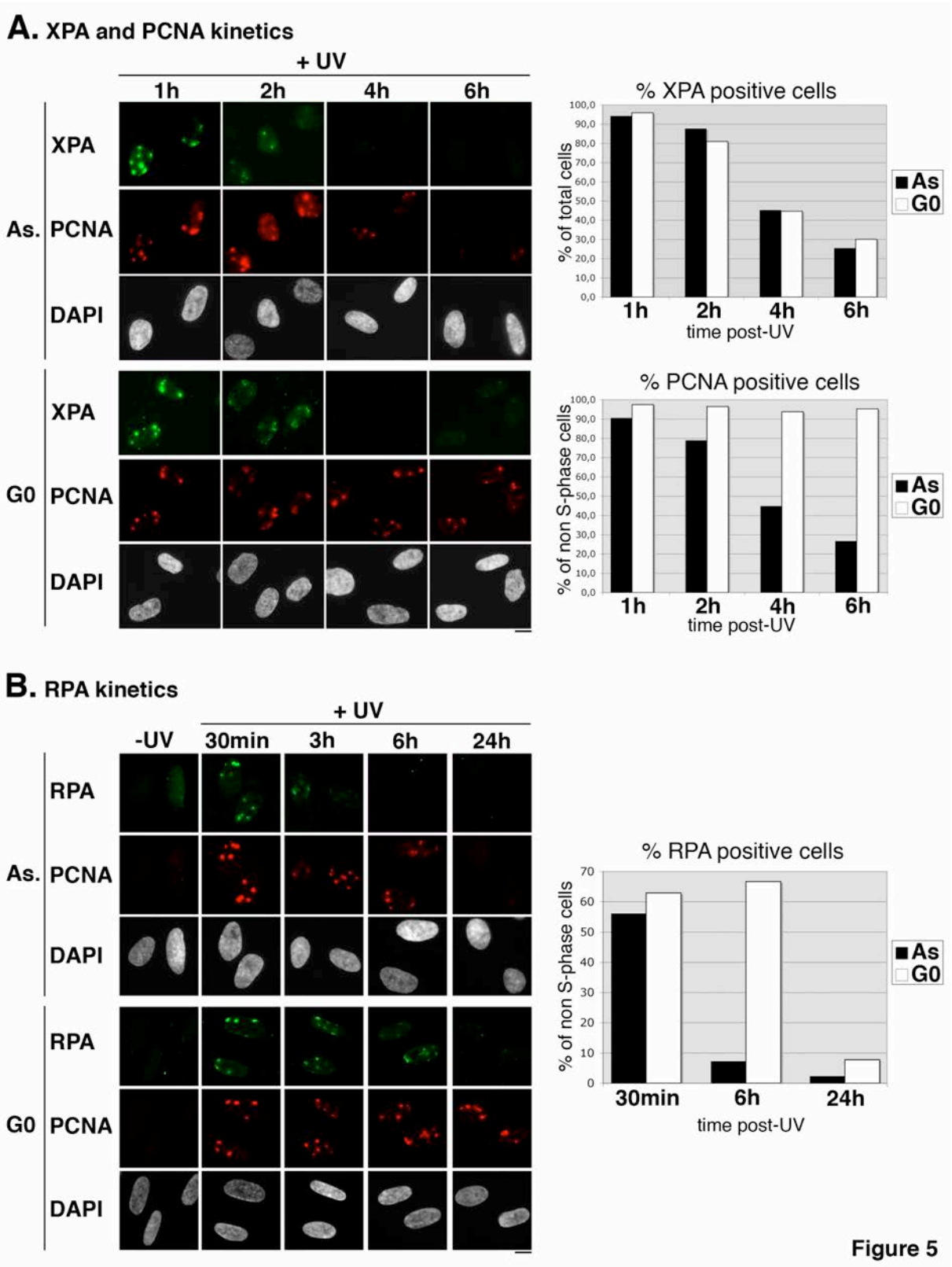
A. XPA and PCNA kinetics
+ UV
1h
2h
4h
6h
As.
XPA
PCNA
DAPI
G0
XPA
PCNA
DAPI
% XPA positive cells
% of total cells
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
1h
2h
4h
6h
time post-UV
As
G0
% PCNA positive cells
% of non S-phase cells
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
1h
2h
4h
6h
time post-UV
As
G0
B. RPA kinetics
-UV
+ UV
30min
3h
6h
24h
As.
RPA
PCNA
DAPI
G0
RPA
PCNA
DAPI
% RPA positive cells
% of non S-phase cells
70
60
50
40
30
20
10
0
30min
6h
24h
time post-UV
As
G0

Figure 5

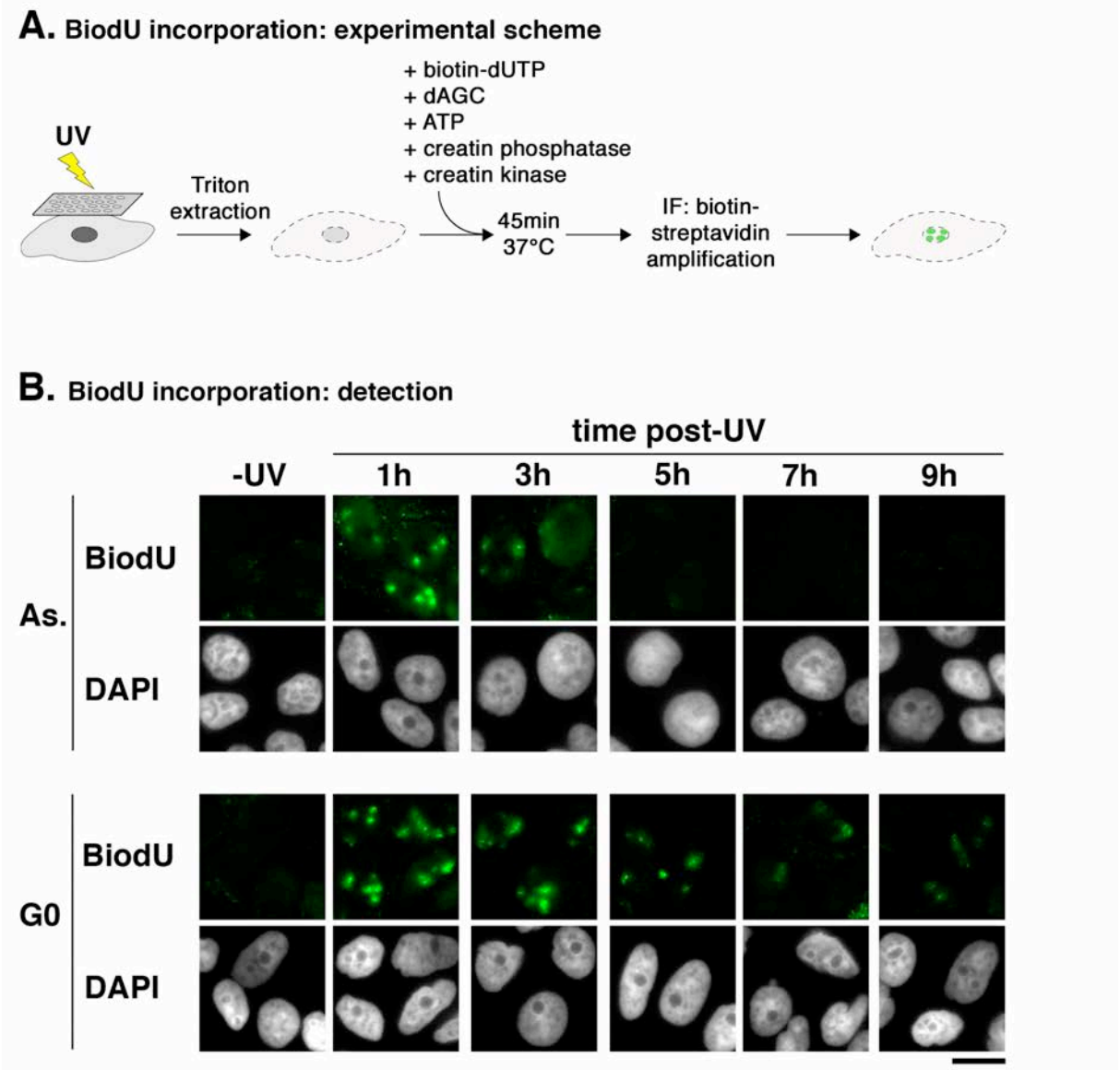
A. BiodU incorporation: experimental scheme
UV
Triton extraction
+ biotin-dUTP
+ dAGC
+ ATP
+ creatin phosphatase
+ creatin kinase
45min 37°C
IF: biotin-streptavidin amplification
B. BiodU incorporation: detection
time post-UV
-UV
1h
3h
5h
7h
9h
As.
BiodU
DAPI
G0
BiodU
DAPI

Figure 6

SUPPLEMENTARY FIGURES

Figure S1: CAF-1 expression is not induced upon UV irradiation in quiescent cells

A. Expression of CAF-1 subunits, PCNA and NER factors analyzed by Western Blot in total protein extracts from asynchronous (As) and G0 arrested MCF7 cells. We used β–actin as a loading control. ***B.*** Expression of CAF-1 p150 and p60 analyzed by immunofluorescence in asynchronous and G0 arrested MCF7 cells at the indicated times after global UV irradiation compared to unirradiated cells (-). The percentages of cells expressing CAF-1 are indicated below. Note that up to 8% of the cells escape G0 arrest. ***C.*** Expression of CAF-1 p150 and p60 analyzed by Western Blot on total extracts from asynchronous (As.) and G0 arrested MCF7 cells at the indicated times after global UV irradiation compared to unirradiated cells (-). We used βactin as a loading control. ***D***. Control of UV irradiation by immunodetection of UV lesions (CPD) in UV irradiated cells (+: 2h post UV irradiation) compared to unirradiated cells (-). ***E.*** Expression of CAF-1 p150 and p60 analyzed by immunofluorescence on asynchronous (As.) and G0 arrested WI38 primary cells at the indicated times after global UV irradiation compared to unirradiated cells (-). The percentages of cells expressing CAF-1 are indicated below. ***F.*** Expression of CAF-1 p150 and p60 analyzed by Western Blot on total extracts from asynchronous (As.) and G0 arrested Wi38 primary cells at the indicated times after global UV irradiation compared to unirradiated cells (-). Scale bars: 20μm.

Figure S2: The limited pool of CAF-1 in quiescent cells is sufficient for a local recruitment to UV damage sites in primary BJ and WI38 cells.

A. Recruitment of CAF-1 p60 to UV damage sites analyzed by immunofluorescence in asynchronous (As.) and G0 BJ primary cells, 1h after local UV irradiation. We permeabilized the cells with Triton prior to fixation to remove soluble nuclear components. NER sites are revealed by staining for CPD lesions. ***B.*** Recruitment of CAF-1 p150 to UV damage sites in asynchronous (As.) and G0 BJ primary cells, 1h after local UV irradiation. Cells were permeabilized with Triton prior to fixation. NER sites were visualized by staining for CPD lesions. ***C.*** Recruitment of CAF-1 p150, together with PCNA, to UV damage sites in asynchronous (As.) and G0 BJ primary cells, 1 hour after

local UV irradiation. We permeabilized the cells with Triton prior to fixation. ***D.*** Flow cytometry analysis of asynchronous (As.) and G0 BJ primary cells.
E. Recruitment of CAF-1 p60 subunit to UV damage sites in asynchronous (As.) and G0 Wi38 primary cells at the indicated times after local UV irradiation. We permeabilized the cells with Triton. NER sites are revealed by staining for CPD lesions. ***F.*** Recruitment of CAF-1 p150, together with PCNA, to UV damage sites in asynchronous (As.) and G0 arrested Wi38 primary cells at the indicated times after local UV irradiation. We permeabilized cells with Triton prior to fixation. Scale bars, 10 μm.

Figure S3: Quiescent BJ primary cells efficiently repair 6,4-PP lesions but show delayed CPD repair

A. Kinetics of 6,4 photoproducts (6,4-PP) removal in asynchronous (As., *upper panel*) and G0 (*lower panel*) BJ primary cells, visualized by immunofluorescence at different time points after local UV irradiation. *Right panel*: quantification of the percentage of cells displaying detectable 6,4-PP signal in asynchronous and G0 cells. ***B.*** Kinetics of cyclobutane pyrimidine dimer (CPD) removal in asynchronous (As., *upper panel*) and G0 (*lower panel*) BJ primary cells, visualized by immunofluorescence at different time points after local UV irradiation. Scale bar: 10μm. *Right panel*: quantification of the percentage of cells displaying detectable CPD or PCNA signal in asynchronous and G0 cells.

Figure S4: Delayed CPD repair in confluent, non-serum starved primary cells

A. Kinetics of cyclobutane pyrimidine dimer (CPD) removal in asynchronous (As., *upper panel*) and confluent (*lower panel*) BJ primary cells, visualized by immunofluorescence at different time points after local UV irradiation. *Right panel*: quantification of the percentage of cells displaying detectable CPD signal in asynchronous and confluent BJ cells. Note that the filter used for local irradiation locally rips off confluent cells, inducing sub-confluency and thus cell cycle entry of ± 20% of the cells, resulting in more rapid CPD repair than in serum-starved cells (compare with Fig S6B).

Figure S5: Delayed CPD repair is not dependent on UV dose

A. Kinetics of cyclobutane pyrimidine dimer (CPD) removal in asynchronous (As., *upper panel*) and G0 (*lower panel*) BJ primary cells, visualized by immunofluorescence at

different time points after local UV irradiation with 40J/m^2. ***B.*** Kinetics of CPD removal in asynchronous (As., *upper panel*) and G0 (*lower panel*) BJ primary cells, visualized by immunofluorescence at different time points after local UV irradiation with 80J/m^2. ***C.*** Kinetics of CPD removal in asynchronous (As., *upper panel*) and G0 (*lower panel*) BJ primary cells, visualized by immunofluorescence at different time points after local UV irradiation with 120J/m^2.

Figure S6: CPD repair kinetics are not affected by downregulation of CAF-1 p60

A. Experimental scheme. BJ primary cells are transfected by nucleofection with control siRNA (siGFP) or siCAF-1 p60 and arrested in quiescence. We exposed the cells to local UV irradiation 48 hours after transfection and harvested at different times after irradiation. ***B.*** Expression of CAF-1 p60 is analyzed by Western Blot in asynchronous (As.) and G0 BJ cells, transfected with control siRNA (siGFP) or sip60, both at the moment of UV irradiation and 32h after irradiation. We used βactin as a loading control. ***C.*** Kinetics of cyclobutane pyrimidine dimer (CPD) removal and PCNA dissociation in asynchronous (As., *upper panel*) and G0 (*lower panel*) BJ primary cells, transfected with control siRNA (siGFP) or sip60 and visualized by immunofluorescence at different time points after local UV irradiation. ***D.*** Quantification of the percentage of cells displaying detectable CPD signal in asynchronous (As.) and G0 arrested BJ cells, transfected with control siRNA (siGFP) or sip60, at indicated times after local UV irradiation.

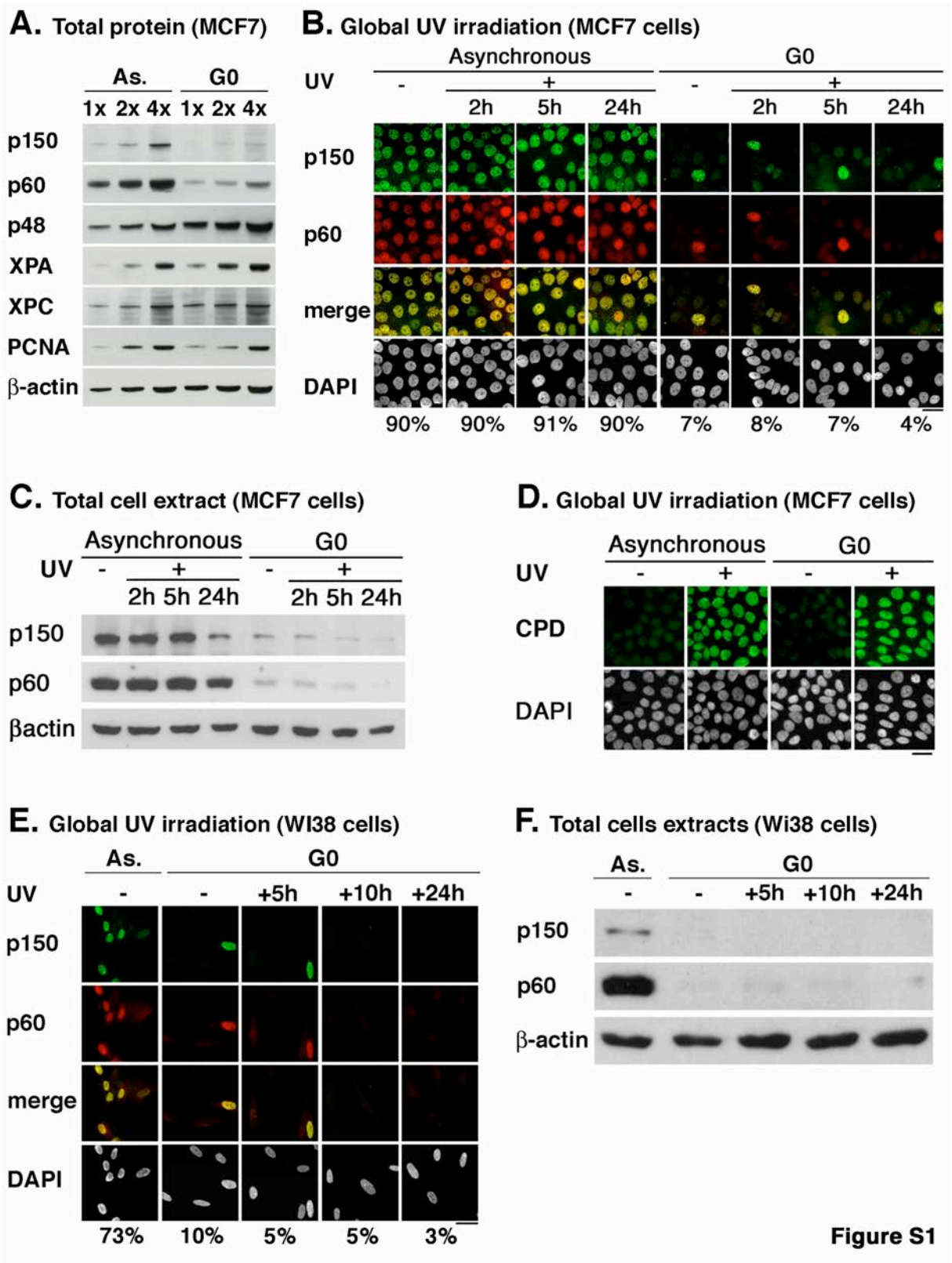
A. Total protein (MCF7)
As.
G0
1x 2x 4x 1x 2x 4x
p150
p60
p48
XPA
XPC
PCNA
β-actin
B. Global UV irradiation (MCF7 cells)
Asynchronous
G0
UV
-
+
2h
5h
24h
p150
p60
merge
DAPI
90%
90%
91%
90%
7%
8%
7%
4%
C. Total cell extract (MCF7 cells)
Asynchronous
G0
UV
2h 5h 24h
p150
p60
βactin
D. Global UV irradiation (MCF7 cells)
CPD
E. Global UV irradiation (WI38 cells)
+5h
+10h
+24h
73%
10%
5%
5%
3%
F. Total cells extracts (Wi38 cells)

Figure S1

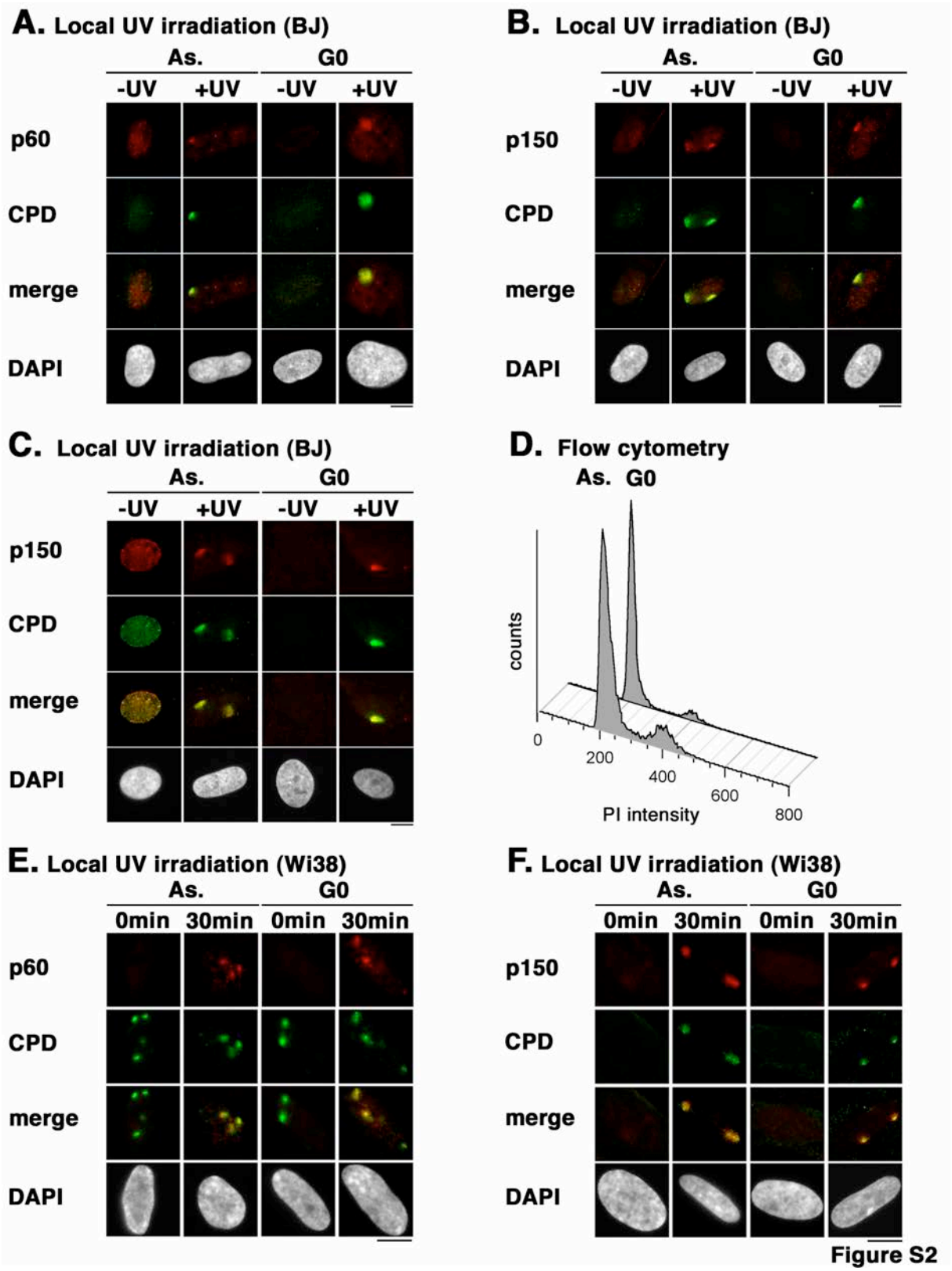
A. Local UV irradiation (BJ)
As.
G0
-UV
+UV
-UV
+UV
p60
CPD
merge
DAPI
B. Local UV irradiation (BJ)
As.
G0
-UV
+UV
-UV
+UV
p150
CPD
merge
DAPI
C. Local UV irradiation (BJ)
As.
G0
-UV
+UV
-UV
+UV
p150
CPD
merge
DAPI
D. Flow cytometry
As.
G0
counts
0
200
400
600
800
PI intensity
E. Local UV irradiation (Wi38)
As.
G0
0min
30min
0min
30min
p60
CPD
merge
DAPI
F. Local UV irradiation (Wi38)
As.
G0
0min
30min
0min
30min
p150
CPD
merge
DAPI

Figure S2

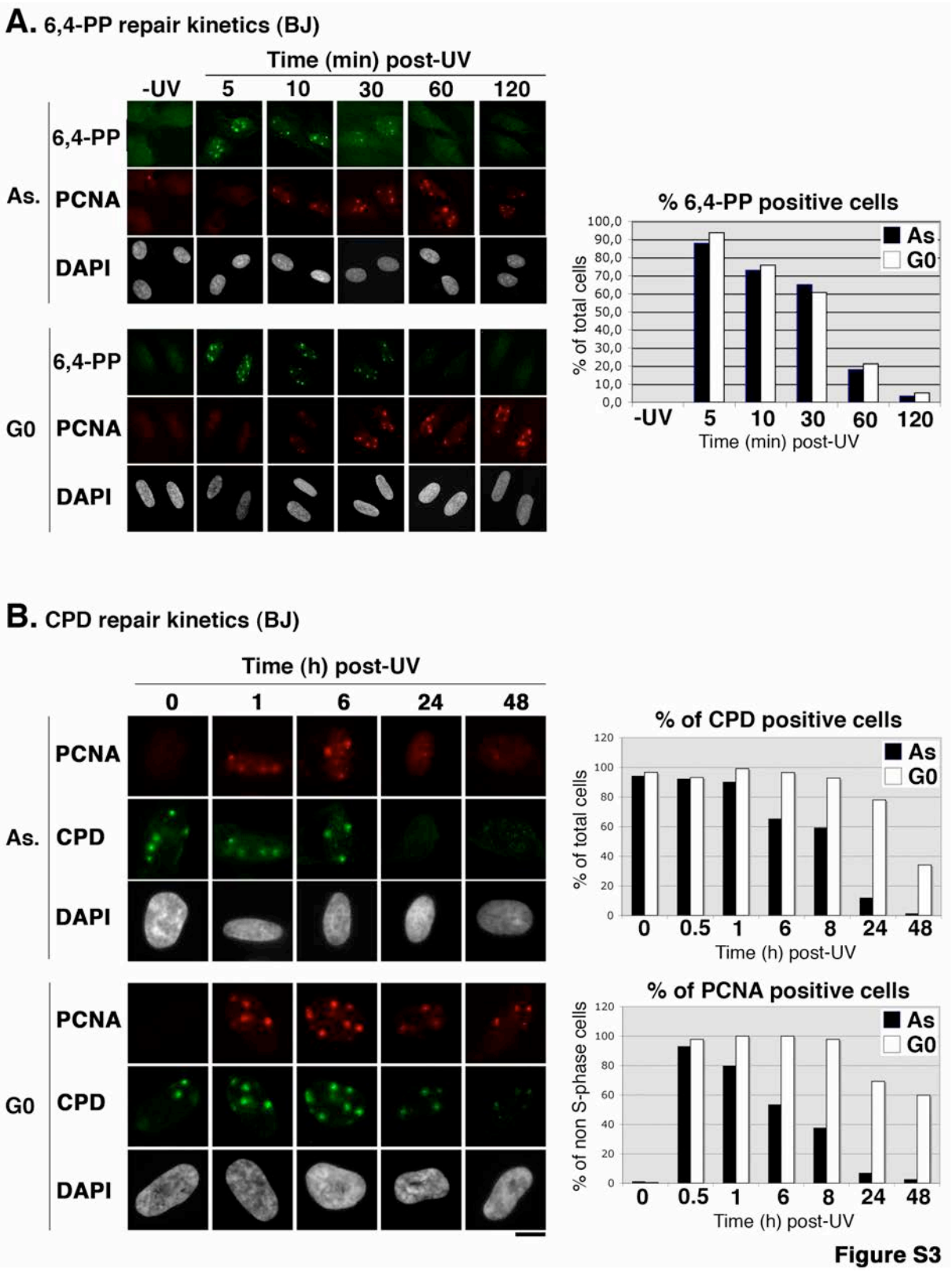
A. 6,4-PP repair kinetics (BJ)
Time (min) post-UV
-UV
5
10
30
60
120
As.
6,4-PP
PCNA
DAPI
G0
6,4-PP
PCNA
DAPI
% 6,4-PP positive cells
% of total cells
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
As
G0
-UV
5
10
30
60
120
Time (min) post-UV
B. CPD repair kinetics (BJ)
Time (h) post-UV
0
1
6
24
48
As.
PCNA
CPD
DAPI
G0
PCNA
CPD
DAPI
% of CPD positive cells
% of total cells
120
100
80
60
40
20
0
As
G0
0
0.5
1
6
8
24
48
Time (h) post-UV
% of PCNA positive cells
% of non S-phase cells
120
100
80
60
40
20
0
As
G0
0
0.5
1
6
8
24
48
Time (h) post-UV

Figure S3

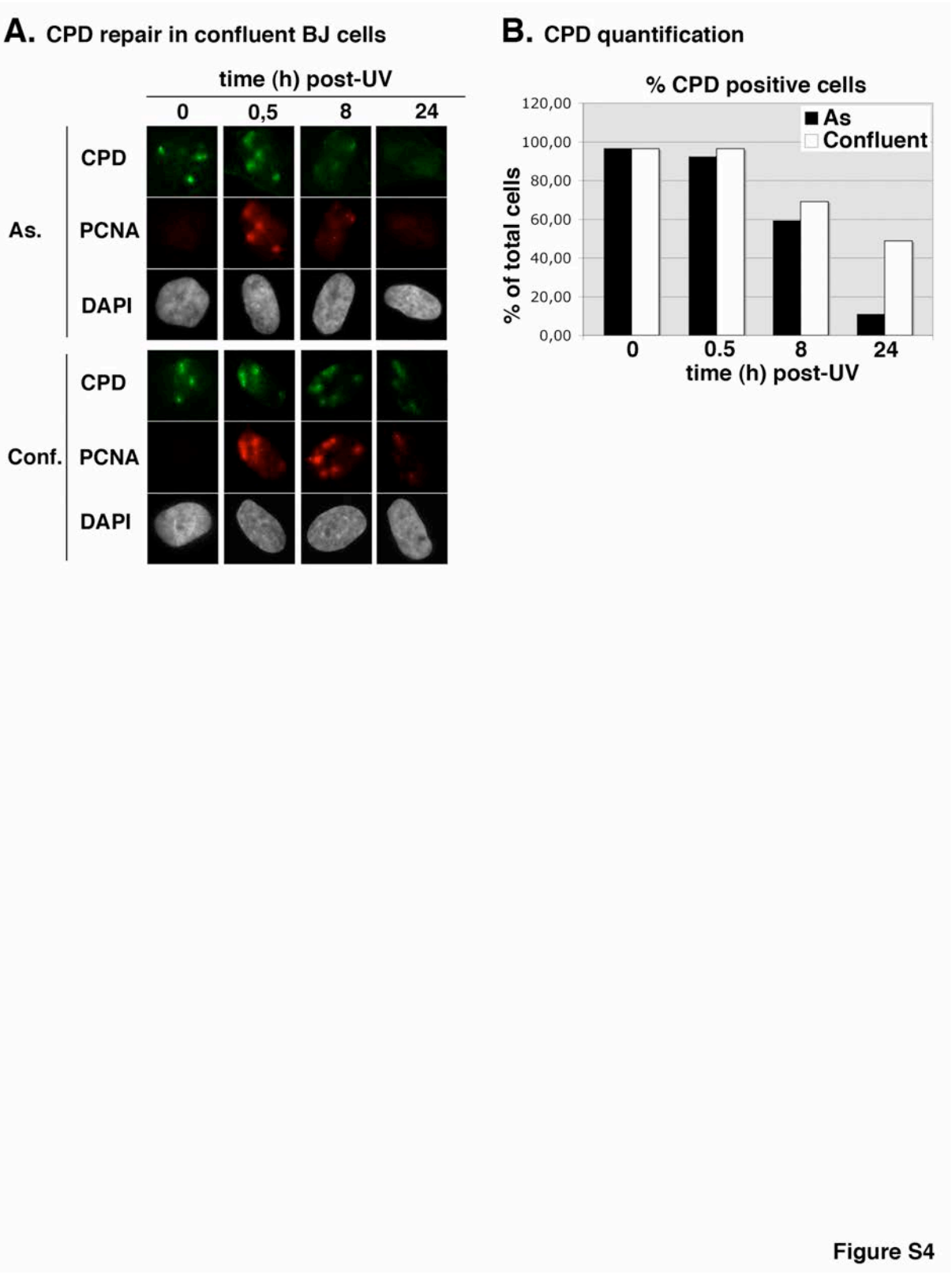
A. CPD repair in confluent BJ cells
time (h) post-UV
0
0,5
8
24
As.
CPD
PCNA
DAPI
Conf.
CPD
PCNA
DAPI
B. CPD quantification
% CPD positive cells
As
Confluent
% of total cells
120,00
100,00
80,00
60,00
40,00
20,00
0,00
0
0.5
8
24
time (h) post-UV

Figure S4

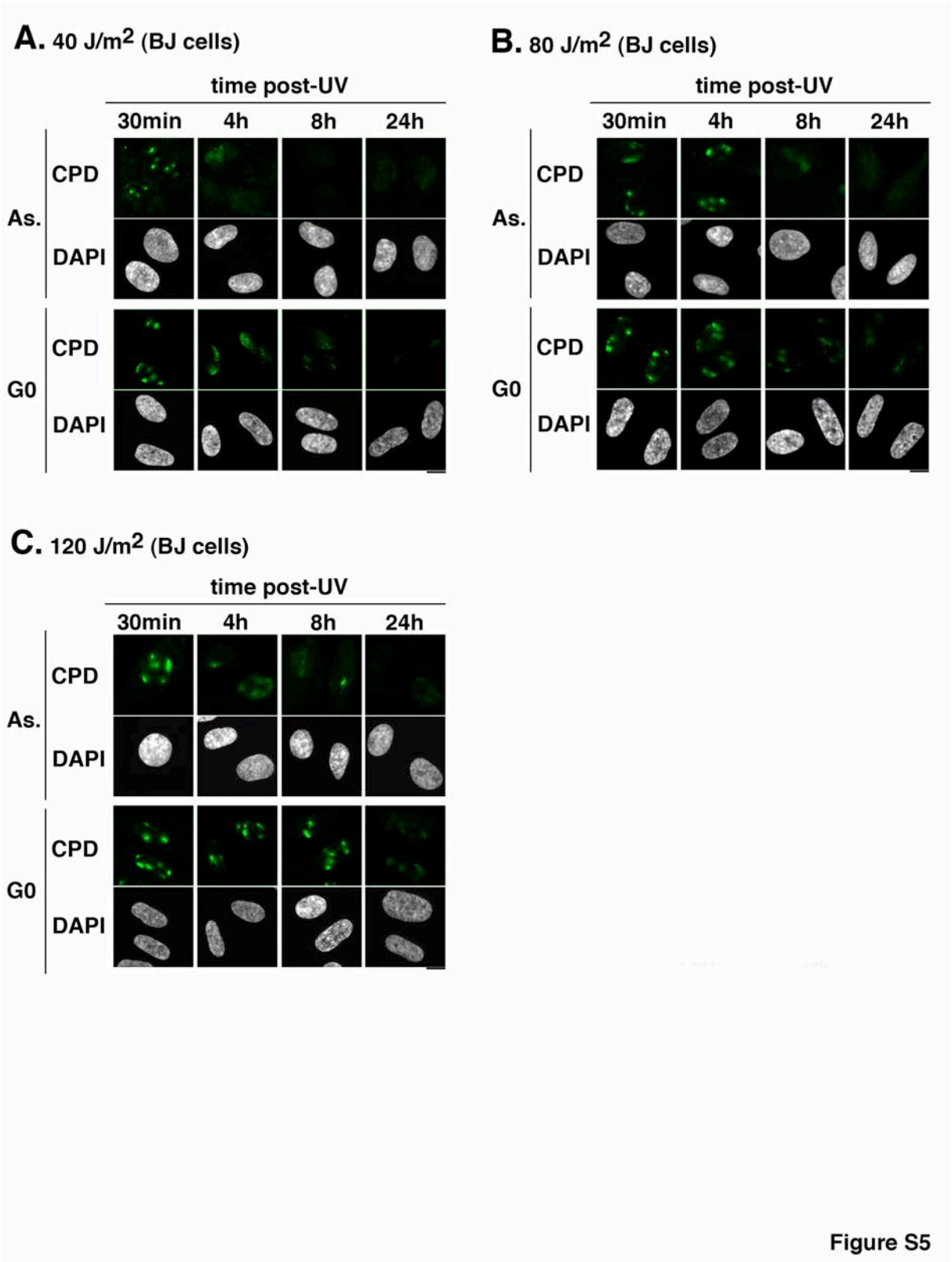
A. 40 J/m2 (BJ cells)
time post-UV
30min
4h
8h
24h
As.
CPD
DAPI
G0
CPD
DAPI
B. 80 J/m2 (BJ cells)
time post-UV
30min
4h
8h
24h
As.
CPD
DAPI
G0
CPD
DAPI
C. 120 J/m2 (BJ cells)
time post-UV
30min
4h
8h
24h
As.
CPD
DAPI
G0
CPD
DAPI

Figure S5

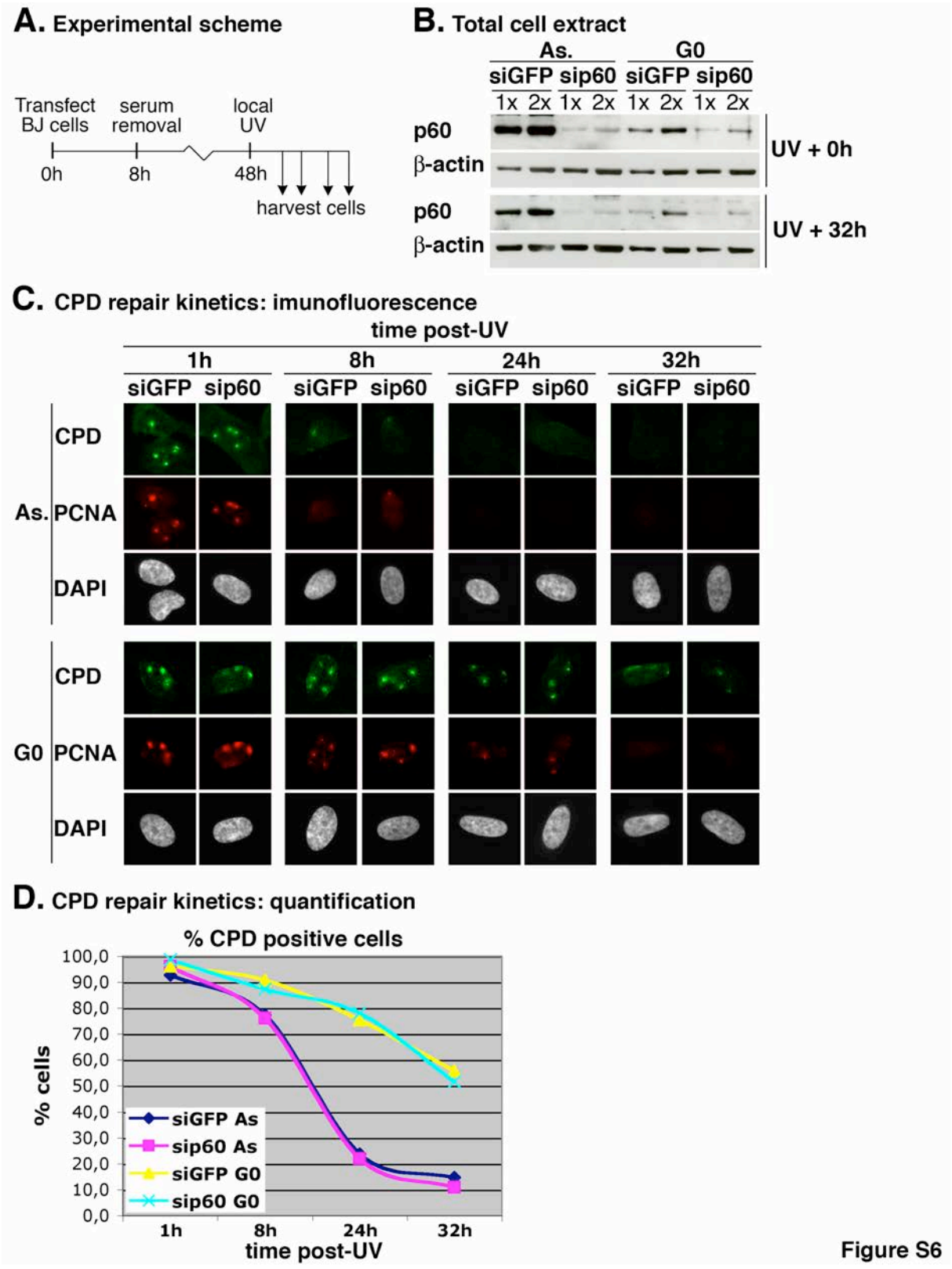
A. Experimental scheme
Transfect BJ cells
serum removal
local UV
0h
8h
48h
harvest cells
B. Total cell extract
As.
G0
siGFP sip60 siGFP sip60
1x 2x 1x 2x 1x 2x 1x 2x
p60
β-actin
UV + 0h
p60
β-actin
UV + 32h
C. CPD repair kinetics: imunofluorescence
time post-UV
1h
8h
24h
32h
siGFP sip60 siGFP sip60 siGFP sip60 siGFP sip60
As.
CPD
PCNA
DAPI
G0
CPD
PCNA
DAPI
D. CPD repair kinetics: quantification
% CPD positive cells
% cells
100,0
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
siGFP As
sip60 As
siGFP G0
sip60 G0
1h
8h
24h
32h
time post-UV

Figure S6

Additional results:

CAF-1 p60 is essential for cell cycle entry upon release from quiescence in human primary cells

DE KONING Leanne, ROCHE Danièle, ALMOUZNI Geneviève

CAF-1 is a highly conserved protein complex composed of p150, p60 (Kaufman et al, 1995a) and p48 (Verreault et al, 1996) in human cells. The complex is, as its name indicates, involved in the process of chromatin assembly. CAF-1 specifically associates with the replicative histone variant H3.1 (Tagami et al, 2004) and mediates deposition of this histone variant onto newly synthesized DNA. CAF-1 is recruited to sites of DNA synthesis through the direct interaction of p150 with the polymerase accessory factor PCNA (Shibahara & Stillman, 1999). Thus, CAF-1 is involved in chromatin assembly coupled to DNA replication (Shibahara & Stillman, 1999; Stillman, 1986). Depletion of CAF-1 p150 in human or murine cells results in a cell cycle arrest in mid to late S-phase (Hoek & Stillman, 2003; Quivy et al, 2004; Ye et al, 2003). This arrest coincides with the replication of pericentric heterochromatin and depends on the interaction of p150 with HP1 (Heterochromatin protein 1) (Quivy et al, 2008; Quivy et al, 2004). These data suggest that the chromatin assembly activity of CAF-1 is not essential for DNA replication *per se*. Indeed, depletion of CAF-1 p60 in proliferating human and murine cells does not affect cell cycle progression (Polo et al, 2006; Quivy et al, 2008). In line with a major involvement in DNA replication, CAF-1 levels dramatically drop in upon cell cycle exit (Polo et al, 2004). Inversely, exit from the quiescent state coincides with the synthesis of large amounts of CAF-1 p60 (Polo et al, 2004). This raises the intriguing question if this massive upregulation of CAF-1 is crucial for cell cycle entry, proliferation and viability.

To study the role of CAF-1 p60 in the exit from quiescence, we set up siRNA transfection in cells that can be arrested in quiescence. For this, we chose BJ primary foreskin fibroblasts, which can be transfected using nucleofection and arrested in quiescence by serum starvation. First, we confirmed that CAF-1 p60 is not essential for normal cell proliferation in these cells. Indeed, as reported previously (Polo et al, 2006;

Quivy et al, 2008), depletion of CAF-1 p60 did not alter cell cycle profiles as assessed by flow cytometry (Fig 1).

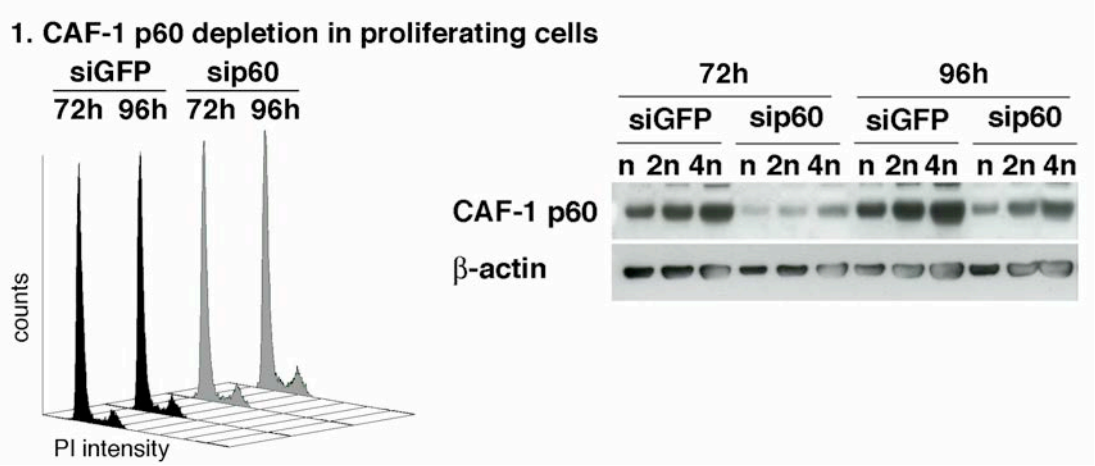

Figure 1: Depletion of CAF-1 p60 (sip60) does not alter cell cycle profiles at 72 or 96 hours after transfection compared to control cells (siGFP). Left panel: flow cytometry analysis. Right panel: Western Blot analysis of total cell extracts. Different amounts (n) were loaded and β-actin serves as loading control.

Next, we addressed whether depletion of CAF-1 p60 would interfere with exit from the quiescent state. We depleted p60 in primary BJ cells and released cells from quiescence 72h after transfection, when CAF-1 p60 levels were minimal. We followed cell cycle entry by flow cytometry and demonstrated that p60 depletion leads to a delay in cell cycle entry (Fig 2). This delay is particularly visible at 24h post-transfection, when a large fraction of control (siGFP) cells have already completed S-phase, while cells depleted for CAF-1 p60 just initiate replication. At 44h post-transfection, sip60 cells are again indistinguishable from control cells, either because they overcome the initial delay or because p60 starts to be reexpressed at this time-point.

To determine if the degree of cell cycle delay is dependent on p60 concentrations, we used a second siRNA sequence against CAF-1 p60, which leads to a less efficient depletion. This siRNA gives rise to a less pronounced cell cycle delay upon exit from G0, showing a concentration-dependent phenotype (Fig 3).

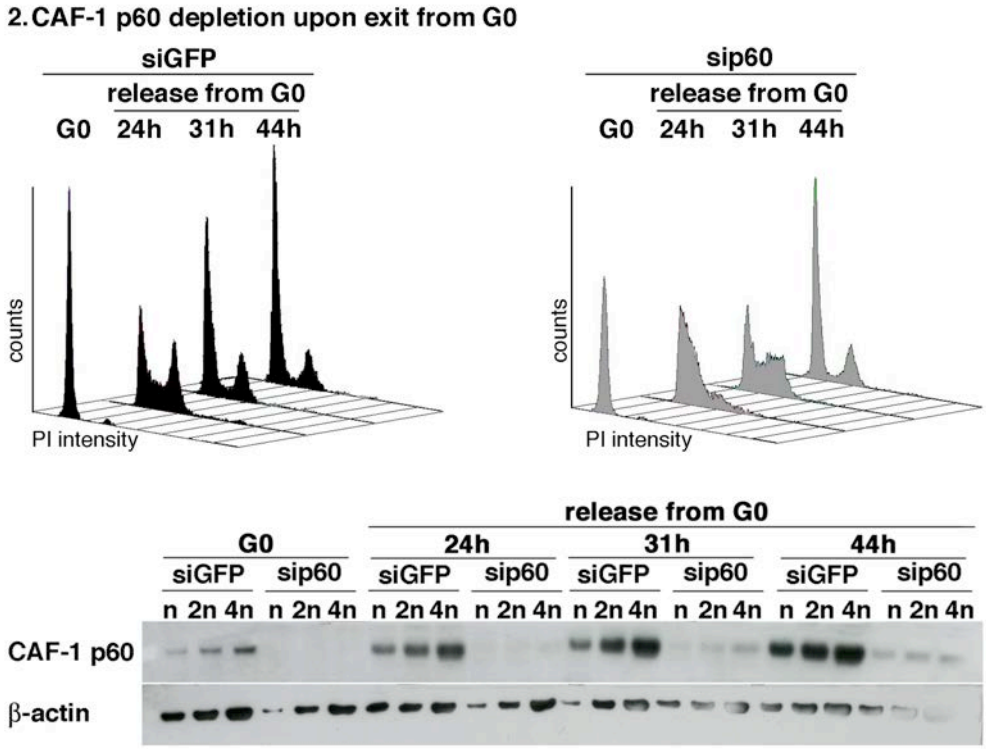

Figure 2: CAF-1 p60 depletion (sip60) impairs cell cycle entry from quiescence compared to control cells (siGFP). Upper panel: flow cytometry analysis of quiescent (G0) cells and cells released from quiescence during the indicated time periods. Lower panel: Western Blot analysis of total cell extracts. Different amounts (n) were loaded and β-actin serves as loading control.

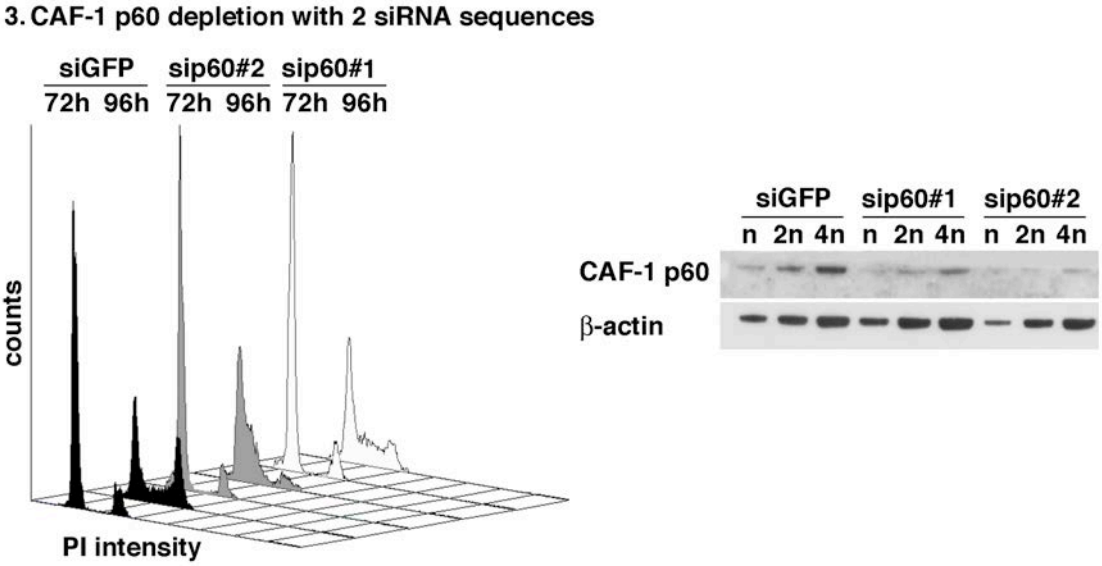

Figure 3: The delayed cell cycle entry upon p60 depletion (sip60) compared to control cells (siGFP) is reproduced with a second siRNA sequence and is dose dependent. Left panel: flow cytometry analysis. Right panel: Western Blot analysis of total cell extracts. Different amounts (n) were loaded and β-actin serves as loading control.

Since depletion of CAF-1 p150 is known to arrest cells in mid to late S-phase, we wished to determine whether p60 depletion induces a similar cell cycle delay within S-phase after release from quiescence. Using BrdU incorporation to quantify the percentage of cells actively replicating, we show that, in the time period where control cells initiate replication, p60 depleted cells do not show any BrdU incorporation (Fig 4). This observation suggests that, in contrast to p150 depletion, p60 depletion delays cells before or at the very moment of replication initiation.

The delayed cell cycle entry has a clear effect on cell survival and proliferation, since depletion of CAF-1 p60 inhibits the formation of colonies upon release from quiescence while colony formation is efficient in asynchronously proliferating cells or in cells released ten days after transfection, when the siRNA is no longer efficient (Fig 5). In conclusion, CAF-1 p60 is essential specifically in the context of cell cycle entry from quiescence, before or at the time of replication initiation.

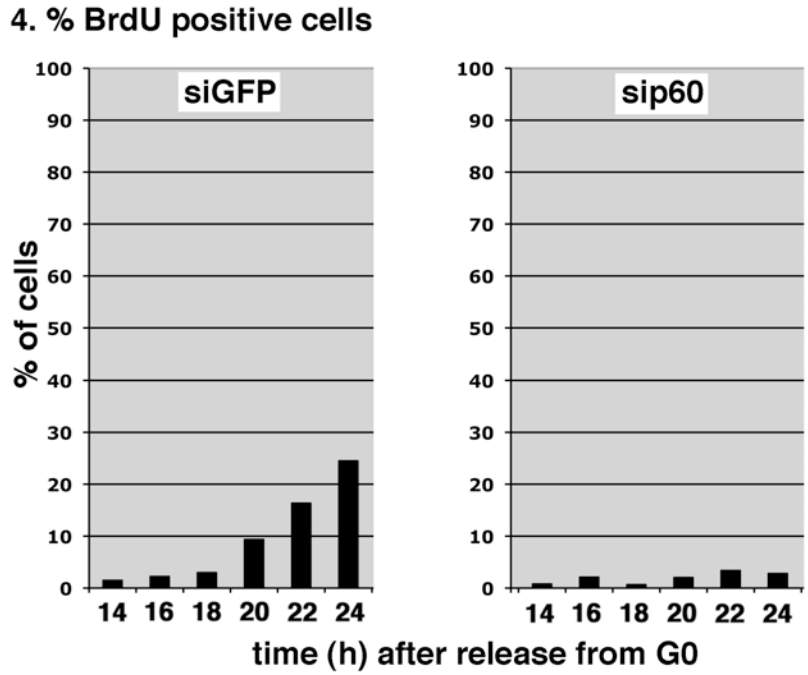

Figure 4: Depletion of p60 (sip60) delays S-phase initiation compared to control cells (siGFP), as determined by the percentage of cells showing BrdU incorporation in immunofluorescence staining.

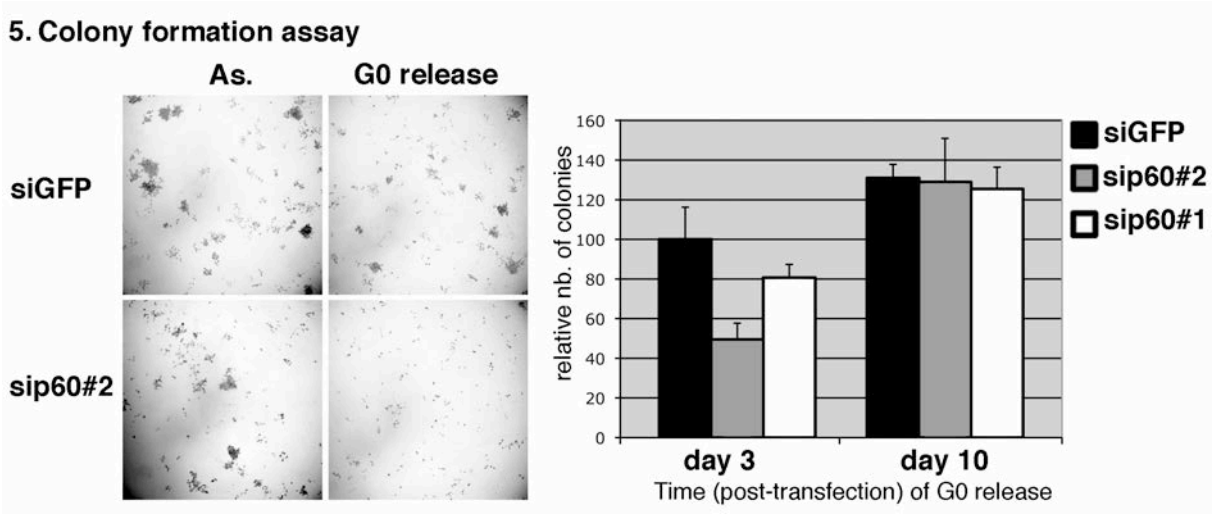

Figure 5: Depletion of CAF-1 p60 (sip60) inhibits cell survival and proliferation upon exit from quiescence compared to control cells (siGFP), as assessed by the colony formation capacity of the cells. Left panel: colonies obtained from asynchronous or G0-released cells. Right panel: quantification of colonies obtained after exit from G0, either at day 3 or at day 10 after transfection. Note that the siRNA was no longer efficient at day 10 (not shown). The number of colonies in siGFP cells released at day 3 was set to 100.

Discussion

We showed that CAF-1 p60 is required for the exit from the quiescent state in primary fibroblasts. Upon depletion of p60, cell cycle entry is delayed before or at the very moment of replication initiation. Cells in which p60 has been depleted, but that are released when the siRNA is no longer efficient, can enter the cell cycle normally (Fig 5). This suggests that the delay is not due to a defect in the quiescent state that subsequently affects cell cycle entry, but that p60 is crucial specifically during the process of cell cycle entry. To our knowledge, this is the first observation implying CAF-1 p60 in the initiation of cell proliferation. We have not been able to address if depletion of CAF-1 p150 leads to a similar delay in cell cycle entry, since no efficient depletion could be obtained in these primary cells. Thus, we cannot tell so far if the entire CAF-1 complex is required or if it concerns a unique function specific for the p60 subunit.

Interestingly, a similar phenotype has been observed in mouse embryonic fibroblasts in which both Cyclin E1 and Cyclin E2 were knocked out (Geng et al, 2007; Geng et al, 2003). In contrast to wild-type cells, the Cyclin E1/2 knock-out cells were unable to enter S-phase within 24h after release from serum starvation. The delay seems to occur during G1 phase and affects the loading of factors involved in replication

initiation. Indeed, while the licensing factor Cell Division Cycle 6 (Cdc6) is loaded normally onto chromatin, the MCM proteins are not loaded onto chromatin in Cyclin E1/2 knock-out cells at least within the first 24h after exit from quiescence. Given the interaction between CAF-1 p60 and Asf1 (Mello et al, 2002), which in turn interacts with the MCM complex (Groth et al, 2007a), it would be interesting to study if the recruitment of Cdc6 and MCM proteins after G0 release is delayed upon p60 depletion. This could support the view according to which a correct histone deposition network is not only required for replication progression, as was recently proposed in the context of Asf1 (Groth et al, 2007a), but also for replication initiation, potentially in a manner coupled to the loading of pre-replication factors in G1.

Our findings are of great interest for the field of clinical oncology. Indeed, CAF-1 p60 is highly overexpressed in many types of cancers and correlates with patient outcome (Polo et al, 2004 and unpublished data). Our results suggest that very early steps of tumorigenesis, which require cell cycle entry from a quiescent state, already require CAF-1 p60 upregulation. This implies that p60 could be of particular use as a marker to detect very early cancerous lesions. Analysis of CAF-1 p60 expression levels in murine or cellular models of tumorigenesis would therefore be of great interest.

III. Discussion

During my PhD, I have been interested in how factors involved in chromatin organization could be of importance in cell proliferation, tumorigenesis and, more generally, genome stability. For this, I particularly focused on two factors involved in chromatin organization, which can interact with each other: HP1 and CAF-1. In addition, we have studied the histone chaperone Asf1, which can act as a histone donor for CAF-1. Here, I will summarize the main results of my studies and discuss how my findings have given rise to new questions for future investigations.

HP1, CAF-1 and Asf1: partners in chromatin organization and breast cancer?

In my first study, I have investigated how the three human isoforms of HP1 relate to cell proliferation and tumorigenesis. HP1 interacts with CAF-1 (Murzina et al, 1999), the p60 subunit of which was previously identified in our laboratory as a proliferation marker in breast tumors (Polo et al, 2004). Using cell line models and human tissue samples, we showed that the HP1α isoform, but not HP1β or γ, is proliferation-dependent and crucial for faithful mitosis in cancer cells. HP1α is upregulated in cancer samples and significantly correlates with disease progression and patient survival. Thus, we put forward HP1α as a new prognostic marker in breast cancer and potentially other types of cancers. These results gave rise to a publication (De Koning et al., *Heterochromatin Protein 1 alpha: a hallmark of cell proliferation relevant in clinical oncology*, EMBO Molecular Medecine, Vol 1 (issue 3), p 178-191, 2009) and a European patent (appendix #2: *HP1alpha as a prognostic marker in human cancer*). Next the analysis was extended to another binding partner of CAF-1: the two isoforms of Asf1. Both Asf1a and b can serve as a histone donor for CAF-1 (Mello et al, 2002), while only the Asf1a isoform interacts with HIRA (Tagami et al, 2004; Zhang et al, 2005). Thus, Asf1b could specifically be involved in CAF-1 dependent chromatin assembly coupled to DNA synthesis. In line with this hypothesis, the expression of Asf1b was found to be more related to the cell proliferation status than the expression of Asf1a. In addition, in early-stage breast cancer patient samples, the expression levels of Asf1b, but not Asf1a, correlate with the tumor size, the grade, the mitotic index and global patient survival (Corpet et al., *Distinct functions for the two Asf1 isoforms in relation to proliferation*, in prep.).

At this stage, a comparison of the expression levels of our three newly identified markers for breast cancer prognosis (CAF-1, HP1α and Asf1b) will be important. Although these studies are still in progress, the correlations between the expression levels of the different genes are already revealing specific trends (Table VII). Expression levels of both HP1α and Asf1b show a strong correlation with the levels of CAF-1 (p150 and p60). However, HP1α and Asf1b only weakly correlate with each other. In addition, comparison of the expression levels with the percentage of cells that stained positive for the proliferation marker Ki67 in immunohistochemistry shows that Asf1b and p60 significantly correlate with Ki67 levels, while this is less pronounced for p150. In contrast, HP1α and Ki67 did not show a significant correlation, at least in this series of patient samples.

Table VII: Correlation between expression levels of genes in early-stage breast cancers

Genes:		p-value:	Significance
HP1α	CAF-1 p60	$p=7.9x10^{-14}$	***
HP1α	CAF-1 p150	$p<2.2x10^{-16}$	***
HP1α	Asf1b	p=0.0012	**
HP1α	Ki67	p=0.71	
CAF-1 p60	CAF-1 p150	$p<2.2x10^{-16}$	***
CAF-1 p60	Asf1b	$p=2.4x10^{-10}$	***
CAF-1 p60	Ki67	p=0.0016	**
CAF-1 p150	Asf1b	$p=3.6x10^{-11}$	***
CAF-1 p150	Ki67	p=0.04	*
Asf1b	Ki67	$p=3.6x10^{-7}$	***

In conclusion, based in these expression levels, two groups of genes emerge: (i) Asf1b, Ki67 and CAF-1 (particularly the p60 subunit) are specifically linked to replication and cell proliferation; (ii) HP1α and CAF-1 (particularly the p150 subunit) rather form a separate group, less related to Asf1b, Ki67 and cell proliferation and with a stronger link to heterochromatin formation, genome stability and gene regulation. Thus, although the expression of HP1α is clearly proliferation dependent in our cell line models, the way in which these proteins can be involved in (breast) tumorigenesis may exploit other aspects of the tumorigenic phenotype. This suggests that the expression

levels of HP1α may provide information for cancer prognosis that could be distinct from and rather complementary to standard proliferation markers, such as Ki67 or the mitotic index. Indeed, in our series of early stage breast cancers, we did not detect a significant correlation between HP1α and the mitotic index. Yet, HP1α proved highly predictive for disease progression and global survival. To get a better understanding concerning the information provided by HP1α, it will be of interest to study its expression levels in the different classes of breast tumors that have been defined by microarray transcriptome studies (reviewed in Sotiriou & Piccart, 2007). Indeed, a preliminary analysis of transcriptome data from 160 breast cancer samples and 19 healthy samples indicates that HP1α overexpression could be particularly significant in basal-like breast cancers (collaboration with T. Dubois and S. Roman-Roman, Translational Research Department, Institut Curie). These breast tumors, predominantly ER, PR and HER2 negative, are clinically aggressive carcinomas for which effective therapies and prognostic markers are currently lacking (reviewed in Rakha & Ellis, 2009). Although it is necessary to confirm these observations in a larger set of basal-like tumors, determining the value of HP1α as a prognostic marker in this type of breast cancer seems clinically relevant. For this, we aim to develop additional investigations at the protein level, using the high throughput approach of Reverse-Phase Protein Arrays (RRPA), in collaboration with the Translational Research Department of the Institut Curie. Indeed, cell lysates from the different types of breast tumors are spotted on arrays and the levels of the HP1α protein in each tumor can thus be assessed in a highly quantitative manner. Since the differences in HP1α expression are systematically larger at the protein levels than at the mRNA level, this approach is expected to provide more discriminative and significant data than transcriptome or Q-PCR analysis and should confirm if determining HP1α expression levels is indeed of clinical relevance in basal-like breast cancers.

Interestingly, the hypothesis has been put forward that basal-like breast cancers could originate from the most pluripotent mammary stem cells, while other less aggressive breast cancer types would develop from progenitor cells at more advanced stages of differentiation (reviewed in Stingl & Caldas, 2007; Yehiely et al, 2006). Indeed, basal-like tumors are generally poorly differentiated and show gene expression profiles highly similar to those obtained from pluripotent breast cancer stem cells with self-renewing capacity. Such breast cancer stem cells, of which the current definition is

based on (i) the presence of specific cell-surface markers (particularly $CD44^{high}$/ $CD24^{low}$), (ii) their capacity to form spherical colonies in suspension and (iii) their tumorigenic potential in xenograft models, are thought to drive tumor growth and recurrence due to their intrinsic resistance to most anti-cancer drugs (Charafe-Jauffret et al, 2009). The identification of drugs that would specifically affect the pluripotency of breast cancer stem cells and induce epithelial differentiation is currently a major challenge in the field (Gupta et al, 2009). Importantly, pluripotency and differentiation are largely orchestrated by epigenetic mechanisms (reviewed in Reik, 2007; Spivakov & Fisher, 2007) and HP1α has been involved in the terminal differentiation of both neurons (Panteleeva et al, 2007) and muscle cells (Yahi et al, 2008). Given that basal-like cancers show similarities to cancer stem cells and seem to express particularly high levels of HP1α, it would be extremely interesting to study in more detail the role of HP1 proteins in the context of mammary stem cells, pluripotency and differentiation.

The role of HP1α overexpression in cancer cells

My observations suggest that the overexpression of the HP1α isoform, specifically, could constitute an advantage for cancer cells. Given all the possible nuclear functions ascribed to HP1α so far (Figure 29), a key issue from a fundamental aspect is to determine which of these functions, individually or in combination, actually contribute to cancer progression, and at which step of the tumorigenesis process.

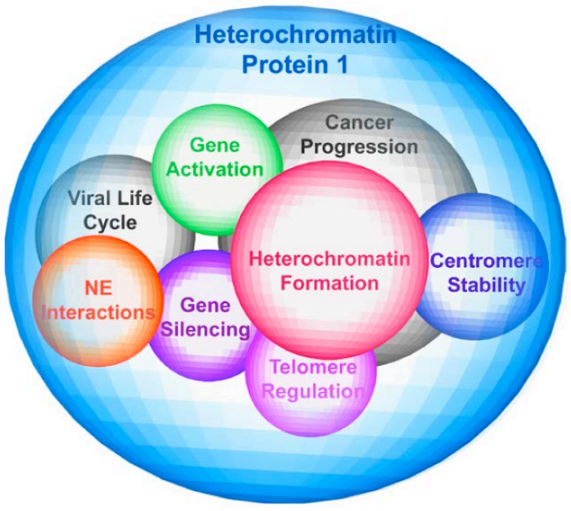

Figure 29: Involvement of HP1 in different nuclear processes. Overlapping spheres indicates that the processes seem related. Image from Dialynas et al, 2008.

My data concerning the localization of HP1α in a mammary cell line model provided a first indication. Indeed, immunofluorescence staining showed that, in tumoral cells, HP1α concentrates at centromeric regions, thus underlining a potential centromere related function. Furthermore, in line with a role in chromosome segregation, siRNA-mediated depletion of HP1α, but not HP1β or γ, led to discrete defects in mitosis. While cancer cells underwent aberrant mitosis in the absence of HP1α, primary human cells did not show aberrant mitotic figures but seemed to spend more time in (pro-)metaphase before actual chromosome segregation. This difference between cancer and primary cells suggests that the cellular context will determine the outcome upon a defect in HP1α function.

With respect to this centromeric function, it is possible that the mitotic defects upon HP1α depletion reflects decreased cohesion between the sister chromatids, as had been proposed in *S. pombe* (Bernard et al, 2001). However, in contrast to the HP1 homologue in *S. pombe*, there is now increasing evidence that mammalian HP1 does not interact with or promote the recruitment of cohesins (Koch et al, 2008; Serrano et al, 2009). It should be kept in mind, though, that the detection of endogenous centromeric cohesins by immunostaining is technically challenging, which explains why most experiments carried out so far have been using overexpression of tagged cohesin subunits. However, since tagged proteins not always recapitulate all functions of endogenous proteins, we cannot formally exclude a role for HP1α in the recruitment of endogenous cohesins. Two reports showed that HP1 interacts with Shugoshin, a protein that protects centromeric cohesins from cleavage and thus prevents precocious chromosome segregation (Serrano et al, 2009; Yamagishi et al, 2008). Yamagishi and colleagues report that Shugoshin is no longer recruited in the absence of HP1 upon prolonged metaphase arrest by nocodazole treatment. However, Serrano et al were not able to confirm this observation using less extreme metaphase arrest conditions. Thus, the cell context and the conditions applied to induce cell cycle arrest may be of importance. In conclusion, clarifying the exact involvement of HP1 in mitotic cohesion under normal cell cycle conditions will necessitate further investigations.

In my experiments, tumoral and healthy cells clearly show different mitotic phenotypes after HP1α depletion. This could be due to distinct levels of checkpoint activation. Indeed, cancer cells often display a reduced efficiency of the mitotic checkpoint (Weaver & Cleveland, 2005), which allows them to pass mitosis in the

presence of some misaligned chromosomes. Thus, it would be important to explore if HP1α depletion impacts on the activation of the mitotic checkpoint, and whether overexpression would have the opposite effect. Indeed, altering the organization of pericentric heterochromatin, which serves as a platform for the recruitment of kinetochore factors, could affect the stability and the dynamics of microtubule attachments to the kinetochores. These attachments create the tensions that turn on and off the spindle checkpoint (May & Hardwick, 2006). An attractive hypothesis that can therefore be put forward is that the overexpression of HP1α in cancer cells, and its localization to centromeric regions, could improve the rapid attachment of microtubules and thus allow cells to pass mitosis in the context of a decreased mitotic checkpoint activity.

In addition to a role in mitosis as discussed above, we should also consider that HP1α has been linked to the invasive potential of cancer cells, potentially through the silencing of pro-invasive genes (Dialynas et al, 2008). Indeed, Kirschmann and colleagues (Kirschmann et al, 2000) showed that established transformed cell lines with a high *in vitro* invasive potential (Hs578T and MB-231) express lower HP1α levels than those with a lower invasive potential (MCF7 and T-45D). However, without a comparison with normal (primary) cells, nor a careful consideration of the particular biological properties of the used breast cancer cell lines, it is difficult to draw clear-cut conclusions from this study. Indeed, while the MCF7 cell line is still hormone responsive (Carroll et al, 2000), the Hs578T cell line is considered as a basal-like cell line (Charafe-Jauffret et al, 2006). Thus, besides the invasive potential, a link between the differentiation status of the cells and HP1α levels should also be considered in this context. The authors put forward that the expression levels of HP1α and the invasive potential of cancer cells are functionally related. Their conclusions are based on (i) data from cell line models, in which they show that downregulation of HP1α increases the *in vitro* invasive potential while HP1α overexpression decreases the invasive potential; and (ii) data from human tissue sections, in which they show that 9 metastatic tissues present less intense HP1α immunohistochemistry staining than 6 primary invasive breast cancers. However, since the proliferation indexes of the used tissues are not indicated, it is difficult to draw conclusions. Thus, while these data may appear contradictory to my findings, they can be reconciled if one considers the inverse

correlation that has been suggested between proliferation and invasion (Berglund & Landberg, 2006). Indeed, metastasis requires the acquisition of invasive potential and the adaptation to a new environment, which are often incompatible with high proliferation rates. A temporal slow-down of tumor proliferation, accompanied by downregulation of HP1α, might therefore permit the expression of pro-invasive genes and the occurrence of metastasis. Thus, although I found that high HP1α expression levels in early-stage breast cancers are predictive for an increased occurrence of metastasis, this does not exclude that a downregulation of HP1α might be found specifically in the few cells that are able to escape the primary tumor niche. Indeed, a single cell can give rise to metastasis, but the expression profile of this single cell will be undetectable within the entire population of tumor cells and the detection of disseminated tumor cells currently constitutes a major challenge in tumor biology (Fehm et al, 2008). Clearly, the link between HP1α expression *in vivo* and the potential of cancer cells to proliferate or, on the other hand, metastasize, deserves to be looked into more carefully.

The role of CAF-1 in DNA repair and early tumorigenesis

In my second main project, I showed that the repair of UV-induced CPD, but not 6,4-PP, lesions is delayed in quiescent cells. Interestingly, this delay seems to affect specifically the late repair steps, involving DNA synthesis and chromatin restoration. Since inefficient DNA synthesis in quiescence would delay the repair of both CPDs and 6,4-PPs, these results favor the hypothesis of impaired chromatin dynamics in quiescent cells. Indeed, CPD lesions can occur within nucleosomes, while 6,4-PP are mostly restricted to linker DNA (Gale & Smerdon, 1990; Mitchell et al, 1990; Suquet et al, 1995). This suggests that the nucleosomal CPD lesions might require additional chromatin dynamics, which would be the limiting step in quiescence, potentially due to the extremely low levels of CAF-1 in these cells. Whether these additional chromatin dynamics concern the disassembly of the nucleosome or the reassembly capacity during chromatin restoration, or both, remains an open question. However, our observations suggest that CAF-1 and PCNA recruitment occur normally. Since CAF-1 is recruited only if XPG and XPF are present at both sides of the lesion (Miura et al, 1996) (Green & Almouzni, 2003b; Staresincic et al, 2009), this indicates that the repair machinery has access to the lesion. Thus, the chromatin dynamics required to provide access, including nucleosome disassembly, seem to occur normally. Therefore, quiescent cells might be

affected specifically in nucleosome reassembly during chromatin restoration. This would particularly affect the repair of lesions localized within nucleosomal DNA, since the repair of lesions in linker DNA might not necessitate histone eviction but rely rather on nucleosome sliding to extent the accessible region.

To test this hypothesis, I aim to perform *in vitro* experiments, which allow to assess separately lesion removal, DNA synthesis and nucleosome assembly. Indeed, incubation of UV-irradiated plasmid with human cell extracts gives rise to a process of DNA repair and chromatin assembly that closely resembles the *in vivo* situation (Martini et al, 1998). While cytosolic cell extracts are proficient for DNA repair, they are unable to promote chromatin assembly. The addition of nuclear extracts is required to provide, among other proteins, CAF-1 and thus to induce nucleosome assembly coupled to DNA repair. Using such cell extracts from both asynchronous and quiescent cells, it will be possible to determine if quiescent cell extracts show delayed CPD repair within naked DNA, in the presence or absence of concurrent nucleosome assembly. This approach will therefore allow us to better understand the limiting step of CPD repair in quiescent cells *in vivo* and the role of CAF-1 therein.

If chromatin dynamics is indeed the limiting step for CPD repair in quiescence, this would be one of the first indications that lesion removal, DNA repair synthesis and chromatin dynamics can show a certain degree of crosstalk. Thus, although CAF-1 is not required for DNA repair *per se* (Game & Kaufman, 1999; Kim & Haber, 2009; Polo et al, 2006), its absence might lead to a delayed repair of specific lesions located within nucleosomal DNA.

An interesting question to be addressed concerns the impact of the delayed CPD repair on cell survival in quiescent versus proliferating cells. Indeed, CPD lesions, but not 6,4-PP lesions, significantly contribute to cell death by apoptosis at least in proliferating cells (de Lima-Bessa et al, 2008). Thus, the delayed CPD repair might make quiescent cells more sensitive to UV irradiation. On the other hand, however, quiescent cells cannot be arrested by cell cycle checkpoints and might dispose of more time to repair the lesion before apoptosis is induced.

Next, it will be of interest to determine to what extent these finding apply to other types of damage, particularly those induced by anti-cancer radiotherapy and chemotherapy. Indeed, a differential repair capacity between proliferative cells and quiescent cells could impact on the efficiency and the toxicity of these genotoxic

treatments. In addition, how differentiated quiescent cells perform DNA repair compared to pluripotent stem cells, which could be at the origin of treatment resistance and local recurrences in many tumors (Morrison et al, 2008), is an exciting question of clinical importance. In this context, it would be of great interest to assess if CAF-1 can predict the response of (breast) tumors to genotoxic anti-cancer treatments, in addition to its value as a proliferation marker. This issue could be assessed for example in pre-menopausal breast cancer patient samples, in which the proliferation rates alone do not predict disease progression (appendix #3), thus allowing the proliferation aspect and the role of CAF-1 in DNA repair to be assessed separately.

Finally, I have presented preliminary data suggesting that CAF-1 p60 is essential for the normal exit from the quiescent state and initiation of proliferation. Depletion of CAF-1 seems to delay cell cycle entry before the onset of replication. To our knowledge, CAF-1 p60 is the first chromatin-related factor identified for being involved in cell cycle entry. This transition in cell cycle status, and the cellular processes that are required, are still poorly understood (Coller, 2007). Whether the whole CAF-1 complex is involved, or only the p60 subunit, and which binding partners are associated with CAF-1 during this transition, remains to be addressed. Given the tight correlation between the expression levels of CAF-1 p60 and Asf1b in breast cancer samples (see above), it would be particularly interesting to study if depletion of Asf1b leads to similar defects in cell cycle entry. These approaches should allow to determine if the crucial function of p60 in cell cycle entry is related to its role in chromatin assembly or rather to another, yet to be identified, function.

The implications of these findings for the field of oncology are obvious: cell cycle entry represents one of the very first events in tumorigenesis. Thus, one could imagine that CAF-1 p60 upregulation would be a crucial event for the initiation of proliferation. This implies that CAF-1 p60 might be a marker for very early benign neoplastic lesions. Since human tissues presenting this type of lesions are extremely rare, mouse models for breast cancer could prove useful to study the stage at which CAF-1 p60 overexpression is acquired.

Concluding remarks

The results obtained during my PhD have shed new light on how chromatin assembly and heterochromatin organization relate to cell proliferation and tumorigenesis, in the context of both fundamental and clinical aspects. Based on my results, HP1α can be considered as a new prognostic marker in breast cancer and additional proof has been provided for its specific function in mitosis. In addition, I have shown that quiescent cells show delayed repair of specific DNA lesions, which is probably linked to impaired chromatin dynamics due to low levels of CAF-1. Finally, I have shown that CAF-1 p60 is important for the exit from quiescence. Its overexpression might therefore constitute one of the very earliest steps in tumorigenesis. In conclusion, my results did not only bring answers to the initial questions, but also raised new questions and opened up new perspectives for future investigations, both in the context of fundamental research and clinical applications.

"*I never see what has been done; I only see what remains to be done*"

Marie Curie, letter to her brother, 1894

IV. References

Aagaard L, Laible G, Selenko P, Schmid M, Dorn R, Schotta G, Kuhfittig S, Wolf A, Lebersorger A, Singh PB, Reuter G, Jenuwein T (1999) Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31. *Embo J* **18**(7)**:** 1923-1938.

Aasland R, Stewart AF (1995) The chromo shadow domain, a second chromo domain in heterochromatin-binding protein 1, HP1. *Nucleic Acids Res* **23**(16)**:** 3168-3173

Aboussekhra A, Biggerstaff M, Shivji MK, Vilpo JA, Moncollin V, Podust VN, Protic M, Hubscher U, Egly JM, Wood RD (1995) Mammalian DNA nucleotide excision repair reconstituted with purified protein components. *Cell* **80**(6)**:** 859-868

Abreu AP, Milani C, Katayama ML, Barbosa KC, Gomes da Fonseca L, Goes JC, Brentani MM, Koike Folgueira MA (2008) Expression of heterochromatin protein 1 in the primary tumor of breast cancer patients in the presence or absence of occult metastatic cells in the bone marrow. *Int J Biol Markers* **23**(4)**:** 219-224

Adams PD (2007) Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. *Gene* **397**(1-2)**:** 84-93

Aebi S, Gelber S, Castiglione-Gertsch M, Gelber RD, Collins J, Thurlimann B, Rudenstam CM, Lindtner J, Crivellari D, Cortes-Funes H, Simoncini E, Werner ID, Coates AS, Goldhirsch A (2000) Is chemotherapy alone adequate for young women with oestrogen-receptor-positive breast cancer? *Lancet* **355**(9218)**:** 1869-1874

Agarwal N, Hardt T, Brero A, Nowak D, Rothbauer U, Becker A, Leonhardt H, Cardoso MC (2007) MeCP2 interacts with HP1 and modulates its heterochromatin association during myogenic differentiation. *Nucleic Acids Res* **35**(16)**:** 5402-5408

Agger K, Christensen J, Cloos PA, Helin K (2008) The emerging functions of histone demethylases. *Curr Opin Genet Dev* **18**(2)**:** 159-168

Agrawal A, Murphy RF, Agrawal DK (2007) DNA methylation in breast and colorectal cancers. *Mod Pathol* **20**(7)**:** 711-721

Agrup M, Stal O, Olsen K, Wingren S (2000) C-erbB-2 overexpression and survival in early onset breast cancer. *Breast Cancer Res Treat* **63**(1)**:** 23-29

Ainsztein AM, Kandels-Lewis SE, Mackay AM, Earnshaw WC (1998) INCENP centromere and spindle targeting: identification of essential conserved motifs and involvement of heterochromatin protein HP1. *J Cell Biol* **143**(7)**:** 1763-1774.

Ait-Si-Ali S, Guasconi V, Fritsch L, Yahi H, Sekhri R, Naguibneva I, Robin P, Cabon F, Polesskaya A, Harel-Bellan A (2004) A Suv39h-dependent mechanism for silencing S-phase genes in differentiating but not in cycling cells. *Embo J* **23**(3)**:** 605-615

Almouzni G, Mechali M (1988a) Assembly of spaced chromatin involvement of ATP and DNA topoisomerase activity. *Embo J* **7**(13)**:** 4355-4365

Almouzni G, Mechali M (1988b) Assembly of spaced chromatin promoted by DNA synthesis in extracts from Xenopus eggs. *Embo J* **7**(3)**:** 665-672

Althaus FR (1992) Poly ADP-ribosylation: a histone shuttle mechanism in DNA excision repair. *J Cell Sci* **102 (Pt 4):** 663-670

Ameyar-Zazoua M, Souidi M, Fritsch L, Robin P, Thomas A, Hamiche A, Percipalle P, Ait-Si-Ali S, Harel-Bellan A (2009) Physical and functional interaction between Heterochromatin Protein 1 alpha and the RNA binding protein hnRNP U. *J Biol Chem*

Anders CK, Acharya CR, Hsu DS, Broadwater G, Garman K, Foekens JA, Zhang Y, Wang Y, Marcom K, Marks JR, Mukherjee S, Nevins JR, Blackwell KL, Potti A (2008a) Age-specific differences in oncogenic pathway deregulation seen in human breast tumors. *PLoS ONE* **3**(1)**:** e1373

Anders CK, Hsu DS, Broadwater G, Acharya CR, Foekens JA, Zhang Y, Wang Y, Marcom PK, Marks JR, Febbo PG, Nevins JR, Potti A, Blackwell KL (2008b) Young age at diagnosis correlates with worse prognosis and defines a subset of breast cancers with shared patterns of gene expression. *J Clin Oncol* **26**(20)**:** 3324-3330

Andre F, Khalil A, Slimane K, Massard C, Mathieu MC, Vignot S, Assi H, Delaloge S, Spielmann M (2005) Mitotic Index and Benefit of Adjuvant Anthracycline-Based Chemotherapy in Patients With Early Breast Cancer. *J Clin Oncol* **23**(13)**:** 2996-3000

Antonini N, Jones H, Horiot JC, Poortmans P, Struikmans H, den Bogaert WV, Barillot I, Fourquet A, Jager J, Hoogenraad W, Collette L, Pierart M, Hart G, Bartelink H (2006) Effect of age and radiation dose on local control after breast conserving treatment: EORTC trial 22881-10882. *Radiother Oncol*

Araki M, Masutani C, Maekawa T, Watanabe Y, Yamada A, Kusumoto R, Sakai D, Sugasawa K, Ohkuma Y, Hanaoka F (2000) Reconstitution of damage DNA excision reaction from SV40 minichromosomes with purified nucleotide excision repair proteins. *Mutat Res* **459**(2)**:** 147-160

Arents G, Moudrianakis EN (1995) The histone fold: a ubiquitous architectural motif utilized in DNA compaction and protein dimerization. *Proc Natl Acad Sci U S A* **92**(24)**:** 11170-11174

Arnold GE, Dunker AK, Smerdon MJ (1987) Limited nucleosome migration can completely randomize DNA repair patches in intact human cells. *J Mol Biol* **196**(2)**:** 433-436

Auth T, Kunkel E, Grummt F (2006) Interaction between HP1alpha and replication proteins in mammalian cells. *Exp Cell Res* **28:** 28

Avery OT, MacLeod CM, McCarty M (1944) Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Inductions of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J Exp Med* **79:** 137-158

Ayoub N, Jeyasekharan AD, Bernal JA, Venkitaraman AR (2008) HP1-beta mobilization promotes chromatin changes that initiate the DNA damage response. *Nature* **453**(7195)**:** 682-686. Epub 2008 Apr 2027.

Ayoub N, Jeyasekharan AD, Bernal JA, Venkitaraman AR (2009) Paving the way for H2AX phosphorylation: chromatin changes in the DNA damage response. *Cell Cycle* **8**(10)**:** 1494-1500

Ayyanathan K, Lechner MS, Bell P, Maul GG, Schultz DC, Yamada Y, Tanaka K, Torigoe K, Rauscher FJ, 3rd (2003) Regulated recruitment of HP1 to a euchromatic gene induces mitotically heritable, epigenetic gene silencing: a mammalian cell culture model of gene variegation. *Genes Dev* **17**(15)**:** 1855-1869

Balaton AL, Coindre JM, Collin F, Ettore F, Fiche M, Jacquemier J, Le Doussal V, Mandard AM, Mathieu MC, Migeon C, Simony-Lafontaine J, Treilleux I, Verriele V, Vilain MO, Zafrani B (1996) [Recommendations for the immunohistochemical evaluation of hormone receptors on paraffin sections of breast cancer. Study Group on Hormone Receptors using Immunohistochemistry FNCLCC/AFAQAP. National Federation of Centres to Combat Cancer/French Association for Quality Assurance in Pathology]. *Ann Pathol* **16**(2)**:** 144-148

Ball LJ, Murzina NV, Broadhurst RW, Raine AR, Archer SJ, Stott FJ, Murzin AG, Singh PB, Domaille PJ, Laue ED (1997) Structure of the chromatin binding (chromo) domain from mouse modifier protein 1. *Embo J* **16**(9)**:** 2473-2481

Ballestar E, Paz MF, Valle L, Wei S, Fraga MF, Espada J, Cigudosa JC, Huang TH, Esteller M (2003) Methyl-CpG binding proteins identify novel sites of epigenetic inactivation in human cancer. *Embo J* **22**(23)**:** 6335-6345

Bannister AJ, Zegerman P, Partridge JF, Miska EA, Thomas JO, Allshire RC, Kouzarides T (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**(6824)**:** 120-124.

Barnard NJ, Hall PA, Lemoine NR, Kadar N (1987) Proliferative index in breast carcinoma determined in situ by Ki67 immunostaining and its relationship to clinical and pathological variables. *J Pathol* **152**(4)**:** 287-295

Bartelink H, Horiot JC, Poortmans P, Struikmans H, Van den Bogaert W, Barillot I, Fourquet A, Borger J, Jager J, Hoogenraad W, Collette L, Pierart M (2001) Recurrence rates after treatment of breast cancer with standard radiotherapy with or without additional radiation. *N Engl J Med* **345**(19)**:** 1378-1387.

Batty DP, Wood RD (2000) Damage recognition in nucleotide excision repair of DNA. *Gene* **241**(2)**:** 193-204.

Baxter J, Sauer S, Peters A, John R, Williams R, Caparros ML, Arney K, Otte A, Jenuwein T, Merkenschlager M, Fisher AG (2004) Histone hypomethylation is an indicator of epigenetic plasticity in quiescent lymphocytes. *Embo J* **23**(22)**:** 4462-4472

Benetti R, Gonzalo S, Jaco I, Munoz P, Gonzalez S, Schoeftner S, Murchison E, Andl T, Chen T, Klatt P, Li E, Serrano M, Millar S, Hannon G, Blasco MA (2008) A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. *Nat Struct Mol Biol* **15**(3)**:** 268-279

Benetti R, Gonzalo S, Jaco I, Schotta G, Klatt P, Jenuwein T, Blasco MA (2007) Suv4-20h deficiency results in telomere elongation and derepression of telomere recombination. *J Cell Biol* **178**(6)**:** 925-936

Berezney R, Dubey DD, Huberman JA (2000) Heterogeneity of eukaryotic replicons, replicon clusters, and replication foci. *Chromosoma* **108**(8)**:** 471-484

Berger AB, Cabal GG, Fabre E, Duong T, Buc H, Nehrbass U, Olivo-Marin JC, Gadal O, Zimmer C (2008) High-resolution statistical mapping reveals gene territories in live yeast. *Nat Methods* **5**(12)**:** 1031-1037

Berglund P, Landberg G (2006) Cyclin e overexpression reduces infiltrative growth in breast cancer: yet another link between proliferation control and tumor invasion. *Cell Cycle* **5**(6)**:** 606-609. Epub 2006 Mar 2015.

Bernard P, Maure JF, Partridge JF, Genier S, Javerzat JP, Allshire RC (2001) Requirement of heterochromatin for cohesion at centromeres. *Science* **294**(5551)**:** 2539-2542. Epub 2001 Oct 2511.

Bertucci F, Houlgatte R, Benziane A, Granjeaud S, Adelaide J, Tagett R, Loriod B, Jacquemier J, Viens P, Jordan B, Birnbaum D, Nguyen C (2000) Gene expression profiling of primary breast carcinomas using arrays of candidate genes. *Hum Mol Genet* **9**(20)**:** 2981-2991

Bhaumik SR, Smith E, Shilatifard A (2007) Covalent modifications of histones during development and disease pathogenesis. *Nat Struct Mol Biol* **14**(11)**:** 1008-1016

Bode J, Goetze S, Heng H, Krawetz SA, Benham C (2003) From DNA structure to gene expression: mediators of nuclear compartmentalization and dynamics. *Chromosome Res* **11**(5)**:** 435-445

Bollet MA, Sigal-Zafrani B, Mazeau V, Savignoni A, de la Rochefordiere A, Vincent-Salomon A, Salmon R, Campana F, Kirova YM, Dendale R, Fourquet A (2007a) Age remains the first prognostic factor for loco-regional breast cancer recurrence in young (<40 years) women treated with breast conserving surgery first. *Radiother Oncol* **82**(3)**:** 272-280. Epub 2007 Feb 2006.

Bollet MA, Sigal-Zafrani B, Mazeau V, Savignoni A, de la Rochefordiere A, Vincent-Salomon A, Salmon R, Campana F, Kirova YM, Dendale R, Fourquet A (2007b) Age

remains the first prognostic factor for loco-regional breast cancer recurrence in young (<40 years) women treated with breast conserving surgery first. *Radiother Oncol* **82**(3)**:** 272-280

Bolzer A, Kreth G, Solovei I, Koehler D, Saracoglu K, Fauth C, Muller S, Eils R, Cremer C, Speicher MR, Cremer T (2005) Three-dimensional maps of all chromosomes in human male fibroblast nuclei and prometaphase rosettes. *PLoS Biol* **3**(5)**:** e157

Branco MR, Pombo A (2007) Chromosome organization: new facts, new models. *Trends Cell Biol* **17**(3)**:** 127-134

Brasher SV, Smith BO, Fogh RH, Nietlispach D, Thiru A, Nielsen PR, Broadhurst RW, Ball LJ, Murzina NV, Laue ED (2000) The structure of mouse HP1 suggests a unique mode of single peptide recognition by the shadow chromo domain dimer. *Embo J* **19**(7)**:** 1587-1597

Brehm A, Tufteland KR, Aasland R, Becker PB (2004) The many colours of chromodomains. *Bioessays* **26**(2)**:** 133-140

Brown SW (1966) Heterochromatin. *Science* **151**(709)**:** 417-425

Bustamante C, Zuccheri G, Leuba SH, Yang G, Samori B (1997) Visualization and analysis of chromatin by scanning force microscopy. *Methods* **12**(1)**:** 73-83

Byvoet P, Shepherd GR, Hardin JM, Noland BJ (1972) The distribution and turnover of labeled methyl groups in histone fractions of cultured mammalian cells. *Arch Biochem Biophys* **148**(2)**:** 558-567

Carroll JS, Prall OW, Musgrove EA, Sutherland RL (2000) A pure estrogen antagonist inhibits cyclin E-Cdk2 activity in MCF-7 breast cancer cells and induces accumulation of p130-E2F4 complexes characteristic of quiescence. *J Biol Chem* **275**(49)**:** 38221-38229

Chambeyron S, Bickmore WA (2004) Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. *Genes Dev* **18**(10)**:** 1119-1130

Chambeyron S, Da Silva NR, Lawson KA, Bickmore WA (2005) Nuclear re-organisation of the Hoxb complex during mouse embryonic development. *Development* **132**(9)**:** 2215-2223

Charafe-Jauffret E, Ginestier C, Birnbaum D (2009) Breast cancer stem cells: tools and models to rely on. *BMC Cancer* **9:** 202

Charafe-Jauffret E, Ginestier C, Monville F, Finetti P, Adelaide J, Cervera N, Fekairi S, Xerri L, Jacquemier J, Birnbaum D, Bertucci F (2006) Gene expression profiling of breast cell lines identifies potential new basal markers. *Oncogene* **25**(15)**:** 2273-2284

Chen JQ, Brown TR, Yager JD (2008) Mechanisms of hormone carcinogenesis: evolution of views, role of mitochondria. *Adv Exp Med Biol* **630:** 1-18

Chen S, Zhou D, Okubo T, Kao YC, Yang C (1999) Breast tumor aromatase: functional role and transcriptional regulation. *Endocr Relat Cancer* **6**(2)**:** 149-156

Chetrite GS, Cortes-Prieto J, Philippe JC, Wright F, Pasqualini JR (2000) Comparison of estrogen concentrations, estrone sulfatase and aromatase activities in normal, and in cancerous, human breast tissues. *J Steroid Biochem Mol Biol* **72**(1-2)**:** 23-27

Cheutin T, McNairn AJ, Jenuwein T, Gilbert DM, Singh PB, Misteli T (2003) Maintenance of stable heterochromatin domains by dynamic HP1 binding. *Science* **299**(5607)**:** 721-725.

Chin HG, Esteve PO, Pradhan M, Benner J, Patnaik D, Carey MF, Pradhan S (2007) Automethylation of G9a and its implication in wider substrate specificity and HP1 binding. *Nucleic Acids Res* **35**(21)**:** 7313-7323

Choi YJ, Choi YL, Cho EY, Shin YK, Sung KW, Hwang YK, Lee SJ, Kong G, Lee JE, Kim JS, Kim JH, Yang JH, Nam SJ (2009) Expression of Bmi-1 protein in tumor tissues is associated with favorable prognosis in breast cancer patients. *Breast Cancer Res Treat* **113**(1)**:** 83-93

Chuang LS, Ian HI, Koh TW, Ng HH, Xu G, Li BF (1997) Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* **277**(5334)**:** 1996-2000

Clarke M, Collins R, Darby S, Davies C, Elphinstone P, Evans E, Godwin J, Gray R, Hicks C, James S, MacKinnon E, McGale P, McHugh T, Peto R, Taylor C, Wang Y (2005) Effects of radiotherapy and of differences in the extent of surgery for early breast cancer on local recurrence and 15-year survival: an overview of the randomised trials. *Lancet* **366**(9503)**:** 2087-2106

Cleaver JE (2005) Cancer in xeroderma pigmentosum and related disorders of DNA repair. *Nat Rev Cancer* **5**(7)**:** 564-573

Clegg NJ, Honda BM, Whitehead IP, Grigliatti TA, Wakimoto B, Brock HW, Lloyd VK, Sinclair DA (1998) Suppressors of position-effect variegation in Drosophila melanogaster affect expression of the heterochromatic gene light in the absence of a chromosome rearrangement. *Genome* **41**(4)**:** 495-503

Cloos PA, Christensen J, Agger K, Maiolica A, Rappsilber J, Antal T, Hansen KH, Helin K (2006) The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. *Nature* **442**(7100)**:** 307-311

Coller HA (2007) What's taking so long? S-phase entry from quiescence versus proliferation. *Nat Rev Mol Cell Biol* **8**(8)**:** 667-670.

Coller HA, Sang L, Roberts JM (2006) A new description of cellular quiescence. *PLoS Biol* **4**(3)**:** e83. Epub 2006 Mar 2007.

Coppock DL, Kopman C, Scandalis S, Gilleran S (1993) Preferential gene expression in quiescent human lung fibroblasts. *Cell Growth Differ* **4**(6)**:** 483-493.

Corpet A, Almouzni G (2009) Making copies of chromatin: the challenge of nucleosomal organization and epigenetic information. *Trends Cell Biol* **19**(1)**:** 29-41

Costa RM, Chigancas V, Galhardo Rda S, Carvalho H, Menck CF (2003) The eukaryotic nucleotide excision repair pathway. *Biochimie* **85**(11)**:** 1083-1099

Cowell IG, Sunter NJ, Singh PB, Austin CA, Durkacz BW, Tilby MJ (2007) gammaH2AX foci form preferentially in euchromatin after ionising-radiation. *PLoS ONE* **2**(10)**:** e1057

Cowieson NP, Partridge JF, Allshire RC, McLaughlin PJ (2000) Dimerisation of a chromo shadow domain and distinctions from the chromodomain as revealed by structural analysis. *Curr Biol* **10**(9)**:** 517-525

Cox DR, Oakes D (1984) *Analysis of survival data.*, London: Chapman & Hall.

Craig JM (2005) Heterochromatin--many flavours, common themes. *Bioessays* **27**(1)**:** 17-28

Cremer T, Cremer M, Dietzel S, Muller S, Solovei I, Fakan S (2006) Chromosome territories--a functional nuclear landscape. *Curr Opin Cell Biol* **18**(3)**:** 307-316

Cryderman DE, Grade SK, Li Y, Fanti L, Pimpinelli S, Wallrath LL (2005) Role of Drosophila HP1 in euchromatic gene expression. *Dev Dyn* **232**(3)**:** 767-774

D'Errico M, Teson M, Calcagnile A, Proietti De Santis L, Nikaido O, Botta E, Zambruno G, Stefanini M, Dogliotti E (2003) Apoptosis and efficient repair of DNA damage protect human keratinocytes against UVB. *Cell Death Differ* **10**(6)**:** 754-756

Daganzo SM, Erzberger JP, Lam WM, Skordalakes E, Zhang R, Franco AA, Brill SJ, Adams PD, Berger JM, Kaufman PD (2003) Structure and function of the conserved core of histone deposition protein Asf1. *Curr Biol* **13**(24)**:** 2148-2158

Davey CA, Sargent DF, Luger K, Maeder AW, Richmond TJ (2002) Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. *J Mol Biol* **319**(5)**:** 1097-1113

de Cremoux P, Bieche I, Tran-Perennou C, Vignaud S, Boudou E, Asselain B, Lidereau R, Magdelenat H, Becette V, Sigal-Zafrani B, Spyratos F (2004) Inter-laboratory quality control for hormone-dependent gene expression in human breast tumors using real-time reverse transcription-polymerase chain reaction. *Endocr Relat Cancer* **11**(3)**:** 489-495

de Cremoux P, Jourdan-Da-Silva N, Couturier J, Tran-Perennou C, Schleiermacher G, Fehlbaum P, Doz F, Mosseri V, Delattre O, Klijanienko J, Vielh P, Michon J (2007) Role of chemotherapy resistance genes in outcome of neuroblastoma. *Pediatr Blood Cancer* **48**(3)**:** 311-317

De Koning L, Corpet A, Haber JE, Almouzni G (2007) Histone chaperones: an escort network regulating histone traffic. *Nat Struct Mol Biol* **14**(11)**:** 997-1007

de la Cruz CC, Kirmizis A, Simon MD, Isono K, Koseki H, Panning B (2007) The polycomb group protein SUZ12 regulates histone H3 lysine 9 methylation and HP1 alpha distribution. *Chromosome Res* **15**(3)**:** 299-314

de Lima-Bessa KM, Armelini MG, Chigancas V, Jacysyn JF, Amarante-Mendes GP, Sarasin A, Menck CF (2008) CPDs and 6-4PPs play different roles in UV-induced cell death in normal and NER-deficient human cells. *DNA Repair (Amst)* **7**(2)**:** 303-312

de Wit E, Greil F, van Steensel B (2007) High-resolution mapping reveals links of HP1 with active and inactive chromatin components. *PLoS Genet* **3**(3)**:** e38

Delcuve GP, Rastegar M, Davie JR (2009) Epigenetic control. *J Cell Physiol* **219**(2)**:** 243-250

Dialynas GK, Terjung S, Brown JP, Aucott RL, Baron-Luhr B, Singh PB, Georgatos SD (2007) Plasticity of HP1 proteins in mammalian cells. *J Cell Sci* **120**(Pt 19)**:** 3415-3424. Epub 2007 Sep 3412.

Dialynas GK, Vitalini MW, Wallrath LL (2008) Linking Heterochromatin Protein 1 (HP1) to cancer progression. *Mutat Res* **647**(1-2)**:** 13-20

Djupedal I, Ekwall K (2009) Epigenetics: heterochromatin meets RNAi. *Cell Res* **19**(3)**:** 282-295

Dohke K, Miyazaki S, Tanaka K, Urano T, Grewal SI, Murakami Y (2008) Fission yeast chromatin assembly factor 1 assists in the replication-coupled maintenance of heterochromatin. *Genes Cells* **13**(10)**:** 1027-1043

Dorigo B, Schalch T, Kulangara A, Duda S, Schroeder RR, Richmond TJ (2004) Nucleosome arrays reveal the two-start organization of the chromatin fiber. *Science* **306**(5701)**:** 1571-1573

Duan Q, Chen H, Costa M, Dai W (2008) Phosphorylation of H3S10 blocks the access of H3K9 by specific antibodies and histone methyltransferase. Implication in regulating chromatin dynamics and epigenetic inheritance during mitosis. *J Biol Chem* **283**(48)**:** 33585-33590

Dunleavy EM, Roche D, Tagami H, Lacoste N, Ray-Gallet D, Nakamura Y, Daigo Y, Nakatani Y, Almouzni-Pettinotti G (2009) HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. *Cell* **137**(3)**:** 485-497

Eissenberg JC, James TC, Foster-Hartnett DM, Hartnett T, Ngan V, Elgin SC (1990) Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **87**(24)**:** 9923-9927

Ekwall K, Javerzat JP, Lorentz A, Schmidt H, Cranston G, Allshire R (1995) The chromodomain protein Swi6: a key component at fission yeast centromeres. *Science* **269**(5229)**:** 1429-1431.

Elkhuizen PH, Voogd AC, van den Broek LC, Tan IT, van Houwelingen HC, Leer JW, van de Vijver MJ (1999) Risk factors for local recurrence after breast-conserving therapy for invasive carcinomas: a case-control study of histological factors and alterations in oncogene expression. *Int J Radiat Oncol Biol Phys* **45**(1)**:** 73-83

Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* **19**(5)**:** 403-410

Eltsov M, Maclellan KM, Maeshima K, Frangakis AS, Dubochet J (2008) Analysis of cryo-electron microscopy images does not support the existence of 30-nm chromatin fibers in mitotic chromosomes in situ. *Proc Natl Acad Sci U S A* **105**(50)**:** 19732-19737

Endo M, Ishikawa Y, Osakabe K, Nakayama S, Kaya H, Araki T, Shibahara K, Abe K, Ichikawa H, Valentine L, Hohn B, Toki S (2006) Increased frequency of homologous recombination and T-DNA integration in Arabidopsis CAF-1 mutants. *Embo J* **25**(23)**:** 5579-5590

Engel J, Kerr J, Schlesinger-Raab A, Sauer H, Holzel D (2004) Quality of life following breast-conserving therapy or mastectomy: results of a 5-year prospective study. *Breast J* **10**(3)**:** 223-231

Esteller M (2007) Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* **8**(4)**:** 286-298

Evans TR, Rowlands MG, Silva MC, Law M, Coombes RC (1993) Prognostic significance of aromatase and estrone sulfatase enzymes in human breast cancer. *J Steroid Biochem Mol Biol* **44**(4-6)**:** 583-587

Eymery A, Callanan M, Vourc'h C (2009) The secret message of heterochromatin: new insights into the mechanisms and function of centromeric and pericentric repeat sequence transcription. *Int J Dev Biol* **53**(2-3)**:** 259-268

Fahrner JA, Eguchi S, Herman JG, Baylin SB (2002) Dependence of histone modifications and gene expression on DNA hypermethylation in cancer. *Cancer Res* **62**(24)**:** 7213-7218

Fehm T, Muller V, Alix-Panabieres C, Pantel K (2008) Micrometastatic spread in breast cancer: detection, molecular characterization and clinical relevance. *Breast Cancer Res* **10 Suppl 1:** S1

Festenstein R, Pagakis SN, Hiragami K, Lyon D, Verreault A, Sekkali B, Kioussis D (2003) Modulation of heterochromatin protein 1 dynamics in primary Mammalian cells. *Science* **299**(5607)**:** 719-721.

Fischle W, Tseng BS, Dormann HL, Ueberheide BM, Garcia BA, Shabanowitz J, Hunt DF, Funabiki H, Allis CD (2005) Regulation of HP1-chromatin binding by histone H3 methylation and phosphorylation. *Nature* **438**(7071)**:** 1116-1122. Epub 2005 Oct 1112.

Fischle W, Wang Y, Jacobs SA, Kim Y, Allis CD, Khorasanizadeh S (2003) Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes Dev* **17**(15)**:** 1870-1881

Fisher TS, Zakian VA (2005) Ku: a multifunctional protein involved in telomere maintenance. *DNA Repair (Amst)* **4**(11)**:** 1215-1226

Flemming W (1882) *Cell substance, Nucleus and Cell Division*, Leipzig: Vogel.

Folco HD, Pidoux AL, Urano T, Allshire RC (2008) Heterochromatin and RNAi are required to establish CENP-A chromatin at centromeres. *Science* **319**(5859)**:** 94-97.

Foltz DR, Jansen LE, Bailey AO, Yates JR, 3rd, Bassett EA, Wood S, Black BE, Cleveland DW (2009) Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. *Cell* **137**(3)**:** 472-484

Font-Burgada J, Rossell D, Auer H, Azorin F (2008) Drosophila HP1c isoform interacts with the zinc-finger proteins WOC and Relative-of-WOC to regulate gene expression. *Genes Dev* **22**(21)**:** 3007-3023

Fousteri M, Mullenders LH (2008) Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. *Cell Res* **18**(1)**:** 73-84

Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, Bonaldi T, Haydon C, Ropero S, Petrie K, Iyer NG, Perez-Rosado A, Calvo E, Lopez JA, Cano A, Calasanz MJ, Colomer D, Piris MA, Ahn N, Imhof A, Caldas C, Jenuwein T, Esteller M (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat Genet* **37**(4)**:** 391-400. Epub 2005 Mar 2013.

Fukagawa T, Nogami M, Yoshikawa M, Ikeno M, Okazaki T, Takami Y, Nakayama T, Oshimura M (2004) Dicer is essential for formation of the heterochromatin structure in vertebrate cells. *Nat Cell Biol* **6**(8)**:** 784-791

Fuks F, Hurd PJ, Deplus R, Kouzarides T (2003) The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Res* **31**(9)**:** 2305-2312.

Gaillard PH, Martini EM, Kaufman PD, Stillman B, Moustacchi E, Almouzni G (1996) Chromatin assembly coupled to DNA repair: a new role for chromatin assembly factor I. *Cell* **86**(6)**:** 887-896.

Gale JM, Smerdon MJ (1990) UV induced (6-4) photoproducts are distributed differently than cyclobutane dimers in nucleosomes. *Photochem Photobiol* **51**(4)**:** 411-417

Galea MH, Blamey RW, Elston CE, Ellis IO (1992) The Nottingham Prognostic Index in primary breast cancer. *Breast Cancer Res Treat* **22**(3)**:** 207-219

Galmiche G, Corvaisier S, Kottler ML (2006) Aromatase gene expression and regulation in the female rat pituitary. *Ann N Y Acad Sci* **1070:** 286-292

Game JC, Kaufman PD (1999) Role of Saccharomyces cerevisiae chromatin assembly factor-I in repair of ultraviolet radiation damage in vivo. *Genetics* **151**(2)**:** 485-497.

Garinis GA, Patrinos GP, Spanakis NE, Menounos PG (2002) DNA hypermethylation: when tumour suppressor genes go silent. *Hum Genet* **111**(2)**:** 115-127

Gaubatz JW, Cutler RG (1990) Mouse satellite DNA is transcribed in senescent cardiac muscle. *J Biol Chem* **265**(29)**:** 17753-17758

Gehring M, Reik W, Henikoff S (2009) DNA demethylation by DNA repair. *Trends Genet* **25**(2)**:** 82-90

Geng Y, Lee YM, Welcker M, Swanger J, Zagozdzon A, Winer JD, Roberts JM, Kaldis P, Clurman BE, Sicinski P (2007) Kinase-independent function of cyclin E. *Mol Cell* **25**(1)**:** 127-139

Geng Y, Yu Q, Sicinska E, Das M, Schneider JE, Bhattacharya S, Rideout WM, Bronson RT, Gardner H, Sicinski P (2003) Cyclin E ablation in the mouse. *Cell* **114**(4)**:** 431-443

Gerard A, Koundrioukoff S, Ramillon V, Sergere JC, Mailand N, Quivy JP, Almouzni G (2006a) The replication kinase Cdc7-Dbf4 promotes the interaction of the p150 subunit of chromatin assembly factor 1 with proliferating cell nuclear antigen. *EMBO Rep* **7**(8)**:** 817-823

Gerard A, Polo SE, Roche D, Almouzni G (2006b) Methods for studying chromatin assembly coupled to DNA repair. *Methods Enzymol* **409:** 358-374

Gérard A, Polo SE, Roche D, Almouzni G (2006) Methods for studying chromatin assembly coupled to DNA repair. *Methods in Enzymology* **409:** 358-374

Geyer FC, Marchio C, Reis-Filho JS (2009) The role of molecular analysis in breast cancer. *Pathology* **41**(1)**:** 77-88

Gilbert N, Boyle S, Sutherland H, de Las Heras J, Allan J, Jenuwein T, Bickmore WA (2003) Formation of facultative heterochromatin in the absence of HP1. *Embo J* **22**(20)**:** 5540-5550.

Girton JR, Johansen KM (2008) Chromatin structure and the regulation of gene expression: the lessons of PEV in Drosophila. *Adv Genet* **61:** 1-43

Godde JS, Ura K (2008) Cracking the enigmatic linker histone code. *J Biochem* **143**(3)**:** 287-293

Gong F, Kwon Y, Smerdon MJ (2005) Nucleotide excision repair in chromatin and the right of entry. *DNA Repair (Amst)* **4**(8)**:** 884-896

Gontijo AM, Green CM, Almouzni G (2003) Repairing DNA damage in chromatin. *Biochimie* **85**(11)**:** 1133-1147.

Goodarzi AA, Noon AT, Deckbar D, Ziv Y, Shiloh Y, Lobrich M, Jeggo PA (2008) ATM signaling facilitates repair of DNA double-strand breaks associated with heterochromatin. *Mol Cell* **31**(2)**:** 167-177

Gotta M, Laroche T, Formenton A, Maillet L, Scherthan H, Gasser SM (1996) The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae. *J Cell Biol* **134**(6)**:** 1349-1363

Green CA, Peter MB, Speirs V, Shaaban AM (2008) The potential role of ER beta isoforms in the clinical management of breast cancer. *Histopathology* **53**(4)**:** 374-380

Green CM, Almouzni G (2002) When repair meets chromatin. First in series on chromatin dynamics. *EMBO Rep* **3**(1)**:** 28-33.

Green CM, Almouzni G (2003a) Local action of the chromatin assembly factor CAF-1 at sites of nucleotide excision repair in vivo. *Embo J* **22**(19)**:** 5163-5174.

Green CM, Almouzni G (2003b) Local action of the chromatin assembly factor CAF-1 at sites of nucleotide excision repair in vivo. *Embo J* **22**(19)**:** 5163-5174.

Grewal SI, Jia S (2007) Heterochromatin revisited. *Nat Rev Genet* **8**(1)**:** 35-46

Grigoryev SA, Nikitina T, Pehrson JR, Singh PB, Woodcock CL (2004) Dynamic relocation of epigenetic chromatin markers reveals an active role of constitutive heterochromatin in the transition from proliferation to quiescence. *J Cell Sci* **117**(Pt 25)**:** 6153-6162

Groth A, Corpet A, Cook AJ, Roche D, Bartek J, Lukas J, Almouzni G (2007a) Regulation of replication fork progression through histone supply and demand. *Science* **318**(5858)**:** 1928-1931

Groth A, Ray-Gallet D, Quivy JP, Lukas J, Bartek J, Almouzni G (2005) Human Asf1 regulates the flow of S phase histones during replicational stress. *Mol Cell* **17**(2)**:** 301-311.

Groth A, Rocha W, Verreault A, Almouzni G (2007b) Chromatin challenges during DNA replication and repair. *Cell* **128**(4)**:** 721-733.

Guenatri M, Bailly D, Maison C, Almouzni G (2004) Mouse centric and pericentric satellite repeats form distinct functional heterochromatin. *J Cell Biol* **166**(4)**:** 493-505. Epub 2004 Aug 2009.

Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, Lander ES (2009) Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell* **138**(4)**:** 645-659

Hackett AJ, Smith HS, Springer EL, Owens RB, Nelson-Rees WA, Riggs JL, Gardner MB (1977) Two syngeneic cell lines from human breast tissue: the aneuploid mammary epithelial (Hs578T) and the diploid myoepithelial (Hs578Bst) cell lines. *J Natl Cancer Inst* **58**(6)**:** 1795-1806.

Hakansson P, Hofer A, Thelander L (2006) Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem* **281**(12)**:** 7834-7841

Hamelers IH, Steenbergh PH (2003) Interactions between estrogen and insulin-like growth factor signaling pathways in human breast tumor cells. *Endocr Relat Cancer* **10**(2)**:** 331-345

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* **100**(1)**:** 57-70

Happel N, Doenecke D (2009) Histone H1 and its isoforms: contribution to chromatin structure and function. *Gene* **431**(1-2)**:** 1-12

Hara R, Mo J, Sancar A (2000) DNA damage in the nucleosome core is refractory to repair by human excision nuclease. *Mol Cell Biol* **20**(24)**:** 9173-9181

Harel J, Hanania N, Tapiero H, Harel L (1968) RNA replication by nuclear satellite DNA in different mouse cells. *Biochem Biophys Res Commun* **33**(4)**:** 696-701

Hartley MC, McKinley BP, Rogers EA, Kalbaugh CA, Messich HS, Blackhurst DW, Lokey JS, Trocha SD (2006) Differential expression of prognostic factors and effect on survival in young (< or =40) breast cancer patients: a case-control study. *Am Surg* **72**(12)**:** 1189-1194; discussion 1194-1185

Hayakawa T, Haraguchi T, Masumoto H, Hiraoka Y (2003) Cell cycle behavior of human HP1 subtypes: distinct molecular domains of HP1 are required for their centromeric localization during interphase and metaphase. *J Cell Sci* **116**(Pt 16)**:** 3327-3338. Epub 2003 Jul 3322.

Hayashi S, Sakamoto T, Inoue A, Yoshida N, Omoto Y, Yamaguchi Y (2003) Estrogen and growth factor signaling pathway: basic approaches for clinical application. *J Steroid Biochem Mol Biol* **86**(3-5)**:** 433-442

Heard E, Disteche CM (2006) Dosage compensation in mammals: fine-tuning the expression of the X chromosome. *Genes Dev* **20**(14)**:** 1848-1867

Hearn MG, Hedrick A, Grigliatti TA, Wakimoto BT (1991) The effect of modifiers of position-effect variegation on the variegation of heterochromatic genes of Drosophila melanogaster. *Genetics* **128**(4)**:** 785-797

Heitz E (1928) Das heterochromatin der moose. *JB Wiss Bot* **69:** 728

Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA (2008) DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer* **8**(3)**:** 193-204

Hinshelwood RA, Clark SJ (2008) Breast cancer epigenetics: normal human mammary epithelial cells as a model system. *J Mol Med* **86**(12)**:** 1315-1328

Hirota T, Lipp JJ, Toh BH, Peters JM (2005) Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin. *Nature* **438**(7071)**:** 1176-1180. Epub 2005 Oct 1112.

Hoeijmakers JH (2001) Genome maintenance mechanisms for preventing cancer. *Nature* **411**(6835)**:** 366-374

Hoek M, Stillman B (2003) Chromatin assembly factor 1 is essential and couples chromatin assembly to DNA replication in vivo. *Proc Natl Acad Sci U S A* **100**(21)**:** 12183-12188. Epub 12003 Sep 12130.

Holli K, Isola J (1997) Effect of age on the survival of breast cancer patients. *Eur J Cancer* **33**(3)**:** 425-428

Houlard M, Berlivet S, Probst AV, Quivy JP, Hery P, Almouzni G, Gerard M (2006) CAF-1 is essential for heterochromatin organization in pluripotent embryonic cells. *PLoS Genet* **2**(11)**:** e181. Epub 2006 Sep 2011.

Hsu TC, Cooper JE, Mace ML, Jr., Brinkley BR (1971) Arrangement of centromeres in mouse cells. *Chromosoma* **34**(1)**:** 73-87

Hudis CA (2007) Trastuzumab--mechanism of action and use in clinical practice. *N Engl J Med* **357**(1)**:** 39-51

Inoue A, Hyle J, Lechner MS, Lahti JM (2008) Perturbation of HP1 localization and chromatin binding ability causes defects in sister-chromatid cohesion. *Mutat Res* **657**(1)**:** 48-55

Issa JP (2004) CpG island methylator phenotype in cancer. *Nat Rev Cancer* **4**(12)**:** 988-993

Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JC, Trent JM, Staudt LM, Hudson J, Jr., Boguski MS, Lashkari D, Shalon D, Botstein D, Brown PO (1999) The transcriptional program in the response of human fibroblasts to serum. *Science* **283**(5398)**:** 83-87.

James TC, Elgin SC (1986) Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene. *Mol Cell Biol* **6**(11)**:** 3862-3872.

Jansen LE, Black BE, Foltz DR, Cleveland DW (2007) Propagation of centromeric chromatin requires exit from mitosis. *J Cell Biol* **176**(6)**:** 795-805

Jensen KA, Smerdon MJ (1990) DNA repair within nucleosome cores of UV-irradiated human cells. *Biochemistry* **29**(20)**:** 4773-4782

Jenuwein T, Allis CD (2001) Translating the histone code. *Science* **293**(5532)**:** 1074-1080

Jeppesen P, Mitchell A, Turner B, Perry P (1992) Antibodies to defined histone epitopes reveal variations in chromatin conformation and underacetylation of centric heterochromatin in human metaphase chromosomes. *Chromosoma* **101**(5-6)**:** 322-332

Jin C, Felsenfeld G (2007) Nucleosome stability mediated by histone variants H3.3 and H2A.Z. *Genes Dev* **21**(12)**:** 1519-1529

Jones DO, Cowell IG, Singh PB (2000) Mammalian chromodomain proteins: their role in genome organisation and expression. *Bioessays* **22**(2)**:** 124-137

Jones PA, Baylin SB (2007) The epigenomics of cancer. *Cell* **128**(4)**:** 683-692.

Kamakaka RT, Bulger M, Kaufman PD, Stillman B, Kadonaga JT (1996) Postreplicative chromatin assembly by Drosophila and human chromatin assembly factor 1. *Mol Cell Biol* **16**(3)**:** 810-817

Kantidze OL, Razin SV (2009) Chromatin loops, illegitimate recombination, and genome evolution. *Bioessays* **31**(3)**:** 278-286

Kaufman PD, Kobayashi R, Kessler N, Stillman B (1995a) The p150 and p60 subunits of chromatin assembly factor I: a molecular link between newly synthesized histones and DNA replication. *Cell* **81**(7)**:** 1105-1114.

Kaufman PD, Kobayashi R, Kessler N, Stillman B (1995b) The p150 and p60 subunits of chromatin assembly factor I: a molecular link between newly synthesized histones and DNA replication. *Cell* **81**(7)**:** 1105-1114

Kaufman PD, Kobayashi R, Stillman B (1997) Ultraviolet radiation sensitivity and reduction of telomeric silencing in Saccharomyces cerevisiae cells lacking chromatin assembly factor-I. *Genes Dev* **11**(3)**:** 345-357.

Kaya H, Shibahara KI, Taoka KI, Iwabuchi M, Stillman B, Araki T (2001) FASCIATA genes for chromatin assembly factor-1 in arabidopsis maintain the cellular organization of apical meristems. *Cell* **104**(1)**:** 131-142

Kim DH, Villeneuve LM, Morris KV, Rossi JJ (2006) Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nat Struct Mol Biol* **13**(9)**:** 793-797

Kim JA, Haber JE (2009) Chromatin assembly factors Asf1 and CAF-1 have overlapping roles in deactivating the DNA damage checkpoint when DNA repair is complete. *Proc Natl Acad Sci U S A* **106**(4)**:** 1151-1156

Kim JA, Kruhlak M, Dotiwala F, Nussenzweig A, Haber JE (2007) Heterochromatin is refractory to gamma-H2AX modification in yeast and mammals. *J Cell Biol* **178**(2)**:** 209-218

Kim JK, Choi BS (1995) The solution structure of DNA duplex-decamer containing the (6-4) photoproduct of thymidylyl(3'-->5')thymidine by NMR and relaxation matrix refinement. *Eur J Biochem* **228**(3)**:** 849-854

Kirik A, Pecinka A, Wendeler E, Reiss B (2006) The chromatin assembly factor subunit FASCIATA1 is involved in homologous recombination in plants. *Plant Cell* **18**(10)**:** 2431-2442

Kirschmann DA, Lininger RA, Gardner LM, Seftor EA, Odero VA, Ainsztein AM, Earnshaw WC, Wallrath LL, Hendrix MJ (2000) Down-regulation of HP1Hsalpha expression is associated with the metastatic phenotype in breast cancer. *Cancer Res* **60**(13)**:** 3359-3363

Klapholz B, Dietrich BH, Schaffner C, Heredia F, Quivy JP, Almouzni G, Dostatni N (2009) CAF-1 is required for efficient replication of euchromatic DNA in Drosophila larval endocycling cells. *Chromosoma* **118**(2)**:** 235-248

Klose RJ, Bird AP (2006) Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* **31**(2)**:** 89-97

Koch B, Kueng S, Ruckenbauer C, Wendt KS, Peters JM (2008) The Suv39h-HP1 histone methylation pathway is dispensable for enrichment and protection of cohesin at centromeres in mammalian cells. *Chromosoma* **117**(2)**:** 199-210. Epub 2007 Dec 2011.

Kornberg RD (1977) Structure of chromatin. *Annu Rev Biochem* **46:** 931-954.

Kouros-Mehr H, Kim JW, Bechis SK, Werb Z (2008) GATA-3 and the regulation of the mammary luminal cell fate. *Curr Opin Cell Biol* **20**(2)**:** 164-170

Krawitz DC, Kama T, Kaufman PD (2002) Chromatin assembly factor I mutants defective for PCNA binding require Asf1/Hir proteins for silencing. *Mol Cell Biol* **22**(2)**:** 614-625

Kreike B, Halfwerk H, Kristel P, Glas A, Peterse H, Bartelink H, van de Vijver MJ (2006) Gene expression profiles of primary breast carcinomas from patients at high risk for local recurrence after breast-conserving therapy. *Clin Cancer Res* **12**(19)**:** 5705-5712

Krude T (1995) Chromatin assembly factor 1 (CAF-1) colocalizes with replication foci in HeLa cell nuclei. *Exp Cell Res* **220**(2)**:** 304-311

Kruithof M, Chien FT, Routh A, Logie C, Rhodes D, van Noort J (2009) Single-molecule force spectroscopy reveals a highly compliant helical folding for the 30-nm chromatin fiber. *Nat Struct Mol Biol* **16**(5)**:** 534-540

Kwon SH, Workman JL (2008) The heterochromatin protein 1 (HP1) family: put away a bias toward HP1. *Mol Cells* **26**(3)**:** 217-227

Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**(6824)**:** 116-120.

Larionov A, Krause A, Miller W (2005) A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics* **6:** 62

Laskey RA, Honda BM, Mills AD, Finch JT (1978) Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. *Nature* **275**(5679)**:** 416-420.

Laskey RA, Mills AD, Morris NR (1977) Assembly of SV40 chromatin in a cell-free system from Xenopus eggs. *Cell* **10**(2)**:** 237-243

Leatherwood J, Vas A (2003) Connecting ORC and heterochromatin: why? *Cell Cycle* **2**(6)**:** 573-575

Lee WL, Cheng MH, Chao HT, Wang PH (2008) The role of selective estrogen receptor modulators on breast cancer: from tamoxifen to raloxifene. *Taiwan J Obstet Gynecol* **47**(1)**:** 24-31

Lehmann AR (2003) DNA repair-deficient diseases, xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy. *Biochimie* **85**(11)**:** 1101-1111

Lewis LK, Karthikeyan G, Cassiano J, Resnick MA (2005) Reduction of nucleosome assembly during new DNA synthesis impairs both major pathways of double-strand break repair. *Nucleic Acids Res* **33**(15)**:** 4928-4939. Print 2005.

Lidonnici MR, Rossi R, Paixao S, Mendoza-Maldonado R, Paolinelli R, Arcangeli C, Giacca M, Biamonti G, Montecucco A (2004) Subnuclear distribution of the largest subunit of the human origin recognition complex during the cell cycle. *J Cell Sci* **117**(Pt 22)**:** 5221-5231

Lin CH, Li B, Swanson S, Zhang Y, Florens L, Washburn MP, Abmayr SM, Workman JL (2008) Heterochromatin protein 1a stimulates histone H3 lysine 36 demethylation by the Drosophila KDM4A demethylase. *Mol Cell* **32**(5)**:** 696-706

Linger J, Tyler JK (2005) The yeast histone chaperone chromatin assembly factor 1 protects against double-strand DNA-damaging agents. *Genetics* **171**(4)**:** 1513-1522

Liu X, Smerdon MJ (2000) Nucleotide excision repair of the 5 S ribosomal RNA gene assembled into a nucleosome. *J Biol Chem* **275**(31)**:** 23729-23735

Lomberk G, Bensi D, Fernandez-Zapico ME, Urrutia R (2006a) Evidence for the existence of an HP1-mediated subcode within the histone code. *Nat Cell Biol* **8**(4)**:** 407-415. Epub 2006 Mar 2012.

Lomberk G, Wallrath L, Urrutia R (2006b) The Heterochromatin Protein 1 family. *Genome Biol* **7**(7)**:** 228.

Loyola A, Almouzni G (2004) Histone chaperones, a supporting role in the limelight. *Biochim Biophys Acta* **1677**(1-3)**:** 3-11

Loyola A, Almouzni G (2007) Marking histone H3 variants: how, when and why? *Trends Biochem Sci* **32**(9)**:** 425-433

Loyola A, Bonaldi T, Roche D, Imhof A, Almouzni G (2006) PTMs on H3 variants before chromatin assembly potentiate their final epigenetic state. *Mol Cell* **24**(2)**:** 309-316

Loyola A, Tagami H, Bonaldi T, Roche D, Quivy JP, Imhof A, Nakatani Y, Dent SY, Almouzni G (2009) The HP1alpha-CAF1-SetDB1-containing complex provides H3K9me1 for Suv39-mediated K9me3 in pericentric heterochromatin. *EMBO Rep*

Lu BY, Emtage PC, Duyf BJ, Hilliker AJ, Eissenberg JC (2000) Heterochromatin protein 1 is required for the normal expression of two heterochromatin genes in Drosophila. *Genetics* **155**(2)**:** 699-708

Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**(6648)**:** 251-260.

Luijsterburg MS, Dinant C, Lans H, Stap J, Wiernasz E, Lagerwerf S, Warmerdam DO, Lindh M, Brink MC, Dobrucki JW, Aten JA, Fousteri MI, Jansen G, Dantuma NP, Vermeulen W, Mullenders LH, Houtsmuller AB, Verschure PJ, van Driel R (2009) Heterochromatin protein 1 is recruited to various types of DNA damage. *J Cell Biol* **185**(4)**:** 577-586

Luo J, Su F, Chen D, Shiloh A, Gu W (2000) Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature* **408**(6810)**:** 377-381

Ma DK, Guo JU, Ming GL, Song H (2009) DNA excision repair proteins and Gadd45 as molecular players for active DNA demethylation. *Cell Cycle* **8**(10)

Mahy NL, Perry PE, Bickmore WA (2002) Gene density and transcription influence the localization of chromatin outside of chromosome territories detectable by FISH. *J Cell Biol* **159**(5)**:** 753-763

Maison C, Almouzni G (2004) HP1 and the dynamics of heterochromatin maintenance. *Nat Rev Mol Cell Biol* **5**(4)**:** 296-304.

Maison C, Bailly D, Peters AH, Quivy JP, Roche D, Taddei A, Lachner M, Jenuwein T, Almouzni G (2002) Higher-order structure in pericentric heterochromatin involves a distinct pattern of histone modification and an RNA component. *Nat Genet* **30**(3)**:** 329-334

Mandrioli M, Borsatti F (2006) DNA methylation of fly genes and transposons. *Cell Mol Life Sci* **63**(17)**:** 1933-1936

Martini E, Roche DM, Marheineke K, Verreault A, Almouzni G (1998) Recruitment of phosphorylated chromatin assembly factor 1 to chromatin after UV irradiation of human cells. *J Cell Biol* **143**(3)**:** 563-575.

Mateescu B, Bourachot B, Rachez C, Ogryzko V, Muchardt C (2008) Regulation of an inducible promoter by an HP1beta-HP1gamma switch. *EMBO Rep* **9**(3)**:** 267-272

Matsumoto M, Yaginuma K, Igarashi A, Imura M, Hasegawa M, Iwabuchi K, Date T, Mori T, Ishizaki K, Yamashita K, Inobe M, Matsunaga T (2007) Perturbed gap-filling synthesis in nucleotide excision repair causes histone H2AX phosphorylation in human quiescent cells. *J Cell Sci* **120**(Pt 6)**:** 1104-1112

May KM, Hardwick KG (2006) The spindle checkpoint. *J Cell Sci* **119**(Pt 20)**:** 4139-4142

Meehan RR, Kao CF, Pennings S (2003) HP1 binding to native chromatin in vitro is determined by the hinge region and not by the chromodomain. *Embo J* **22**(12)**:** 3164-3174

Mehra R, Varambally S, Ding L, Shen R, Sabel MS, Ghosh D, Chinnaiyan AM, Kleer CG (2005) Identification of GATA3 as a breast cancer prognostic marker by global gene expression meta-analysis. *Cancer Res* **65**(24)**:** 11259-11264

Mello JA, Almouzni G (2001) The ins and outs of nucleosome assembly. *Curr Opin Genet Dev* **11**(2)**:** 136-141

Mello JA, Sillje HH, Roche DM, Kirschner DB, Nigg EA, Almouzni G (2002) Human Asf1 and CAF-1 interact and synergize in a repair-coupled nucleosome assembly pathway. *EMBO Rep* **3**(4)**:** 329-334

Meshorer E, Yellajoshula D, George E, Scambler PJ, Brown DT, Misteli T (2006) Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. *Dev Cell* **10**(1)**:** 105-116.

Miki Y, Suzuki T, Tazawa C, Yamaguchi Y, Kitada K, Honma S, Moriya T, Hirakawa H, Evans DB, Hayashi S, Ohuchi N, Sasano H (2007) Aromatase localization in human breast cancer tissues: possible interactions between intratumoral stromal and parenchymal cells. *Cancer Res* **67**(8)**:** 3945-3954

Miller WR, Hawkins RA, Forrest AP (1982) Significance of aromatase activity in human breast cancer. *Cancer Res* **42**(8 Suppl)**:** 3365s-3368s

Minc E, Allory Y, Courvalin JC, Buendia B (2001) Immunolocalization of HP1 proteins in metaphasic mammalian chromosomes. *Methods Cell Sci* **23**(1-3)**:** 171-174.

Minc E, Allory Y, Worman HJ, Courvalin JC, Buendia B (1999) Localization and phosphorylation of HP1 proteins during the cell cycle in mammalian cells. *Chromosoma* **108**(4)**:** 220-234.

Mitchell DL, Nguyen TD, Cleaver JE (1990) Nonrandom induction of pyrimidine-pyrimidone (6-4) photoproducts in ultraviolet-irradiated human chromatin. *J Biol Chem* **265**(10)**:** 5353-5356

Mito Y, Henikoff JG, Henikoff S (2005) Genome-scale profiling of histone H3.3 replacement patterns. *Nat Genet* **37**(10)**:** 1090-1097

Miura M, Nakamura S, Sasaki T, Takasaki Y, Shiomi T, Yamaizumi M (1996) Roles of XPG and XPF/ERCC1 endonucleases in UV-induced immunostaining of PCNA in fibroblasts. *Exp Cell Res* **226**(1)**:** 126-132

Mocquet V, Laine JP, Riedl T, Yajin Z, Lee MY, Egly JM (2008) Sequential recruitment of the repair factors during NER: the role of XPG in initiating the resynthesis step. *Embo J* **27**(1)**:** 155-167

Moggs JG, Grandi P, Quivy JP, Jonsson ZO, Hubscher U, Becker PB, Almouzni G (2000) A CAF-1-PCNA-mediated chromatin assembly pathway triggered by sensing DNA damage. *Mol Cell Biol* **20**(4)**:** 1206-1218.

Mone MJ, Volker M, Nikaido O, Mullenders LH, van Zeeland AA, Verschure PJ, Manders EM, van Driel R (2001) Local UV-induced DNA damage in cell nuclei results in local transcription inhibition. *EMBO Rep* **2**(11)**:** 1013-1017

Morris KV, Chan SW, Jacobsen SE, Looney DJ (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**(5688)**:** 1289-1292

Morrison BJ, Schmidt CW, Lakhani SR, Reynolds BA, Lopez JA (2008) Breast cancer stem cells: implications for therapy of breast cancer. *Breast Cancer Res* **10**(4)**:** 210

Mousson F, Ochsenbein F, Mann C (2007) The histone chaperone Asf1 at the crossroads of chromatin and DNA checkpoint pathways. *Chromosoma* **116**(2)**:** 79-93. Epub 2006 Dec 2019.

Moyer A (1997) Psychosocial outcomes of breast-conserving surgery versus mastectomy: a meta-analytic review. *Health Psychol* **16**(3)**:** 284-298

Muchardt C, Guilleme M, Seeler JS, Trouche D, Dejean A, Yaniv M (2002) Coordinated methyl and RNA binding is required for heterochromatin localization of mammalian HP1alpha. *EMBO Rep* **3**(10)**:** 975-981

Mujtaba S, Zeng L, Zhou MM (2007) Structure and acetyl-lysine recognition of the bromodomain. *Oncogene* **26**(37)**:** 5521-5527

Murzina N, Verreault A, Laue E, Stillman B (1999) Heterochromatin dynamics in mouse cells: interaction between chromatin assembly factor 1 and HP1 proteins. *Mol Cell* **4**(4)**:** 529-540

Nabatiyan A, Szuts D, Krude T (2006) Induction of CAF-1 expression in response to DNA strand breaks in quiescent human cells. *Mol Cell Biol* **26**(5)**:** 1839-1849.

Narita M, Nunez S, Heard E, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113**(6)**:** 703-716

Nicholson RI, Gee JM, Knowlden J, McClelland R, Madden TA, Barrow D, Hutcheson I (2003) The biology of antihormone failure in breast cancer. *Breast Cancer Res Treat* **80 Suppl 1:** S29-34; discussion S35

Nielsen AL, Ortiz JA, You J, Oulad-Abdelghani M, Khechumian R, Gansmuller A, Chambon P, Losson R (1999) Interaction with members of the heterochromatin protein 1 (HP1) family and histone deacetylation are differentially involved in transcriptional silencing by members of the TIF1 family. *Embo J* **18**(22)**:** 6385-6395

Nielsen AL, Oulad-Abdelghani M, Ortiz JA, Remboutsika E, Chambon P, Losson R (2001a) Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins. *Mol Cell* **7**(4)**:** 729-739.

Nielsen PR, Nietlispach D, Mott HR, Callaghan J, Bannister A, Kouzarides T, Murzin AG, Murzina NV, Laue ED (2002) Structure of the HP1 chromodomain bound to histone H3 methylated at lysine 9. *Nature* **416**(6876)**:** 103-107

Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D, Firestein R, Cleary M, Jenuwein T, Herrera RE, Kouzarides T (2001b) Rb targets histone H3 methylation and HP1 to promoters. *Nature* **412**(6846)**:** 561-565

Niggli HJ, Cerutti PA (1982) Nucleosomal distribution of thymine photodimers following far- and near-ultraviolet irradiation. *Biochem Biophys Res Commun* **105**(3)**:** 1215-1223

Niméus E, Krogh M, Malmström P, Strand C, Fredriksson I, Karlsson P, Nordenskjöld B, Stål O, Östberg G, Peterson C, Fernö M (2006) Gene expression profiling in primary breast cancer distinguishes patients developing local recurrence despite postoperative radiotherapy after breast conserving surgery. *29th Annual San Antonio Breast Cancer Symposium 2006* **Volume 103, Number 1 / mai 2007:** 115-124

Nolan T, Hands RE, Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. *Nat Protoc* **1**(3)**:** 1559-1582

Norwood LE, Grade SK, Cryderman DE, Hines KA, Furiasse N, Toro R, Li Y, Dhasarathy A, Kladde MP, Hendrix MJ, Kirschmann DA, Wallrath LL (2004) Conserved properties of HP1(Hsalpha). *Gene* **336**(1)**:** 37-46

Norwood LE, Moss TJ, Margaryan NV, Cook SL, Wright L, Seftor EA, Hendrix MJ, Kirschmann DA, Wallrath LL (2006) A requirement for dimerization of HP1Hsalpha in suppression of breast cancer invasion. *J Biol Chem* **28:** 28

Nuyten DS, Kreike B, Hart AA, Chi JT, Sneddon JB, Wessels LF, Peterse HJ, Bartelink H, Brown PO, Chang HY, van de Vijver MJ (2006) Predicting a local recurrence after breast-conserving therapy by gene expression profiling. *Breast Cancer Res* **8**(5)**:** R62

O'Geen H, Squazzo SL, Iyengar S, Blahnik K, Rinn JL, Chang HY, Green R, Farnham PJ (2007) Genome-wide analysis of KAP1 binding suggests autoregulation of KRAB-ZNFs. *PLoS Genet* **3**(6)**:** e89. Epub 2007 Apr 2019.

Obuse C, Iwasaki O, Kiyomitsu T, Goshima G, Toyoda Y, Yanagida M (2004) A conserved Mis12 centromere complex is linked to heterochromatic HP1 and outer kinetochore protein Zwint-1. *Nat Cell Biol* **6**(11)**:** 1135-1141

Oh DS, Troester MA, Usary J, Hu Z, He X, Fan C, Wu J, Carey LA, Perou CM (2006a) Estrogen-regulated genes predict survival in hormone receptor-positive breast cancers. *J Clin Oncol* **24**(11)**:** 1656-1664

Oh JL, Bonnen M, Outlaw ED, Schechter NR, Perkins GH, Strom EA, Babiera G, Oswald MJ, Allen PK, Thames HD, Buchholz TA (2006b) The impact of young age on locoregional recurrence after doxorubicin-based breast conservation therapy in patients 40 years old or younger: How young is "young"? *Int J Radiat Oncol Biol Phys* **65**(5)**:** 1345-1352

Oh JL, Bonnen M, Outlaw ED, Schechter NR, Perkins GH, Strom EA, Babiera G, Oswald MJ, Allen PK, Thames HD, Buchholz TA (2006c) The impact of young age on locoregional recurrence after doxorubicin-based breast conservation therapy in patients 40 years old or younger: How young is "young"? *Int J Radiat Oncol Biol Phys*

Okano S, Lan L, Caldecott KW, Mori T, Yasui A (2003) Spatial and temporal cellular responses to single-strand breaks in human cells. *Mol Cell Biol* **23**(11)**:** 3974-3981.

Olins AL, Olins DE (1974) Spheroid chromatin units (v bodies). *Science* **183**(4122)**:** 330-332

Olins AL, Olins DE, Zentgraf H, Franke WW (1980) Visualization of nucleosomes in thin sections by stereo electron microscopy. *J Cell Biol* **87**(3 Pt 1)**:** 833-836

Ono T, Kaya H, Takeda S, Abe M, Ogawa Y, Kato M, Kakutani T, Mittelsten Scheid O, Araki T, Shibahara K (2006) Chromatin assembly factor 1 ensures the stable maintenance of silent chromatin states in Arabidopsis. *Genes Cells* **11**(2)**:** 153-162

Osley MA (1991) The regulation of histone synthesis in the cell cycle. *Annu Rev Biochem* **60:** 827-861

Oudet P, Gross-Bellard M, Chambon P (1975) Electron microscopic and biochemical evidence that chromatin structure is a repeating unit. *Cell* **4**(4)**:** 281-300

Panteleeva I, Boutillier S, See V, Spiller DG, Rouaux C, Almouzni G, Bailly D, Maison C, Lai HC, Loeffler JP, Boutillier AL (2007) HP1alpha guides neuronal fate by timing E2F-targeted genes silencing during terminal differentiation. *Embo J* **26**(15)**:** 3616-3628. Epub 2007 Jul 3612.

Pauley RJ, Santner SJ, Tait LR, Bright RK, Santen RJ (2000) Regulated CYP19 aromatase transcription in breast stromal fibroblasts. *J Clin Endocrinol Metab* **85**(2)**:** 837-846

Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. *Nature* **406**(6797)**:** 747-752

Peters AH, O'Carroll D, Scherthan H, Mechtler K, Sauer S, Schofer C, Weipoltshammer K, Pagani M, Lachner M, Kohlmaier A, Opravil S, Doyle M, Sibilia M, Jenuwein T (2001) Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* **107**(3)**:** 323-337

Piacentini L, Fanti L, Berloco M, Perrini B, Pimpinelli S (2003) Heterochromatin protein 1 (HP1) is associated with induced gene expression in Drosophila euchromatin. *J Cell Biol* **161**(4)**:** 707-714

Pietersen AM, Horlings HM, Hauptmann M, Langerod A, Ajouaou A, Cornelissen-Steijger P, Wessels LF, Jonkers J, van de Vijver MJ, van Lohuizen M (2008) EZH2 and BMI1 inversely correlate with prognosis and TP53 mutation in breast cancer. *Breast Cancer Res* **10**(6)**:** R109

Pike MC, Spicer DV, Dahmoush L, Press MF (1993) Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. *Epidemiol Rev* **15**(1)**:** 17-35

Plohl M, Luchetti A, Mestrovic N, Mantovani B (2008) Satellite DNAs between selfishness and functionality: structure, genomics and evolution of tandem repeats in centromeric (hetero)chromatin. *Gene* **409**(1-2)**:** 72-82

Polo SE, Almouzni G (2006) Chromatin assembly: a basic recipe with various flavours. *Curr Opin Genet Dev* **16**(2)**:** 104-111.

Polo SE, Roche D, Almouzni G (2006) New histone incorporation marks sites of UV repair in human cells. *Cell* **127**(3)**:** 481-493.

Polo SE, Theocharis SE, Klijanienko J, Savignoni A, Asselain B, Vielh P, Almouzni G (2004) Chromatin assembly factor-1, a marker of clinical value to distinguish quiescent from proliferating cells. *Cancer Res* **64**(7)**:** 2371-2381

Prasanth SG, Prasanth KV, Siddiqui K, Spector DL, Stillman B (2004) Human Orc2 localizes to centrosomes, centromeres and heterochromatin during chromosome inheritance. *Embo J* **23**(13)**:** 2651-2663

Prasanth SG, Prasanth KV, Stillman B (2002) Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. *Science* **297**(5583)**:** 1026-1031

Probst AV, Dunleavy E, Almouzni G (2009) Epigenetic inheritance during the cell cycle. *Nat Rev Mol Cell Biol* **10**(3)**:** 192-206

Prokhortchouk A, Sansom O, Selfridge J, Caballero IM, Salozhin S, Aithozhina D, Cerchietti L, Meng FG, Augenlicht LH, Mariadason JM, Hendrich B, Melnick A,

Prokhortchouk E, Clarke A, Bird A (2006) Kaiso-deficient mice show resistance to intestinal cancer. *Mol Cell Biol* **26**(1)**:** 199-208

Prokhortchouk E, Defossez PA (2008) The cell biology of DNA methylation in mammals. *Biochim Biophys Acta* **1783**(11)**:** 2167-2173

Pusarla RH, Bhargava P (2005) Histones in functional diversification. Core histone variants. *FEBS J* **272**(20)**:** 5149-5168

Pusztai L, Cristofanilli M, Paik S (2007) New generation of molecular prognostic and predictive tests for breast cancer. *Semin Oncol* **34**(2 Suppl 3)**:** S10-16

Quivy JP, Gerard A, Cook AJ, Roche D, Almouzni G (2008) The HP1-p150/CAF-1 interaction is required for pericentric heterochromatin replication and S-phase progression in mouse cells. *Nat Struct Mol Biol*

Quivy JP, Grandi P, Almouzni G (2001) Dimerization of the largest subunit of chromatin assembly factor 1: importance in vitro and during Xenopus early development. *Embo J* **20**(8)**:** 2015-2027

Quivy JP, Roche D, Kirschner D, Tagami H, Nakatani Y, Almouzni G (2004) A CAF-1 dependent pool of HP1 during heterochromatin duplication. *Embo J* **23**(17)**:** 3516-3526

Rakha EA, Ellis IO (2009) Triple-negative/basal-like breast cancer: review. *Pathology* **41**(1)**:** 40-47

Ray-Gallet D, Quivy JP, Sillje HW, Nigg EA, Almouzni G (2007) The histone chaperone Asf1 is dispensable for direct de novo histone deposition in Xenopus egg extracts. *Chromosoma* **116**(5)**:** 487-496

Reik W (2007) Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* **447**(7143)**:** 425-432

Richardson RT, Batova IN, Widgren EE, Zheng LX, Whitfield M, Marzluff WF, O'Rand MG (2000) Characterization of the histone H1-binding protein, NASP, as a cell cycle-regulated somatic protein. *J Biol Chem* **275**(39)**:** 30378-30386

Ridgway P, Almouzni G (2000) CAF-1 and the inheritance of chromatin states: at the crossroads of DNA replication and repair. *J Cell Sci* **113 (Pt 15):** 2647-2658

Robinson PJ, Fairall L, Huynh VA, Rhodes D (2006) EM measurements define the dimensions of the "30-nm" chromatin fiber: evidence for a compact, interdigitated structure. *Proc Natl Acad Sci U S A* **103**(17)**:** 6506-6511

Robinson PJ, Rhodes D (2006) Structure of the '30 nm' chromatin fibre: a key role for the linker histone. *Curr Opin Struct Biol* **16**(3)**:** 336-343

Rogers S, Wells R, Rechsteiner M (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**(4774)**:** 364-368

Routh A, Sandin S, Rhodes D (2008) Nucleosome repeat length and linker histone stoichiometry determine chromatin fiber structure. *Proc Natl Acad Sci U S A* **105**(26)**:** 8872-8877

Ruault M, Dubarry M, Taddei A (2008) Re-positioning genes to the nuclear envelope in mammalian cells: impact on transcription. *Trends Genet* **24**(11)**:** 574-581

Rudert F, Bronner S, Garnier JM, Dolle P (1995) Transcripts from opposite strands of gamma satellite DNA are differentially expressed during mouse development. *Mamm Genome* **6**(2)**:** 76-83

Ryan RF, Schultz DC, Ayyanathan K, Singh PB, Friedman JR, Fredericks WJ, Rauscher FJ, 3rd (1999) KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol Cell Biol* **19**(6)**:** 4366-4378.

Salhab M, Reed MJ, Al Sarakbi W, Jiang WG, Mokbel K (2006) The role of aromatase and 17-beta-hydroxysteroid dehydrogenase type 1 mRNA expression in predicting the clinical outcome of human breast cancer. *Breast Cancer Res Treat* **99**(2)**:** 155-162

Santner SJ, Pauley RJ, Tait L, Kaseta J, Santen RJ (1997) Aromatase activity and expression in breast cancer and benign breast tissue stromal cells. *J Clin Endocrinol Metab* **82**(1)**:** 200-208

Sasaki T, Gilbert DM (2007) The many faces of the origin recognition complex. *Curr Opin Cell Biol* **19**(3)**:** 337-343

Sasano H, Frost AR, Saitoh R, Harada N, Poutanen M, Vihko R, Bulun SE, Silverberg SG, Nagura H (1996) Aromatase and 17 beta-hydroxysteroid dehydrogenase type 1 in human breast carcinoma. *J Clin Endocrinol Metab* **81**(11)**:** 4042-4046

Schmiedeberg L, Weisshart K, Diekmann S, Meyer Zu Hoerste G, Hemmerich P (2004) High- and low-mobility populations of HP1 in heterochromatin of mammalian cells. *Mol Biol Cell* **15**(6)**:** 2819-2833. Epub 2004 Apr 2812.

Schneider C, King RM, Philipson L (1988) Genes specifically expressed at growth arrest of mammalian cells. *Cell* **54**(6)**:** 787-793.

Schonrock N, Exner V, Probst A, Gruissem W, Hennig L (2006) Functional genomic analysis of CAF-1 mutants in Arabidopsis thaliana. *J Biol Chem* **281**(14)**:** 9560-9568

Schotta G, Ebert A, Krauss V, Fischer A, Hoffmann J, Rea S, Jenuwein T, Dorn R, Reuter G (2002) Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *Embo J* **21**(5)**:** 1121-1131.

Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, Reinberg D, Jenuwein T (2004) A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev* **18**(11)**:** 1251-1262

Schotta G, Sengupta R, Kubicek S, Malin S, Kauer M, Callen E, Celeste A, Pagani M, Opravil S, De La Rosa-Velazquez IA, Espejo A, Bedford MT, Nussenzweig A, Busslinger M, Jenuwein T (2008) A chromatin-wide transition to H4K20 monomethylation impairs genome integrity and programmed DNA rearrangements in the mouse. *Genes Dev* **22**(15)**:** 2048-2061

Schultz DC, Ayyanathan K, Negorev D, Maul GG, Rauscher FJ, 3rd (2002) SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev* **16**(8)**:** 919-932

Schwartz YB, Pirrotta V (2008) Polycomb complexes and epigenetic states. *Curr Opin Cell Biol* **20**(3)**:** 266-273

Serrano A, Rodriguez-Corsino M, Losada A (2009) Heterochromatin protein 1 (HP1) proteins do not drive pericentromeric cohesin enrichment in human cells. *PLoS ONE* **4**(4)**:** e5118

Sharma GG, Hwang KK, Pandita RK, Gupta A, Dhar S, Parenteau J, Agarwal M, Worman HJ, Wellinger RJ, Pandita TK (2003) Human heterochromatin protein 1 isoforms HP1(Hsalpha) and HP1(Hsbeta) interfere with hTERT-telomere interactions and correlate with changes in cell growth and response to ionizing radiation. *Mol Cell Biol* **23**(22)**:** 8363-8376.

Shaw PJ, Abranches R, Paula Santos A, Beven AF, Stoger E, Wegel E, Gonzalez-Melendi P (2002) The architecture of interphase chromosomes and nucleolar transcription sites in plants. *J Struct Biol* **140**(1-3)**:** 31-38

Shelby RD, Monier K, Sullivan KF (2000) Chromatin assembly at kinetochores is uncoupled from DNA replication. *J Cell Biol* **151**(5)**:** 1113-1118

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**(7)**:** 941-953

Shibahara K, Stillman B (1999) Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. *Cell* **96**(4)**:** 575-585.

Silva MC, Rowlands MG, Dowsett M, Gusterson B, McKinna JA, Fryatt I, Coombes RC (1989) Intratumoral aromatase as a prognostic factor in human breast carcinoma. *Cancer Res* **49**(10)**:** 2588-2591

Simon JA, Lange CA (2008) Roles of the EZH2 histone methyltransferase in cancer epigenetics. *Mutat Res* **647**(1-2)**:** 21-29

Singh PB, Miller JR, Pearce J, Kothary R, Burton RD, Paro R, James TC, Gaunt SJ (1991) A sequence motif found in a Drosophila heterochromatin protein is conserved in animals and plants. *Nucleic Acids Res* **19**(4)**:** 789-794

Smerdon MJ (1991) DNA repair and the role of chromatin structure. *Curr Opin Cell Biol* **3**(3)**:** 422-428.

Smith IE, Dowsett M (2003) Aromatase inhibitors in breast cancer. *N Engl J Med* **348**(24)**:** 2431-2442

Smith S, Stillman B (1989) Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication in vitro. *Cell* **58**(1)**:** 15-25

Smith S, Stillman B (1991a) Immunological characterization of chromatin assembly factor I, a human cell factor required for chromatin assembly during DNA replication in vitro. *J Biol Chem* **266**(18)**:** 12041-12047.

Smith S, Stillman B (1991b) Immunological characterization of chromatin assembly factor I, a human cell factor required for chromatin assembly during DNA replication in vitro. *J Biol Chem* **266**(18)**:** 12041-12047

Smothers JF, Henikoff S (2000) The HP1 chromo shadow domain binds a consensus peptide pentamer. *Curr Biol* **10**(1)**:** 27-30

Sobin LH, Wittekind C (2002) *TNM classification of malignant tumours.*, New-York: Wiley-Liss.

Song JJ, Garlick JD, Kingston RE (2008) Structural basis of histone H4 recognition by p55. *Genes Dev* **22**(10)**:** 1313-1318

Song K, Jung Y, Jung D, Lee I (2001) Human Ku70 interacts with heterochromatin protein 1alpha. *J Biol Chem* **276**(11)**:** 8321-8327

Song Y, He F, Xie G, Guo X, Xu Y, Chen Y, Liang X, Stagljar I, Egli D, Ma J, Jiao R (2007) CAF-1 is essential for Drosophila development and involved in the maintenance of epigenetic memory. *Dev Biol* **311**(1)**:** 213-222

Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* **98**(19)**:** 10869-10874

Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, Demeter J, Perou CM, Lonning PE, Brown PO, Borresen-Dale AL, Botstein D (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* **100**(14)**:** 8418-8423

Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, Jazaeri A, Martiat P, Fox SB, Harris AL, Liu ET (2003) Breast cancer classification and prognosis based on gene expression profiles from a population-based study. *Proc Natl Acad Sci U S A* **100**(18)**:** 10393-10398

Sotiriou C, Piccart MJ (2007) Taking gene-expression profiling to the clinic: when will molecular signatures become relevant to patient care? *Nat Rev Cancer* **7**(7)**:** 545-553

Sparmann A, van Lohuizen M (2006) Polycomb silencers control cell fate, development and cancer. *Nat Rev Cancer* **6**(11)**:** 846-856

Spivakov M, Fisher AG (2007) Epigenetic signatures of stem-cell identity. *Nat Rev Genet* **8**(4)**:** 263-271

Sripathy SP, Stevens J, Schultz DC (2006) The KAP1 corepressor functions to coordinate the assembly of de novo HP1-demarcated microenvironments of heterochromatin required for KRAB zinc finger protein-mediated transcriptional repression. *Mol Cell Biol* **26**(22)**:** 8623-8638

Staibano S, Mascolo M, Mancini FP, Kisslinger A, Salvatore G, Di Benedetto M, Chieffi P, Altieri V, Prezioso D, Ilardi G, De Rosa G, Tramontano D (2009) Overexpression of chromatin assembly factor-1 (CAF-1) p60 is predictive of adverse behaviour of prostatic cancer. *Histopathology* **54**(5)**:** 580-589

Staibano S, Mignogna C, Lo Muzio L, Mascolo M, Salvatore G, Di Benedetto M, Califano L, Rubini C, De Rosa G (2007) Chromatin assembly factor-1 (CAF-1)-mediated regulation of cell proliferation and DNA repair: a link with the biological behaviour of squamous cell carcinoma of the tongue? *Histopathology* **50**(7)**:** 911-919

Staresincic L, Fagbemi AF, Enzlin JH, Gourdin AM, Wijgers N, Dunand-Sauthier I, Giglia-Mari G, Clarkson SG, Vermeulen W, Scharer OD (2009) Coordination of dual incision and repair synthesis in human nucleotide excision repair. *Embo J*

Stewart MD, Li J, Wong J (2005) Relationship between histone H3 lysine 9 methylation, transcription repression, and heterochromatin protein 1 recruitment. *Mol Cell Biol* **25**(7)**:** 2525-2538.

Stillman B (1986) Chromatin assembly during SV40 DNA replication in vitro. *Cell* **45**(4)**:** 555-565.

Stingl J, Caldas C (2007) Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. *Nat Rev Cancer* **7**(10)**:** 791-799

Stratton MR, Campbell PJ, Futreal PA (2009) The cancer genome. *Nature* **458**(7239)**:** 719-724

Suganuma T, Workman JL (2008) Crosstalk among Histone Modifications. *Cell* **135**(4)**:** 604-607

Sugasawa K, Masutani C, Hanaoka F (1993) Cell-free repair of UV-damaged simian virus 40 chromosomes in human cell extracts. I. Development of a cell-free system detecting excision repair of UV-irradiated SV40 chromosomes. *J Biol Chem* **268**(12)**:** 9098-9104

Suquet C, Mitchell DL, Smerdon MJ (1995) Repair of UV-induced (6-4) photoproducts in nucleosome core DNA. *J Biol Chem* **270**(28)**:** 16507-16509

Suter CM, Martin DI, Ward RL (2004) Germline epimutation of MLH1 in individuals with multiple cancers. *Nat Genet* **36**(5)**:** 497-501

Tachibana M, Sugimoto K, Nozaki M, Ueda J, Ohta T, Ohki M, Fukuda M, Takeda N, Niida H, Kato H, Shinkai Y (2002) G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev* **16**(14)**:** 1779-1791

Taddei A, Maison C, Roche D, Almouzni G (2001) Reversible disruption of pericentric heterochromatin and centromere function by inhibiting deacetylases. *Nat Cell Biol* **3**(2)**:** 114-120

Tagami H, Ray-Gallet D, Almouzni G, Nakatani Y (2004) Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell* **116**(1)**:** 51-61

Takanashi M, Oikawa K, Fujita K, Kudo M, Kinoshita M, Kuroda M (2009) Heterochromatin protein 1gamma epigenetically regulates cell differentiation and exhibits potential as a therapeutic target for various types of cancers. *Am J Pathol* **174**(1)**:** 309-316

Talasz H, Lindner HH, Sarg B, Helliger W (2005) Histone H4-lysine 20 monomethylation is increased in promoter and coding regions of active genes and correlates with hyperacetylation. *J Biol Chem* **280**(46)**:** 38814-38822. Epub 32005 Sep 38815.

Tang MS, Bohr VA, Zhang XS, Pierce J, Hanawalt PC (1989) Quantification of aminofluorene adduct formation and repair in defined DNA sequences in mammalian cells using the UVRABC nuclease. *J Biol Chem* **264**(24)**:** 14455-14462

Temple WJ, Russell ML, Parsons LL, Huber SM, Jones CA, Bankes J, Eliasziw M (2006) Conservation surgery for breast cancer as the preferred choice: a prospective analysis. *J Clin Oncol* **24**(21)**:** 3367-3373

Thoma F, Koller T, Klug A (1979) Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. *J Cell Biol* **83**(2 Pt 1)**:** 403-427

Trojer P, Reinberg D (2007) Facultative heterochromatin: is there a distinctive molecular signature? *Mol Cell* **28**(1)**:** 1-13

Tryndyak VP, Kovalchuk O, Pogribny IP (2006) Loss of DNA methylation and histone H4 lysine 20 trimethylation in human breast cancer cells is associated with aberrant expression of DNA methyltransferase 1, Suv4-20h2 histone methyltransferase and methyl-binding proteins. *Cancer Biol Ther* **5**(1)**:** 65-70

Turner BM (2000) Histone acetylation and an epigenetic code. *Bioessays* **22**(9)**:** 836-845

Turner BM (2002) Cellular memory and the histone code. *Cell* **111**(3)**:** 285-291

Turner BM (2007) Defining an epigenetic code. *Nat Cell Biol* **9**(1)**:** 2-6

Tuusa J, Uitto L, Syvaoja JE (1995) Human DNA polymerase epsilon is expressed during cell proliferation in a manner characteristic of replicative DNA polymerases. *Nucleic Acids Res* **23**(12)**:** 2178-2183

Umehara T, Horikoshi M (2003) Transcription initiation factor IID-interactive histone chaperone CIA-II implicated in mammalian spermatogenesis. *J Biol Chem* **278**(37)**:** 35660-35667

Ura K, Araki M, Saeki H, Masutani C, Ito T, Iwai S, Mizukoshi T, Kaneda Y, Hanaoka F (2001) ATP-dependent chromatin remodeling facilitates nucleotide excision repair of UV-induced DNA lesions in synthetic dinucleosomes. *Embo J* **20**(8)**:** 2004-2014

Vakoc CR, Mandat SA, Olenchock BA, Blobel GA (2005) Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. *Mol Cell* **19**(3)**:** 381-391.

Valladares A, Hernandez NG, Gomez FS, Curiel-Quezada E, Madrigal-Bujaidar E, Vergara MD, Martinez MS, Arenas Aranda DJ (2006) Genetic expression profiles and chromosomal alterations in sporadic breast cancer in Mexican women. *Cancer Genet Cytogenet* **170**(2)**:** 147-151

Van Den Broeck A, Brambilla E, Moro-Sibilot D, Lantuejoul S, Brambilla C, Eymin B, Khochbin S, Gazzeri S (2008) Loss of histone H4K20 trimethylation occurs in preneoplasia and influences prognosis of non-small cell lung cancer. *Clin Cancer Res* **14**(22)**:** 7237-7245

van Driel R, Fransz P (2004) Nuclear architecture and genome functioning in plants and animals: what can we learn from both? *Exp Cell Res* **296**(1)**:** 86-90

van Oosterwijk MF, Versteeg A, Filon R, van Zeeland AA, Mullenders LH (1996) The sensitivity of Cockayne's syndrome cells to DNA-damaging agents is not due to defective transcription-coupled repair of active genes. *Mol Cell Biol* **16**(8)**:** 4436-4444

Venezia TA, Merchant AA, Ramos CA, Whitehouse NL, Young AS, Shaw CA, Goodell MA (2004) Molecular signatures of proliferation and quiescence in hematopoietic stem cells. *PLoS Biol* **2**(10)**:** e301. Epub 2004 Sep 2028.

Verreault A, Kaufman PD, Kobayashi R, Stillman B (1996) Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. *Cell* **87**(1)**:** 95-104.

Verschure PJ, van der Kraan I, de Leeuw W, van der Vlag J, Carpenter AE, Belmont AS, van Driel R (2005) In vivo HP1 targeting causes large-scale chromatin condensation and enhanced histone lysine methylation. *Mol Cell Biol* **25**(11)**:** 4552-4564.

Vincent-Salomon A, Lucchesi C, Gruel N, Raynal V, Pierron G, Goudefroye R, Reyal F, Radvanyi F, Salmon R, Thiery JP, Sastre-Garau X, Sigal-Zafrani B, Fourquet A, Delattre O (2008) Integrated genomic and transcriptomic analysis of ductal carcinoma in situ of the breast. *Clin Cancer Res* **14**(7)**:** 1956-1965

Vincent-Salomon A, Rousseau A, Jouve M, Beuzeboc P, Sigal-Zafrani B, Freneaux P, Rosty C, Nos C, Campana F, Klijanienko J, Al Ghuzlan A, Sastre-Garau X (2004) Proliferation markers predictive of the pathological response and disease outcome of patients with breast carcinomas treated by anthracycline-based preoperative chemotherapy. *Eur J Cancer* **40**(10)**:** 1502-1508

Vitolo B, Lidonnici MR, Montecucco C, Montecucco A (2005) A new monoclonal antibody against DNA ligase I is a suitable marker of cell proliferation in cultured cell and tissue section samples. *Eur J Histochem* **49**(4)**:** 349-354

Voduc D, Cheang M, Nielsen T (2008) GATA-3 expression in breast cancer has a strong association with estrogen receptor but lacks independent prognostic value. *Cancer Epidemiol Biomarkers Prev* **17**(2)**:** 365-373

Volker M, Mone MJ, Karmakar P, van Hoffen A, Schul W, Vermeulen W, Hoeijmakers JH, van Driel R, van Zeeland AA, Mullenders LH (2001) Sequential assembly of the nucleotide excision repair factors in vivo. *Mol Cell* **8**(1)**:** 213-224

Volpe TA, Kidner C, Hall IM, Teng G, Grewal SI, Martienssen RA (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**(5588)**:** 1833-1837

Vrieling C, Collette L, Fourquet A, Hoogenraad WJ, Horiot JC, Jager JJ, Bing Oei S, Peterse HL, Pierart M, Poortmans PM, Struikmans H, Van den Bogaert W, Bartelink H (2003) Can patient-, treatment- and pathology-related characteristics explain the high local recurrence rate following breast-conserving therapy in young patients? *Eur J Cancer* **39**(7)**:** 932-944

Wallace JA, Orr-Weaver TL (2005) Replication of heterochromatin: insights into mechanisms of epigenetic inheritance. *Chromosoma* **114**(6)**:** 389-402

Wang H, Walsh ST, Parthun MR (2008a) Expanded binding specificity of the human histone chaperone NASP. *Nucleic Acids Res* **36**(18)**:** 5763-5772

Wang Y, Ye L, Leung LK (2008b) A positive feedback pathway of estrogen biosynthesis in breast cancer cells is contained by resveratrol. *Toxicology* **248**(2-3)**:** 130-135

Wang ZG, Wu XH, Friedberg EC (1991) Nucleotide excision repair of DNA by human cell extracts is suppressed in reconstituted nucleosomes. *J Biol Chem* **266**(33)**:** 22472-22478

Watson JD, Crick FH (1953) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **171**(4356)**:** 737-738

Weaver BA, Cleveland DW (2005) Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. *Cancer Cell* **8**(1)**:** 7-12.

Weichert W (2009) HDAC expression and clinical prognosis in human malignancies. *Cancer Lett* **280**(2)**:** 168-176

Wheatley SP, Carvalho A, Vagnarelli P, Earnshaw WC (2001) INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis. *Curr Biol* **11**(11)**:** 886-890.

Williams JG, Penman S (1975) The messenger RNA sequences in growing and resting mouse fibroblasts. *Cell* **6**(2)**:** 197-206.

Williams JI, Friedberg EC (1979) Deoxyribonucleic acid excision repair in chromatin after ultraviolet irradiation of human fibroblasts in culture. *Biochemistry* **18**(18)**:** 3965-3972

Williams L, Grafi G (2000) The retinoblastoma protein - a bridge to heterochromatin. *Trends Plant Sci* **5**(6)**:** 239-240.

Witt O, Albig W, Doenecke D (1996) Testis-specific expression of a novel human H3 histone gene. *Exp Cell Res* **229**(2)**:** 301-306

Witt O, Deubzer HE, Milde T, Oehme I (2009) HDAC family: What are the cancer relevant targets? *Cancer Lett* **277**(1)**:** 8-21

Wutz A, Gribnau J (2007) X inactivation Xplained. *Curr Opin Genet Dev* **17**(5)**:** 387-393

Yager JD, Davidson NE (2006) Estrogen carcinogenesis in breast cancer. *N Engl J Med* **354**(3)**:** 270-282

Yahi H, Fritsch L, Philipot O, Guasconi V, Souidi M, Robin P, Polesskaya A, Losson R, Harel-Bellan A, Ait-Si-Ali S (2008) Differential cooperation between heterochromatin protein HP1 isoforms and MyoD in myoblasts. *J Biol Chem* **283**(35)**:** 23692-23700

Yamagishi Y, Sakuno T, Shimura M, Watanabe Y (2008) Heterochromatin links to centromeric protection by recruiting shugoshin. *Nature* **455**(7210)**:** 251-255.

Yamamoto K, Sonoda M (2003) Self-interaction of heterochromatin protein 1 is required for direct binding to histone methyltransferase, SUV39H1. *Biochem Biophys Res Commun* **301**(2)**:** 287-292.

Yamamoto K, Sonoda M, Inokuchi J, Shirasawa S, Sasazuki T (2004) Polycomb group suppressor of zeste 12 links heterochromatin protein 1alpha and enhancer of zeste 2. *J Biol Chem* **279**(1)**:** 401-406

Yang CL, Chang LS, Zhang P, Hao H, Zhu L, Toomey NL, Lee MY (1992) Molecular cloning of the cDNA for the catalytic subunit of human DNA polymerase delta. *Nucleic Acids Res* **20**(4)**:** 735-745

Yang ZQ, Imoto I, Fukuda Y, Pimkhaokham A, Shimada Y, Imamura M, Sugano S, Nakamura Y, Inazawa J (2000) Identification of a novel gene, GASC1, within an amplicon at 9p23-24 frequently detected in esophageal cancer cell lines. *Cancer Res* **60**(17)**:** 4735-4739

Yang ZQ, Imoto I, Pimkhaokham A, Shimada Y, Sasaki K, Oka M, Inazawa J (2001) A novel amplicon at 9p23 - 24 in squamous cell carcinoma of the esophagus that lies proximal to GASC1 and harbors NFIB. *Jpn J Cancer Res* **92**(4)**:** 423-428

Yao J, Weremowicz S, Feng B, Gentleman RC, Marks JR, Gelman R, Brennan C, Polyak K (2006) Combined cDNA array comparative genomic hybridization and serial analysis of gene expression analysis of breast tumor progression. *Cancer Res* **66**(8)**:** 4065-4078

Ye X, Franco AA, Santos H, Nelson DM, Kaufman PD, Adams PD (2003) Defective S phase chromatin assembly causes DNA damage, activation of the S phase checkpoint, and S phase arrest. *Mol Cell* **11**(2)**:** 341-351.

Yehiely F, Moyano JV, Evans JR, Nielsen TO, Cryns VL (2006) Deconstructing the molecular portrait of basal-like breast cancer. *Trends Mol Med* **12**(11)**:** 537-544

Yue W, Wang JP, Hamilton CJ, Demers LM, Santen RJ (1998) In situ aromatization enhances breast tumor estradiol levels and cellular proliferation. *Cancer Res* **58**(5)**:** 927-932

Zarebski M, Wiernasz E, Dobrucki JW (2009) Recruitment of heterochromatin protein 1 to DNA repair sites. *Cytometry A*

Zhang R, Chen W, Adams PD (2007) Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol Cell Biol* **27**(6)**:** 2343-2358. Epub 2007 Jan 2322.

Zhang R, Poustovoitov MV, Ye X, Santos HA, Chen W, Daganzo SM, Erzberger JP, Serebriiskii IG, Canutescu AA, Dunbrack RL, Pehrson JR, Berger JM, Kaufman PD, Adams PD (2005) Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. *Dev Cell* **8**(1)**:** 19-30

Zhang Y, Li N, Caron C, Matthias G, Hess D, Khochbin S, Matthias P (2003a) HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. *Embo J* **22**(5)**:** 1168-1179

Zhang Z, Yamashita H, Toyama T, Omoto Y, Sugiura H, Hara Y, Wu X, Kobayashi S, Iwase H (2003b) Quantitative determination, by real-time reverse transcription polymerase chain reaction, of aromatase mRNA in invasive ductal carcinoma of the breast. *Breast Cancer Res* **5**(6)**:** R250-256

Zhao C, Dahlman-Wright K, Gustafsson JA (2008) Estrogen receptor beta: an overview and update. *Nucl Recept Signal* **6:** e003

Zhu Q, Wani G, Arab HH, El-Mahdy MA, Ray A, Wani AA (2009) Chromatin restoration following nucleotide excision repair involves the incorporation of ubiquitinated H2A at damaged genomic sites. *DNA Repair (Amst)* **8**(2)**:** 262-273

Zinner R, Albiez H, Walter J, Peters AH, Cremer T, Cremer M (2006) Histone lysine methylation patterns in human cell types are arranged in distinct three-dimensional nuclear zones. *Histochem Cell Biol* **125**(1-2)**:** 3-19.

V. Appendices

Appendix #1:

Histone chaperones: an escort network regulating histone traffic

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(*These authors contributed equally to this work)

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In this review, we aimed to give an overview of recent literature concerning histone chaperones, their specificities and their different functions. First, we provide a definition of histone chaperones, and then provide criteria for the further classification as histone deposition factors. Having clarified the general definition of a histone chaperone, we propose a classification of currently known chaperones according to their histone preference and their biochemical properties and functions within enzymatic complexes.

Next, we discuss the structural features that could underlie the selectivity of particular histone chaperones for specific histones or histone variants. We then describe how histone chaperones act within a network of histone chaperones to adapt the flow of histone proteins to cellular needs, and how they provide an interphase with a multitude of interacting partners involved in various cellular processes that can regulate and target chaperone activity.

A last part covers our current knowledge and hypotheses concerning histone chaperone functions in the context of gene transcription and double strand break repair. In this context, we discuss how histone chaperones might be decisive for a correct balance between recycling of old histones and incorporation of newly synthesized histones, and thus play a role in the maintenance and the transmission of epigenetic marks.

CHROMATIN

REVIEW

nature structural & molecular biology

Histone chaperones: an escort network regulating histone traffic

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In eukaryotes, DNA is organized into chromatin in a dynamic manner that enables it to be accessed for processes such as transcription and repair. Histones, the chief protein component of chromatin, must be assembled, replaced or exchanged to preserve or change this organization according to cellular needs. Histone chaperones are key actors during histone metabolism. Here we classify known histone chaperones and discuss how they build a network to escort histone proteins. Molecular interactions with histones and their potential specificity or redundancy are also discussed in light of chaperone structural properties. The multiplicity of histone chaperone partners, including histone modifiers, nucleosome remodelers and cell-cycle regulators, is relevant to their coordination with key cellular processes. Given the current interest in chromatin as a source of epigenetic marks, we address the potential contributions of histone chaperones to epigenetic memory and genome stability.

Histones, key proteins that compact the structure of all eukaryotic DNA in the form of chromatin[1], have recently attracted much attention because of their impact on genome function. They form the core of the repeated unit of chromatin, the nucleosome[2], in which DNA is wrapped around a histone octamer comprising a tetramer of $(H3\text{-}H4)_2$ flanked by two dimers of H2A-H2B, a structure that has been defined at high resolution[3]. This organization contributes to the regulation of all cellular processes operating on DNA. However, during cell metabolism, histones are not necessarily continuously associated with DNA. Indeed, newly synthesized histones have to be transported to the nucleus and targeted to the required location, whereas old or damaged histones have to be discarded. In addition, cellular processes involving DNA can require transient histone eviction and replacement. Although being 'DNA-free' is usually a transient state for histones, it can last longer, as demonstrated by the importance of histone storage during *Xenopus laevis* oogenesis to provide for early development[4]. As histones are highly basic proteins, their presence in the cell could lead to deleterious effects through promiscuous interactions and aggregation. Thus, under most circumstances, when histones are not in association with DNA, they are bound to dedicated proteins called histone chaperones[5]. These escort proteins prevent unintended interactions of histones with other factors and help to control histone supply and incorporation into chromatin. In this way, histone chaperones are crucial in fundamental processes such as transcription, replication and DNA repair. In this review, we first propose a classification of currently known histone chaperones. Next, we discuss how recent molecular and cellular data provide insights into chaperone function and histone selectivity, in connection with key partners such as histone-remodeling factors and histone-modifying enzymes. Descriptions of some chaperone functions are provided, with examples of their involvement in gene transcription and double-strand break (DSB) repair.

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Toward a definition of histone chaperones

Histone chaperones are defined mainly as factors that associate with histones and stimulate a reaction involving histone transfer without being part of the final product (**Box 1**). This fundamental property of histone transfer, shared by all histone chaperones, can be evidenced by *in vitro* nucleosome reconstitution using purified DNA and histones. However, not all histone chaperones are necessarily histone-deposition factors *in vivo*; they can also use their transfer capacity to fulfill other important roles in histone dynamics, such as histone transport, transfer or storage. Thus, a broad range of *in vivo* functions, as summarized in **Box 1**, can be assigned to histone chaperones on the basis of their fundamental properties.

To address the functions of histone chaperones in chromatin assembly beyond their *in vitro* nucleosome-reconstitution capacity, cell-free systems have been developed that approach the complexity encountered *in vivo*. The first cell-free system enabling chromatin assembly was derived from *X. laevis* eggs[6]. Later on, the use of human cytosolic and nuclear extracts to replicate and assemble SV40 mini-chromosomes was combined with biochemical fractionation[7], resulting in a powerful assay that elucidated the role of chromatin-assembly factor-1 (CAF-1; see **Fig. 1**) in replication-coupled histone deposition[8]. A method using *X. laevis* egg extracts was further developed to assess whether or not certain histone chaperones act as deposition factors[9]. These egg extracts contain all factors essential for chromatin assembly during the rapid rounds of DNA replication that occur in early development. When depleted of a putative histone deposition

BOX 1 Histone chaperones: the basics

Definition

Factors that associate with histones and stimulate a reaction involving histone transfer, without being part of the final product.

Functions in histone transfer

- Histone transfer from one chaperone to another
- Histone transfer to enzymes using histones as a substrate
- Histone transfer onto DNA (deposition)
- Histone transfer off DNA (eviction)

Functions in storage

- Storage of histone pools during development
- Buffering transient histone overload

Selectivity

Choice of a histone variant in a given metabolic pathway.

Regulation

- During development, by cell type
- During cell cycle and cell-cycle exit; damage response

factor, they lose their ability to support nucleosome assembly, which can be restored in add-back experiments. This approach clearly demonstrated that CAF-1 and histone regulator A (HIRA; see **Fig. 1**) are true histone deposition factors, promoting chromatin assembly dependent on and independent of DNA synthesis, respectively[10,11]. An example of a histone chaperone that is not necessarily directly involved in histone deposition is anti-silencing function-1 (Asf1; see **Fig. 1**). Indeed, when human ASF1 is added to human cell-free extracts without any additional partners, it does not promote histone deposition[12]. In addition, the results of depletion and add-back experiments in *X. laevis* egg extracts contradict a direct role of Asf1 in chromatin assembly, either replication-dependent or replication-independent[13]. Thus, although Asf1 can promote deposition of purified histones onto naked DNA in a nucleosome-reconstitution experiment *in vitro*, this is not sufficient evidence to conclude that it functions directly as a deposition factor *in vivo*. Rather, current data suggest a role for Asf1 as an H3-H4 histone donor for HIRA and CAF-1, which in turn deposit histones onto DNA[14].

The point made above emphasizes how challenging it is to define the exact function of an individual histone chaperone. As a first step toward this end, we here list known histone chaperones and classify them into three main categories (**Fig. 1**): (i) chaperones that can bind and transport or transfer histones without necessarily involving additional partners—for example, Asf1; (ii) multichaperone complexes that combine several histone chaperone subunits—for example, the CAF-1 complex; and (iii) chaperones that provide histone-binding capacity within large enzymatic complexes—for example, actin-related protein-4 (Arp4) in the INO80 chromatin-remodeling complex[15]. In regard to the latter case, *in vitro* nucleosome-reconstitution capacity has not yet been tested for the several actin-related proteins that bind histones. However, their interaction with histones and their role in stimulating a reaction involving histones (chromatin remodeling or modification) can justify considering them as histone chaperones, rather than histone-binding modules. Similarly, *in vitro* nucleosome-reconstitution tests have not yet been performed with the recently discovered histone chaperone Chz1 (chaperone for H2AZ; see **Fig. 1**), although its *in vitro* capacity to deposit H2AZ in cooperation with the chromatin remodeler Swr1 (ref. 16) strongly suggests a chaperone activity. One particular group of histone chaperones, the human RbAp48 protein and its homologs, should be considered as multiclass chaperones, as they fit into all three chaperone categories defined above. Indeed, RbAp48 can function autonomously and is found in the multichaperone complex CAF-1 as well as in many histone-remodeling and histone-modifying enzymatic complexes—for example, in the ISWI and Mi-2/CHD families of chromatin-remodeling complexes.

Histone chaperones can also be distinguished on the basis of their histone binding selectivity, which differentiates chaperones that preferentially interact with H3-H4 from those that prefer H2A-H2B. Furthermore, particular chaperones show selectivity toward specific histone H3 and H2A isoforms (reviewed in refs. 14,17). These chaperones preferentially bind either replicative variants, which represent the bulk of the histones and are expressed and deposited mostly in a replication-coupled manner during S phase (for example, H3.1 in mammals), or replacement variants, which are expressed constitutively at lower levels and incorporated in a replication-independent fashion (for example, H3.3 in mammals)[18]. *Saccharomyces cerevisiae* has only a single H3 variant, most closely related to H3.3, so cell cycle–specific deposition does not apply. For many replacement histones, how their incorporation is regulated and which chaperone is involved are ongoing subjects of investigation. Another specific category to consider involves histone chaperones that preferentially interact with linker histones. For example, Nasp has been shown to interact with the linker histone H1 in mammalian cells[19], although it is generally considered to be an H3-H4 chaperone (see **Fig. 1**). Similarly, the H2A-H2B chaperone Nap1 binds linker histone B4 in *X. laevis* eggs, as assessed by immunoprecipitation[20]. This underscores that a preferential chaperone-histone interaction is not always a strict specific binding; rather, it may be subject to regulation and should be evaluated context-dependently.

The structural basis of chaperone-histone binding, although not sufficient to account for selective interactions with specific histone variants, has proven informative in many ways. Given the basicity of histones, it is not surprising that long acidic stretches are found in numerous histone chaperones, such as nucleoplasmin[21], yeast Asf1 (ref. 22 and **Fig. 2a**), yeast Nap1 (ref. 23), the Spt16 subunit of hFACT[24] and nucleolin[25] (see **Fig. 1**). Charge neutralization between acidic chaperones and basic histones can thus be achieved[24,25], but this does not account for specificity of the histone interaction. Charge neutralization can even be dispensable for histone chaperone activity[21,23,26–28], as exemplified by mammalian Asf1, which lacks an acidic tail (**Fig. 2a**). Therefore, acidic regions may serve to enhance histone interaction and may be an intrinsic feature in one species—for example, in a chaperone containing an acidic tail—whereas in other species the interaction is regulated through post-translational modification, as exemplified by the polyglutamylation of Nap1 in *Drosophila melanogaster*[29]. Other structural features should also be considered. Recent high-resolution studies of the H2A-H2B chaperones nucleoplasmin[21,30], Nap1 and SET/TAF-1β[23,27] show that these chaperones have a conserved β-structure exposed to the solvent. This structure is also present in the H3-H4 chaperone Asf1 (refs. 26,31) and is predicted to exist in HIRA[32] and CAF-1 (RbAp48 and p60)[33,34]. Recent evidence shows that, for Asf1, this conserved β-structure is involved in H3-H4 binding, underscoring the potential importance of such an architecture for histone binding[31,35–38]. However, it is interesting to note that Chz1 is unstructured when bacterially expressed in the absence of histones, but adopts an α-helical structure when bound to Htz1-H2B (*S. cerevisiae* homologs of H2AZ-H2B)[16].

The intimate interaction between chaperones and their cognate histones and the variety of histone metabolic pathways lead us to propose that each histone chaperone is positioned within an escort

Classification of chaperones		Histone chaperones in species of function identification		Histone selectivity	Conservation	Main functions
Class I: single chaperone 	H3-H4	Asf1 (*D.m.*)[126]		Both H3.1-H4 & H3.3-H4	*S.c.*, Asf1; *S.p.*, Cia1 *A.t.*, Sga1 & Sga2; *D.m.*, Asf1; *X.l.*, Asf1; *M.m.*, Asf1a & Asf1b; *H.s.*, Asf1a & Asf1b	Histone donor for CAF-1 and HIRA
		Fkbp39p (*S.p.*)[127]		H3-H4	*S.c.*, Fpr; *S.p.*, Fkbp39p	rDNA silencing
		HIRA (*X.l.*)[11]		H3.3-H4	*S.c.*, HIR1/HIR2; *S.p.*, Hip1, Sim2 *A.t.*, HIRA; *D.m.*, HIRA; *X.l.*, HIRA; *M.m.*, HIRA; *H.s.*, HIRA	Deposition factor independent of DNA synthesis
		N1/N2 (*X.l.*)[128]		H3-H4[b]	*X.l.*, N1/N2; *M.m.*, tNasp & sNasp; *H.s.*, tNasp & sNasp	H3-H4 storage in *X. laevis* oocytes
		Spt6 (*S.c.*)[129]		H3-H4	*S.c.*, Spt6; *S.p.*, Spt6 *D.m.*, Spt6; *X.l.*, Spt6; *M.m.*, Spt6; *H.s.*, Spt6	Transcription initiation & elongation
		Rtt106 (*S.c.*)[130]		H3-H4	*S.c.*, Rtt106; *S.p.*, SPAC6G9.03c	Heterochromatic silencing
	H2A-H2B	Nucleoplasmin (*X.l.*)[131]/ Nucleophosmin (NPM1) (*H.s.*)[132]		H2A-H2B	*D.m.*, Nip; *X.l.*, Nucleoplasmin; *M.m.*, NMP1, NPM2 & NPM3; *H.s.*, NMP1; NPM2 & NPM3	Storage in *X. laevis* oocytes, cytosolic-nuclear transport, replication, transcription
		Chz1 (*S.c.*)[16]		H2AZ-H2B	*S.c.*, Chz1; *H.s.*, HIRIP3?	Chz1: H2AZ incorporation by SWR1
		Nap1 (*X.l.*)[131] Nap1-related proteins: Nap1L2 (*M.m.*)[133], SET/TAF1b (*H.s.*)[134], CINAP (*H.s.*, *M.m.*)[135] and Vps75 (*S.c.*)[136]		H2A-H2B[b]	*S.c.*, Nap1; *S.p.*, Nap1 & Nap1.2 *A.t.*, NRP1 & NRP2; *D.m.*, Nap1 *X.l.*, Nap1; *M.m.*, Nap1; *H.s.*, Nap1	Cytosolic-nuclear transport Transcription Replication
		Nucleolin (*H.s.*)[25]		H2A-H2B[c]	*S.c.*, NSR1 *A.t.*, Nucleoli; *X.l.*, Nucleolin *M.m.*, Nucleolin; *H.s.*, Nucleolin	Transcription elongation; assist chromatin remodelling
Class II: multi-chaperone complex 	H3-H4	CAF-1 complex (*H.s.*)[8]	p150	H3.1-H4	*S.c.*, Rlf2/Cac1; *S.p.*, SPBC29A10.03C *A.t.*, Fas1; *D.m.*, p180; *X.l.*, p150; *M.m.*, p150; *H.s.*, p150	Deposition factor coupled to DNA synthesis: -Replication -Repair
			p60	H3.1-H4	*S.c.*, Cac2; *S.p.*, SPAC26H5.03 *A.t.*, Fas2; *D.m.*, p150; *X.l.*, p60; *M.m.*, p60; *H.s.*, p60	
			RbAp48[a]	H3-H4	*S.c.*, Msi1/Cac3; *S.p.*, Msi16 *A.t.*, Msi1; *D.m.*, p55; *X.l.*, p48; *M.m.*, RbAp48; *H.s.*, RbAp48	
	H2A-H2B	FACT complex (*H.s.*)[24]	Spt16	H2A-H2B	*S.c.*, Spt16; *S.p.*, Spt16 *A.t.*, Spt16; *D.m.*, Spt16; *X.l.*, Spt16; *M.m.*, Spt16; *H.s.*, Spt16	Transcription elongation
			SSRP1	H3-H4[d]	*S.c.*, Pob3; *S.p.*, Pob3 *A.t.*, SSRP; *D.m.*, SSRP1; *X.l.*, SSRP1; *M.m.*, SSRP1; *H.s.*, SSRP1	
Class III: within enzymatic complexes 		Chaperone	Enzymatic complex			
	H3-H4	Hif1 (*S.c.*)[137]	Hat1p/Hat2 (*S.c.*)	H3-H4	*S.c.*, Hif1	Assist HAT
		Rsf-1 (*H.s.*)[138]	RSF (*H.s.*)	H3-H4	*H.s.*, Rsf-1; *M.m.*, Rsf-1	Assist RC
	ND/ multiple	Arp4 (*S.c.*)[15]	RC INO80 (*S.c.*, *D.m.*, *M.m.*, *H.s.*) RC SWR1 (*S.c.*) HAT NuA4 (*S.c.*) RC+HAT Tip60 (*D.m.*, *M.m.*, *H.s.*)	ND	*S.c.*, Arp4; *S.p.*, Alp5 *A.t.*, Arp4; *D.m.*, BAP55; *M.m.*, BAF53; *H.s.*, BAF53	Assist RC
		Arp7, Arp9 (*S.c.*)[139]	RC SWI/SNF & RSC (*S.c.*): BAF/PBAF & BAP (*D.m.*, *M.m.*, *H.s.*)	ND	*S.c.*, Arp7,9 *A.t.*, Arp7,9; *D.m.*, BAP55; *M.m.*, BAF53; *H.s.*, BAF53	Assist RC
		Arp8 (*S.c.*)[113]	RC INO80 (*S.c.*, *D.m.*, *M.m.*, *H.s.*)	H3-H4	*S.c.*, Arp8 *D.m.*, BAP55; *M.m.*, BAF53; *H.s.*, BAF53	Assist RC
		Acf1 (*D.m.*)[140]	RC ACF/CHRAC (*S.c.*, *D.m.*, *M.m.*)	Both H2A-H2B & H3-H4	*S.c.*, Itc1 *D.m.*, Acf1; *M.m.*, Acf1; *H.s.*, Acf1	Assist RC
Multiclass chaperone	H3-H4	RbAp48[a] (*H.s.*)[8]	1. Single chaperone 2. CAF-1 complex 3. RC, HAT, HDAC, PC	Both H3-H4 & CENPA-H4	*S.c.*, Msi1/Cac3; *S.p.*, Mis16 *A.t.*, Msi1; *D.m.*, p55; *X.l.*, p48; *M.m.*, RbAp48; *H.s.*, RbAp48	1. Centromeric chromatin maintenance 2. Chromatin assembly coupled to DNA synthesis 3. Assist multiple enzymatic activities

Figure 1 Classification of histone chaperones in multiple organisms. For each class, we show in bold one prototype chaperone. The selective interactions with either H3-H4 or H2A-H2B are indicated and specifically preferred histone variants, when known, are underlined. H, histone; RC, remodelling complex; HDAC, histone deacetylase complex; PC, Polycomb complex; ND, not determined; *S.c.*, *S. cerevisiae*; *S.p.*, *S. pombe*; *A.t.*, *Arabidopsis thaliana*; *D.m.*, *D. melanogaster*; *X.l.*, *X. laevis*; *M.m.*, *Mus musculus*; *H.s.*, *Homo sapiens*. [a]RbAp48 (also called Rbbp4) and its homologs can act as single chaperones, as part of the multichaperone CAF-1 complex or within other complexes (RC, HAT, HDAC or PC). [b]Both sNasp[19] and Nap1 (ref. 20) also interact with linker histones (see text). [c]Nucleolin facilitates remodeling of nucleosomes containing macroH2A[25], suggesting specificity for this variant. [d]SSRP1, through its interaction with H3-H4, could assist Spt16 in nucleosome binding[24].

network regulating histone traffic. Careful evaluation will be needed to determine the extent to which histones can substitute for one another within this chaperone network and how the chaperones participate in regulating histone traffic in coordination with cellular processes involving chromatin reorganization.

Histone chaperones build a network of escort functions

Recent crystallographic studies have provided new insights into the relationships between histone chaperones and their cargo, the histones, but also between different histone chaperones. Here, using data on the conserved portion of Asf1 as an example (**Fig. 2a**), we discuss how it is possible to evaluate the contribution of histone chaperones to nucleosome assembly and disassembly mechanisms and to complete the picture of the histone chaperone network—that is, what histone chaperones can do and how they selectively regulate histone dynamics.

The escort-cargo interaction: from structure to function. In the nucleosome, histones H3 and H4 form a tetramer[3], which remains relatively stable when it is free of DNA in solution. This biochemical property led to the long-standing assumption that these histones were

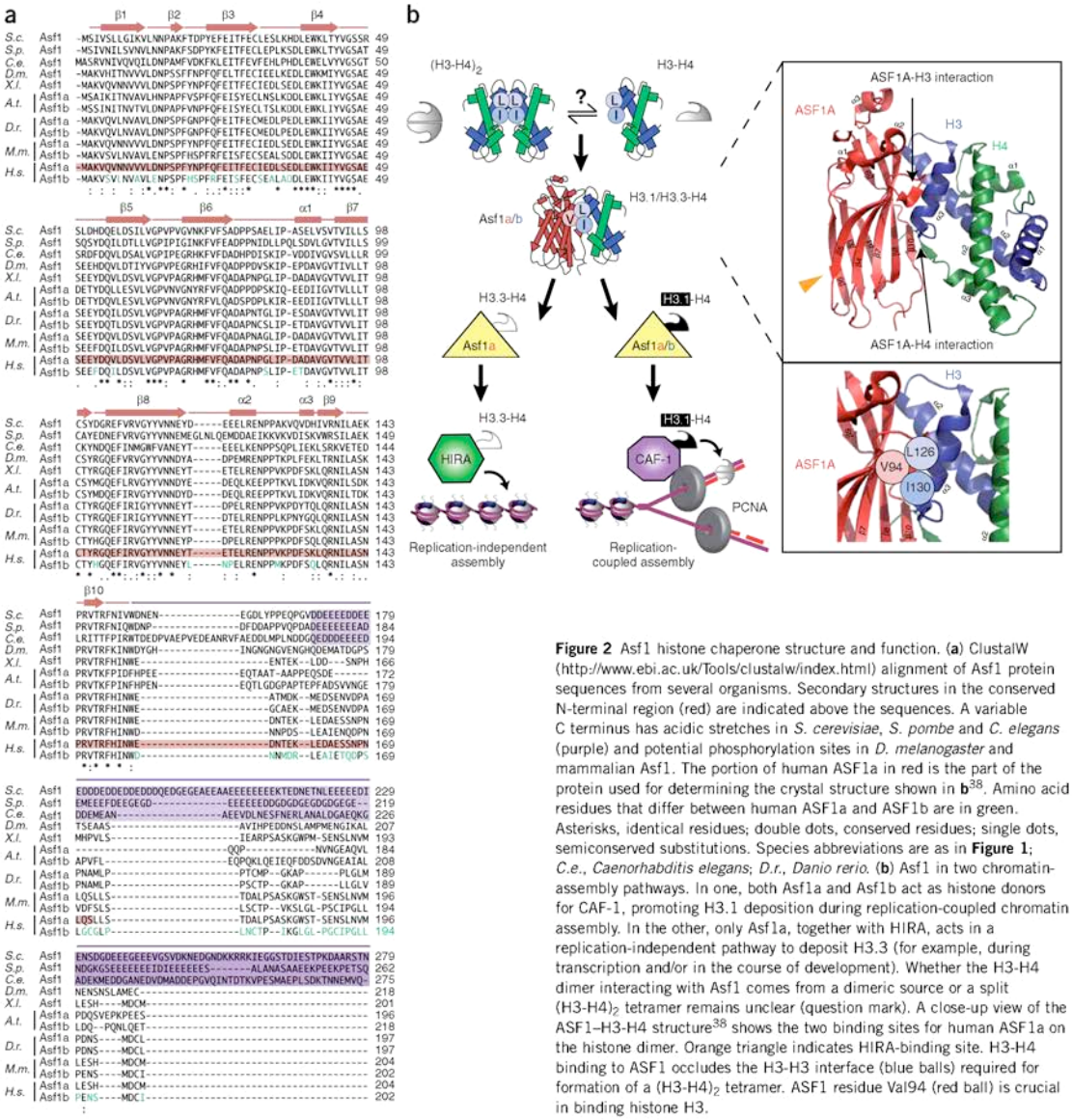

Figure 2 Asf1 histone chaperone structure and function. (**a**) ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html) alignment of Asf1 protein sequences from several organisms. Secondary structures in the conserved N-terminal region (red) are indicated above the sequences. A variable C terminus has acidic stretches in *S. cerevisiae*, *S. pombe* and *C. elegans* (purple) and potential phosphorylation sites in *D. melanogaster* and mammalian Asf1. The portion of human ASF1a in red is the part of the protein used for determining the crystal structure shown in **b**[38]. Amino acid residues that differ between human ASF1a and ASF1b are in green. Asterisks, identical residues; double dots, conserved residues; single dots, semiconserved substitutions. Species abbreviations are as in **Figure 1**; *C.e.*, *Caenorhabditis elegans*; *D.r.*, *Danio rerio*. (**b**) Asf1 in two chromatin-assembly pathways. In one, both Asf1a and Asf1b act as histone donors for CAF-1, promoting H3.1 deposition during replication-coupled chromatin assembly. In the other, only Asf1a, together with HIRA, acts in a replication-independent pathway to deposit H3.3 (for example, during transcription and/or in the course of development). Whether the H3-H4 dimer interacting with Asf1 comes from a dimeric source or a split $(H3\text{-}H4)_2$ tetramer remains unclear (question mark). A close-up view of the ASF1–H3-H4 structure[38] shows the two binding sites for human ASF1a on the histone dimer. Orange triangle indicates HIRA-binding site. H3-H4 binding to ASF1 occludes the H3-H3 interface (blue balls) required for formation of a $(H3\text{-}H4)_2$ tetramer. ASF1 residue Val94 (red ball) is crucial in binding histone H3.

deposited directly as a tetrameric complex. Recent data have challenged this view. A first indication that H3-H4 can also exist as a dimer was found in an analysis of H3 bound to ASF1, HIRA and CAF-1 in complexes isolated from human nuclear cell extracts[18]. This was further confirmed by stoichiometric analysis of the Asf1–H3-H4 complex[39]. These findings raise the question of whether interaction of chaperones with dimeric H3-H4 is a general rule for H3-H4 dynamics in other contexts and for other chaperones.

The high-resolution structure of the conserved N-terminal part of Asf1 (**Fig. 2a**) interacting with a dimer of H3-H4 (refs. 37,38) reveals two major binding sites (**Fig. 2b**): (i) a concave hydrophobic groove of Asf1 surrounded by conserved charged amino acid residues interacts with the C-terminal part of H3, and (ii) the C terminus of H4, which forms a parallel β-sheet with H2A within the nucleosome, undergoes an unexpected major conformational change, rotating about 180° to form an antiparallel β-sheet with the last β-strand of Asf1 (refs. 35–38). Minor structural adjustments in Asf1 accompany this conformational change of H4 and favor the interaction[37]. The results of *in vitro* and *in vivo* mutagenesis of key residues underscore the importance of these binding regions for Asf1 chaperone activity[31,35–38].

The Asf1–H3-H4 structure has important functional implications for histone chaperone activity during nucleosome assembly and disassembly. Notably, the binding sites through which the H3-H4 dimer interacts with Asf1 are also implicated in the formation of $(H3\text{-}H4)_2$ tetramers and the nucleosome[3], providing support for the idea that an H3-H4 dimer can interact with either Asf1 or another H3-H4 dimer[37,38] (**Fig. 2b**). English *et al.*[37] suggest that Asf1 has the capacity to disassemble nucleosomes by a 'strand-capture' mechanism, using the H4 C terminus as a 'handle' to dissociate $(H3\text{-}H4)_2$ tetramers. Consistent with this hypothesis, an *in vitro* tetramer-disrupting activity of ASF1 has also been reported by Natsume *et al.*[38], who showed that addition of an equimolar amount of ASF1 to $(H3\text{-}H4)_2$ tetramers could cause the disappearance of the tetramer species and appearance of an ASF1–H3-H4 complex. Genetic studies in *S. cerevisiae* have shown that Asf1 is required to ensure efficient histone eviction during transcription, providing further evidence *in vivo* that Asf1 has a role in nucleosome disassembly[40,41]. A thorough evaluation of this tetramer-disrupting capacity and its regulation is needed before these findings can be extended to other organisms and to other DNA transactions, such as replication or repair.

It will be interesting to determine how this potential tetramer-disrupting activity of Asf1 is regulated. Whereas the N terminus of Asf1 is highly conserved (**Fig. 2a**), its C terminus is rather divergent and could be a good candidate for the basis of distinct functions both among various species and between the Asf1a and Asf1b isoforms, when they exist[42,43]. In mammals, this C terminus is the target of specific modifications such as phosphorylation[44]. Thus, divergence between species in this region deserves further attention in the attempt to explain species-specific functions of Asf1.

Selectivity of the relationship between the escort and its cargo. The crystal structure of Asf1 bound to H3-H4 also reveals that the binding interface involves only amino acid residues conserved between the two human isoforms ASF1a and ASF1b, suggesting that both interact in the same manner with the H3-H4 dimer (ref. 38). Divergent residues remain exposed to the solvent, thereby potentially providing a specific interface for other factors. Moreover, residues that differ between the H3.1 and H3.3 variants[17], and those targeted by post-translational modifications (such as the acetylation of histone H3 Lys56 (H3K56ac), known to be important in *S. cerevisiae*), are still exposed to the solvent[36,38] (**Fig. 2b**). In *S. cerevisiae*, Asf1 helps to present the H3K56 residue to Rtt109, a newly characterized histone acetyltransferase (HAT), possibly to promote the proper histone acetylation[45–47] before deposition onto DNA[48–50]. As H3K56 acetylation by Rtt109 is conserved in *Schizosaccharomyces pombe*[51], whether this modification is mediated by Asf1 should be assessed. The small amount of H3K56ac in human cells[52] raises the question of whether a corresponding role for Asf1a and Asf1b exists in higher organisms.

Recent discovery of the yeast histone chaperone Chz1, which preferentially binds an H2A variant, also sheds light on the selectivity of the interaction between the escort and its cargo[16]. A conserved domain within Chz1 (the CHZ motif) contains the key determinants for specific Htz1 recognition *in vivo*[16]. It will be interesting to find out whether the human Chz1 homolog, HIRA-interacting protein-3 (HIRIP3, ref. 53; see **Fig. 1**), has similar H2AZ specificity. This might provide a mechanism for coordinated transactions between histone H3.3 incorporation by HIRA and H2AZ deposition during transcription. The selectivity for H2A variants may extend to other chaperones, as shown by a recent study revealing that nucleolin induces SWI/SNF-dependent remodeling of macroH2A but not H2ABbd nucleosomes[25] (see **Fig. 1**).

Histone chaperone interactions and their position in the escort network. The interaction between Asf1 and Hir was initially found in budding yeast[54]. In humans, the interaction of ASF1a with HIRA[18,26,55] requires the conserved N terminus of ASF1a[26], which is recognized by a region called the B-domain in HIRA[55]. The recent crystal structure of the conserved N terminus of ASF1a with a small HIRA peptide further shows that the HIRA B-domain forms an antiparallel β-hairpin, which interacts with the β-sandwich of the ASF1a N-terminal core domain[43]. Intriguingly, although this binding interface is strictly conserved in both ASF1a and ASF1b, it is instead the less conserved regions in the C and N termini of ASF1a that mediate specific recognition. Moreover, binding involves a HIRA-specific domain located outside of the B-domain[43]. Previous biochemical analysis revealed that the binding of ASF1 to HIRA or CAF-1 seems to be mutually exclusive[18] and that both ASF1a and ASF1b interact with CAF-1 p60 (refs. 12,56). In fact, the CAF-1 p60 C terminus contains B-domain–like motifs that resemble those in HIRA and recognize the same region of ASF1a, explaining why these two histone chaperones bind ASF1 in a mutually exclusive manner[43], as was recently confirmed in vertebrate cells[57]. Taking these observations together, we have created a scheme summarizing the current understanding of Asf1's specificity and its role as a donor to HIRA and CAF-1 in chromatin assembly (**Fig. 2b**).

Beyond its function as an accessory factor for CAF-1 and HIRA in chromatin assembly, Asf1 is also connected to various aspects of DNA metabolism through its interactions with several partners. As an example, in budding yeast, Asf1 binds the checkpoint kinase Rad53 under normal conditions[58,59] and is released upon phosphorylation of Rad53 during genotoxic stress. Interestingly, Rad53 is involved in the degradation of excess histones[60]. In mammals, although the corresponding interactions with checkpoint proteins could not be found (A. Groth, J. Bartek, J. Lukas and G.A., unpublished data), ASF1a and ASF1b function as a buffer for excess histones upon replication block[61]. They are phosphorylated by the Tousled-like kinases Tlk1 and Tlk2, which themselves are targets of the checkpoint kinase Chk1 (ref. 44). How this modification contributes to Asf1 function remains to be elucidated. The growing list of partners for Asf1 reveals complex interplay with various functions, which may differ between yeast and mammals (**Fig. 3a**). These multiple interactions contribute to the formation of an intricate network of

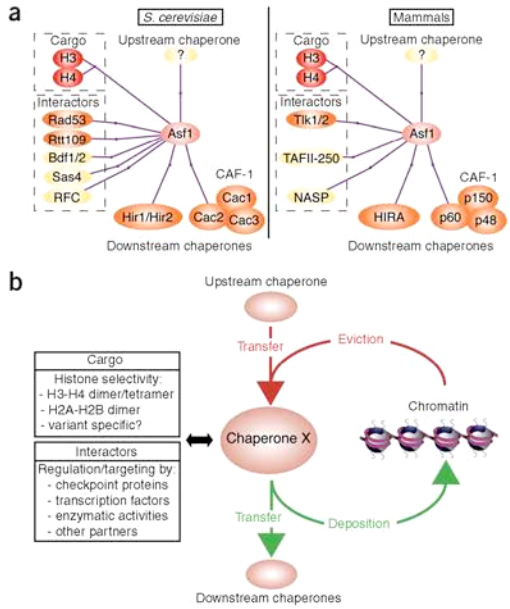

Figure 3 Asf1 is an example of a histone chaperone within a network of binding partners. (**a**) Binding partners are depicted for both *S. cerevisiae* Asf1 and mammalian Asf1 to illustrate that these histone chaperones act within a network of interactors that cooperate in chromatin assembly (for example, CAF-1 and HIRA) and potentially regulate Asf1 function (for example, Rad53). Red-to-yellow gradient represents the amount of evidence supporting each interaction, red being a well-demonstrated interaction and yellow a potential interaction. (**b**) Schematic representation of the relationship of a given histone chaperone to its network of binding partners. The chaperone will have various degrees of selectivity toward specific histone dimers, or possibly tetramers, which it will accept from, or transfer to, other chaperones. Alternatively, the chaperone could also accept histones immediately after their eviction from chromatin or transfer histones directly onto DNA in a deposition reaction. The histone chaperone's function will be regulated and targeted through its binding partners, which are involved in various cellular processes.

different partners (**Fig. 3b**), in which histone chaperones are key actors in the regulation of histone metabolism.

Histone chaperones and histone traffic

Here we discuss how histone chaperones can contribute to local histone dynamics during transcription and DSB repair. For information related to DNA replication or to other DNA damage responses, readers can consult recent reviews[62,63].

Histone chaperones in gene transcription. Chromatin structure can impose constraints on gene transcription, and it has been suggested that local chromatin rearrangements facilitate the passage of RNA polymerase II (Pol II)[64]. This can be achieved by mechanisms such as post-translational modifications of histones, recruitment of remodeling machinery, and actions of histone variants and histone chaperones[65,66]. Recent systematic interactome studies[67] that have made use of large collections of yeast mutants, including those with defective histone chaperones, remodeling machinery and transcription factors, can provide insight into the connection between chaperones and transcription. Nevertheless, there is still a gap between the results of these global analyses and understanding of the mutants' underlying molecular properties.

Histone modifications participate in the regulation of chromatin structure during the process of transcription[65] (**Fig. 4**; see also the review by Bhaumik *et al.*[68] in this issue). One modification, histone acetylation, is achieved via multiple HAT complexes and can occur on various lysine residues. Reports in *S. cerevisiae* show that acetylation of H3K56, located in the globular core domain of H3, weakens the electrostatic interaction between the histone and DNA, leading to increased accessibility of the DNA wrapped around the nucleosome[69]. Recent studies have shown that this acetylation may be involved in transcription elongation[50] and is preferentially enriched at certain active genes[70]. Acetylation of H3K56 in *S. cerevisiae* requires the Rtt109 HAT in complex with a histone chaperone, the Nap1-related protein Vps75 or Asf1 (see refs. 45,47,71 and **Fig. 1**). Formation of such a complex could enhance the binding affinity of Rtt109 for H3, favoring acetylation[45,47,71]. Notably, the Rtt109-Vps75 HAT preferentially acetylates non-nucleosomal newly synthesized H3 (ref. 71), suggesting that H3 could exchange with H3K56-acetylated histones to promote transcription or contribute to reassembly, which in turn could prevent cryptic initiation. In this case, one could anticipate some interaction with the chaperone Spt16, a subunit of the 'facilitates chromatin transcription' (FACT) complex (see **Fig. 1**), which promotes transcription[72], or with Spt6, an H3-H4 chaperone (see below)[73,74]. Furthermore, ATP-dependent chromatin-remodeling machinery has the ability to perturb nucleosome-DNA contacts (see also the review by Cairns[75] in this issue) in ways that can change the accessibility of DNA to transcription factors or assist the polymerase during initiation and elongation[65]. Indeed, remodeling complexes such as SWI/SNF, CHD1 and INO80 participate in RNA Pol II activation or elongation through their remodeling activities (reviewed in ref. 76). Histone-binding proteins present in these complexes, such as Arp7 and Arp9 in SWI/SNF or Arp8 in INO80, could thus have a role in nucleosome mobilization (**Fig. 4**).

Incorporation of histone variants into nucleosomes is also thought to affect transcriptional regulation. The Htz1 variant (yeast homolog of H2AZ) is found in nucleosomes flanking the nucleosome-free region within promoters in yeast and is evicted upon transcriptional elongation[66,77]. Whether the newly identified histone chaperone Chz1, which preferentially interacts with Htz1, contributes to transcriptional activation or repression[16] should be considered. Interestingly, nucleolin is able to promote transcription by inducing remodeling of macroH2A-containing nucleosomes by SWI/SNF[25] (**Fig. 4**).

Various degrees of histone eviction may facilitate transcription *in vitro* and *in vivo* (**Fig. 4**), although the extent to which this occurs probably depends on local chromatin structure and transcription rates[64]. Histone chaperones have been implicated in histone eviction and deposition during transcription. H2A-H2B dimers and the $(H3\text{-}H4)_2$ tetramer occupy distinct positions in the nucleosome. The external dimers are less tightly bound to DNA than is the tetramer, and they are therefore the main candidates for displacement from DNA[78,79]. Histone chaperones such as yeast Nap1 and nucleoplasmin are thought to facilitate transcription factor binding by removing an H2A-H2B dimer[80,81]. FACT, identified on the basis of its requirement during transcriptional elongation on chromatin templates[82,83], promotes the displacement of H2A-H2B dimers to facilitate RNA Pol II passage[24]. Nucleolin is thought to remove H2A-H2B from assembled nucleosomes in a manner similar to FACT[25]. In

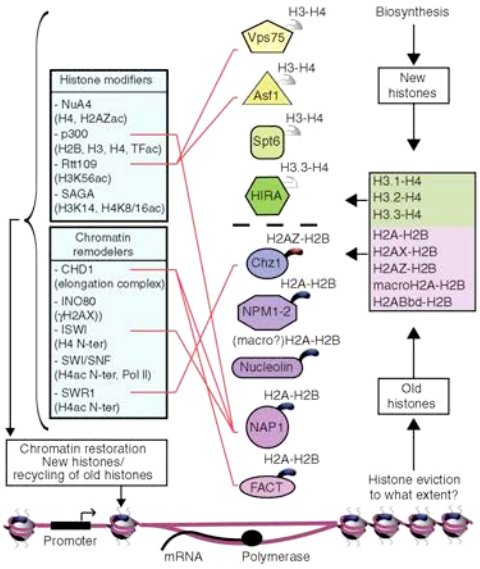

Figure 4 Histone chaperones mediate histone dynamics during transcription elongation. During transcription, histones are thought to be partially or totally evicted from the transcribed DNA. The evicted histones may then be recycled upon chromatin restoration after passage of the polymerase. Alternatively, newly synthesized histones might be incorporated at this step. This cycle of histone eviction and deposition is facilitated by the cooperation (red lines) of the candidate histone chaperones with candidate histone modifiers and remodeling factors that are involved in transcription elongation. The binding of the histone modifiers and remodeling factors to their substrates (indicated in parentheses) may assist in targeting histone chaperones toward sites of active transcription. ac, acetylation; TF, transcription factor; N-ter, N terminus.

addition, other chaperones such as Nap1 and nucleophosmin are also believed to be involved in histone removal during elongation of chromatin templates *in vitro*[84–86]. In yeast, activator-mediated removal of histones H3-H4 from promoter sites seems to be promoted by Asf1 (refs. 41,87). Indeed, ChIP experiments demonstrate that yeast Asf1 travels with RNA Pol II during elongation and is important for efficient eviction of H3 but not H2B[40]. This function of Asf1 is further supported by recent structural studies[37,38] and evidence that tetramer stability is compromised in the presence of ASF1 *in vitro*[38]. That FACT could have a disassembly activity comparable to that of Asf1 is an attractive hypothesis. Histone chaperones can promote transcription elongation simply as a 'histone sink', as shown using nucleoplasmin and *in vitro* transcription on chromatin templates[88], but they could also help to channel transfer of histones, facilitating their recycling or targeting them for other events in histone metabolism.

Reassembly of nucleosomes at promoters and in the wake of RNA Pol II is crucial to restore chromatin structure and to avoid erroneous initiation from internal cryptic sites. New histones are incorporated during this process[89,90], but it is not clear how the recycling of evicted histones and incorporation of new histones are regulated (**Fig. 4**). Spt6, FACT and Asf1 have been implicated in the deposition of histones behind RNA Pol II[24,40,74]. Mutations in the FACT subunits Spt16 or Pob3 in *S. cerevisiae* are lethal when combined with mutations in HIR proteins[91]. Furthermore, delayed reassembly of nucleosomes on promoters in *hir1* and *asf1* mutant budding yeast strains[92], along with a requirement for the histone H3-H4 chaperone Spt6 (refs. 41,73), supports a concerted action of these chaperones, possibly in conjunction with histone-modifying enzymes.

Several mechanisms can direct the targeting of histone chaperones to transcription sites where they can effect histone eviction and deposition. In the case of human SPT6, direct interaction with the elongating RNA Pol II is involved[93]. In a potential alternative mechanism, human ASF1 may be recruited to transcription sites through binding to general transcription factors, as this protein interacts with two bromodomains of human TAFII250, the largest subunit of the transcription factor TFIID[94]. Interaction with chromatin-remodeling machinery provides yet another way to recruit a histone chaperone to its site of action, as shown for FACT, which contacts the chromatin-remodeling complex CHD1 through its Chd1 subunit[95] (**Fig. 4**). Interestingly, CHD1 was recently found to be involved in H3.3 deposition in cooperation with HIRA during *D. melanogaster* development[96]. Obviously, the question of what proportion of histones deposited during transcription are recycled as opposed to newly incorporated (**Fig. 4**) should now be considered in light of findings on 'transcriptional memory'. Indeed, this balance will determine to what extent post-translational histone modifications and/or particular variants can be erased during elongation. Interestingly, high Htz1 levels at gene promoters in yeast have recently been associated with transcriptional memory that can be inherited through several cell generations[97]. A crucial issue to address is the manner in which the donor and acceptor functions of histone chaperones are regulated in various cell types, which may contribute to the specificity of expression profiles. In this respect, the example of phosphorylation changes in nucleoplasmin after oocyte maturation, which promotes nucleoplasmin's histone-donor function[98], illustrates the potential role of post-translational modifications driven by cell-specific factors in controlling cellular function.

Histone chaperones in DSB DNA repair. In the response to a DSB within chromatin, the first challenge is to detect the damage, and the second is to recruit DNA repair factors that should be able to deal with the damage in its particular context. The current picture of the function of histone dynamics during these processes involves histone modifications, chromatin remodeling and histone eviction, occurring in a sequence of events whose relationship to the events of DNA repair itself is not entirely clear (**Fig. 5**; for recent reviews on the DSB repair process, see refs. 99–102). Here we would like to highlight the possible contributions of the different classes of histone chaperones (**Fig. 1**) to histone dynamics during and after DNA repair. Important for this are data from budding yeast, in which chromatin structure has been studied near a DSB induced by site-specific enzymatic cleavage. The recent development of new methods to create specific DSB sites in mammalian cells, as pioneered by Maria Jasin's group[103–105], offers promise for analyzing chromatin dynamics at these sites.

Within minutes of DSB formation, long regions of adjacent chromatin ($\geq$1 megabase of DNA in mammalian cells[106] and about 50–100 kilobases in budding yeast) undergo extensive phosphorylation. This occurs on the H2AX variant in mammals, which is then called γH2AX[106]; in yeast, the phosphorylated histone is highly similar to H2AX, but is called H2A, as it is the predominant H2A form in this organism[107]. For consistency, we will refer to it here as γH2AX. Although H2AX constitutes only about 10% of total mammalian H2A, the rapidity of its phosphorylation suggests that at least some of the phosphorylated H2AX molecules are already present in chromatin. Indeed, H2AX and H2A exist in nonrandom domains such that one

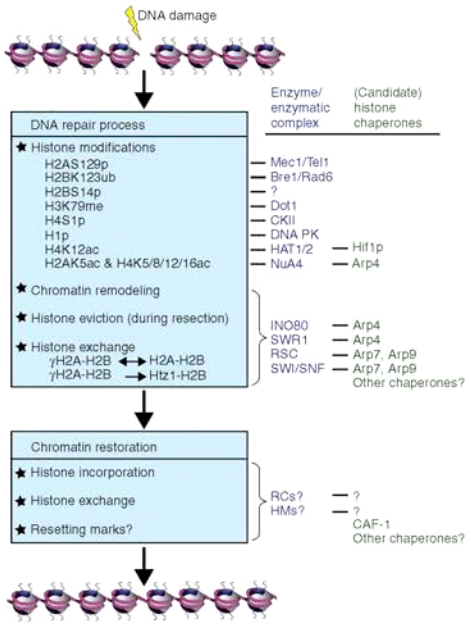

Figure 5 Chromatin rearrangements and histone chaperone activities during DSB repair in *S. cerevisiae*. After DSB formation, H2A (H2AX in higher organisms) is phosphorylated within relatively large chromatin domains. Additional histone modifications then occur, such as phosphorylation of H4 Ser1 by casein kinase II (CKII) and acetylation of γH2AX and H4 by NuA4. Methylation of H3 Lys79 (H3K79me) is not increased upon DSB formation; rather, this modification becomes accessible, which, in combination with γH2AX, facilitates recruitment of Rad9 (53BP1 in mammals)[101]. In fission yeast, recruitment of the Rad9-related protein Crb2 depends on H4 Lys20 methylation (H4K20me), whereas in mammalian cells, both H3K79me and H4K20me might be involved. Chromatin-remodeling complexes, together with NuA4 and possibly histone chaperones, make chromatin more accessible by evicting and replacing histones. γH2AX removal through replacement with the H2AZ variant (Htz1 in *S. cerevisiae*) relies on this process. Restoration of local chromatin organization may rely on histone chaperones such as CAF-1, remodeling complexes and histone modifiers. p, phosphorylation; ub, ubiquitination; me, methylation; ac, acetylation; K, lysine; S, serine; RC, remodeling complexes; HM, histone modifiers.

H2AX domain may be fully modified by phosphorylation[108]. However, exchange of canonical H2A for the H2AX variant could also take place to reinforce initial phosphorylation levels (**Fig. 5**). In respect to this, it is noteworthy that yeast strains lacking the remodeling factor Rsc1 show delayed formation of γH2AX foci[109], suggesting that rapid remodeling occurs before repair-factor recruitment. This might relate to an exchange process and thus require an as yet unknown histone chaperone with selectivity for H2AX. When both the ATR and ATM kinases are inactivated after creation of a DSB, however, no change in γH2AX levels is evident before the onset of resection, suggesting that no major increase or loss of γH2AX occurs after the initial phosphorylation step[110].

Phosphorylated H2AX is thought to provide a docking place not only for several repair factors but also for histone modifiers and chromatin-remodeling complexes, stabilizing their recruitment (**Fig. 5**). For example, in budding yeast, the NuA4 HAT binds γH2AX through its associated chaperone Arp4 (ref. 111), which is also found as a component of the chromatin-remodeling complexes INO80 and SWR1 (**Fig. 1**). It is thus possible that Arp4 could help to bridge the subsequent interaction of these complexes and then initiate an amplification loop for recruitment to γH2AX. However, neither Arp4 nor the Arp8 chaperone in the INO80 complex is responsible for the specific binding of INO80 to γH2AX[112]. Rather, the binding seems to depend on the INO80 subunit Nhp10; this dependence may be either direct or indirect, as a direct interaction of Nhp10 with histones has not yet been demonstrated. Intriguingly, the presence of Nhp10 is not crucial for the INO80 remodeling activity but could contribute to its targeting[113,114]. In addition, the absence of Arp4, Arp8 or Nhp10 does not affect H2AX phosphorylation.

Less well characterized is the local recruitment of the nuclear yeast Hat1–Hat2 complex and its associated chaperone Hif1 (see **Fig. 1**) to DSBs, where this complex is responsible for the increased acetylation of H4K12 near the break[115] (**Fig. 5**). This HAT is believed to acetylate only newly synthesized soluble histones[116], suggesting that break-associated H4K12 acetylation is due to the incorporation of new histones near the break, in the region that is resected. Because the recruitment of Hat1–Hat2 occurs soon after γH2AX formation and does not depend on functional DSB repair by homologous recombination[115], the modification could have an effect early in the repair pathway. Alternatively, the acetylation of histones might represent chromatin-restoration activity, as some HO endonuclease–induced DSB ends are rejoined by nonhomologous end joining. Similarly, transient increases in H3 and H4 acetylation during DSB repair[117] might assist the repair process itself or represent post-repair chromatin restoration.

Of great interest is how γH2AX is removed from chromatin. Surprisingly, it seems that although there is a dedicated phosphatase (PP4C in yeast or PP2A in mammalian cells) that removes this modification, dephosphorylation in budding yeast apparently takes place only after removal of γH2AX (or γH2AX-H2B dimers) from the DNA[118,119]. There seem to be at least two distinct modes for this removal: histone eviction during resection and histone exchange (**Fig. 5**). After creation of a DSB, the broken ends are extensively resected by 5′-to-3′ exonucleases to create long single-stranded regions. In yeast, resection leads to displacement of γH2AX at a rate of about 4 kilobases h^{-1} on each side of the DSB. It has not yet been determined whether the eviction takes place as a result of the resection process itself or is promoted by the action of remodeling complexes such as INO80, or both. To what extent histone eviction takes place, and whether it affects only H2A-H2B dimers or also $(H3\text{-}H4)_2$ tetramers, remain to be clarified as well. Interestingly, during DSB repair in *S. cerevisiae*, the phosphorylation of H4S1 by casein kinase II[120] seems to persist (as assessed by chromatin immunoprecipitation) even when one DNA strand is resected (J.A. Kim and J.E.H., unpublished data). This observation suggests that some H3-H4 may remain associated with single-stranded DNA. Furthermore, the fate of the evicted histones should also be considered, and escort by chaperones may be required to keep them available as a storage pool or to direct them for recycling or elimination.

Alternatively, γH2AX can be removed through histone exchange, which is thought to require active histone eviction and deposition, as well as chaperone activity (**Fig. 5**). So far, we have found that mutations in Asf1, CAF-1 or Nap1 do not prevent the loss of γH2AX, either individually or in combination (J.A. Kim and J.E.H., unpublished data). This excludes an active contribution of these chaperones to eviction but not a possible role for them in proper histone usage after eviction. Chromatin-remodeling complexes and

their associated histone chaperones, in contrast, seem to have a more active role in γH2AX removal. Indeed, in the unusual situation where the chromatin remodeler INO80 is absent, replacement of γH2AX by the histone H2A variant H2AZ can be promoted by the chromatin remodeler Swr1 (ref. 121). However, there is no substantial effect when INO80 is present, and the absence of Htz1 (H2AZ homolog in *S. cerevisiae*) does not seem to affect the DNA damage checkpoint. Nevertheless, a similar mechanism for γH2AX replacement seems to operate in flies, and probably in mammals, through the action of the Tip60 complex (reviewed in ref. 122), which is considered to be the combined homolog of both yeast Swr1 and yeast NuA4. In addition, two remodeling complexes have been directly implicated in DSB repair. The RSC complex seems to be involved mainly in nonhomologous end joining[123]. The Snf2-Snf5 chromatin remodeler seems to have a key function in the process of homologous recombination, specifically in the strand-invasion step during recombination between the resected DSB end and a homologous donor sequence[124].

When the repair is completed, chromatin organization must be restored (**Fig. 5**). This requires histone incorporation, and possibly histone exchange, within the repaired DNA. At this step, the balance between the incorporation of new histones and the recycling of evicted histones could be of importance. In human cells, CAF-1–dependent incorporation of newly synthesized H3.1 histones has been observed *in vivo* at sites of both laser-induced damage, which is known to produce DSBs, and UV-induced damage[125]. The incorporation of new histones could make it difficult to restore chromatin organization to its state before the occurrence of a DSB, in terms of both the proportions of given variants and histone modifications. Indeed, new histones present a set of specific modifications distinct from that of their nucleosomal counterparts. Removal of these modifications and resetting of initial marks probably require *ad hoc* intervention by histone modifiers and remodeling complexes, in addition to other histone chaperones (**Fig. 5**). The regulation of this restoration step is currently a subject of investigation and will be important for understanding a possible cross-generational memory of damage events.

Conclusions

In this review, we have classified currently known histone chaperones on the basis of their structural and functional properties. We emphasize that histone chaperones are not necessarily deposition factors and that they can fulfill various other functions in histone metabolism. Importantly, only a few chaperones showing selectivity toward histone variants have been identified so far. Identifying and documenting chaperone functions associated with histone variants such as H2AX or CENPA will be important steps forward.

We have highlighted three major challenges for the field of epigenetics. First, how and why a given chaperone shows selectivity toward specific histones remains an enigma that structural studies have not fully resolved. Understanding the structural basis of this selectivity will open up a broad range of new perspectives, including those gained from mutational analyses and the identification of new chaperones on the basis of their structural properties. Second, it will be important to identify the functional and molecular interactions of chaperones with each other and with their other partners so as to understand the molecular basis of histone transfer between different chaperones. A hierarchy with key chaperones at crucial nodes would then emerge. Finally, the contribution of histone chaperones to the maintenance of chromatin stability and epigenetic marks is another important question[63]. We have discussed the balance between incorporation of new histones and recycling of old histones in the context of transcription and DSB repair. To preserve and propagate the epigenetic marks that provide identity to a given cell lineage, the original marks should be reset after such processes. If histone marks represent true epigenetic marks, then recycled histones may serve as a template for copying and reestablishing correct marks on new histones, thus favoring transmission of chromatin organization. The potential tetramer-disrupting capacity of Asf1 and other chaperones may favor the recycling of old histones. The precise contribution of histone chaperones to epigenetic memory, however, remains to be determined.

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1. Kornberg, R.D. Structure of chromatin. *Annu. Rev. Biochem.* **46**, 931–954 (1977).
2. Oudet, P., Gross-Bellard, M. & Chambon, P. Electron microscopic and biochemical evidence that chromatin structure is a repeating unit. *Cell* **4**, 281–300 (1975).
3. Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F. & Richmond, T.J. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389**, 251–260 (1997).
4. Woodland, H.R. & Adamson, E.D. The synthesis and storage of histones during the oogenesis of *Xenopus laevis*. *Dev. Biol.* **57**, 118–135 (1977).
5. Loyola, A. & Almouzni, G. Histone chaperones, a supporting role in the limelight. *Biochim. Biophys. Acta* **1677**, 3–11 (2004).
6. Laskey, R.A., Mills, A.D. & Morris, N.R. Assembly of SV40 chromatin in a cell-free system from *Xenopus* eggs. *Cell* **10**, 237–243 (1977).
7. Stillman, B. Chromatin assembly during SV40 DNA replication *in vitro*. *Cell* **45**, 555–565 (1986).
8. Smith, S. & Stillman, B. Purification and characterization of CAF-I, a human cell factor required for chromatin assembly during DNA replication *in vitro*. *Cell* **58**, 15–25 (1989).
9. Ray-Gallet, D. & Almouzni, G. DNA synthesis-dependent and -independent chromatin assembly pathways in *Xenopus* egg extracts. *Methods Enzymol.* **375**, 117–131 (2004).
10. Quivy, J.P., Grandi, P. & Almouzni, G. Dimerization of the largest subunit of chromatin assembly factor 1: importance *in vitro* and during *Xenopus* early development. *EMBO J.* **20**, 2015–2027 (2001).
11. Ray-Gallet, D. *et al.* HIRA is critical for a nucleosome assembly pathway independent of DNA synthesis. *Mol. Cell* **9**, 1091–1100 (2002).
12. Mello, J.A. *et al.* Human Asf1 and CAF-1 interact and synergize in a repair-coupled nucleosome assembly pathway. *EMBO Rep.* **3**, 329–334 (2002).
13. Ray-Gallet, D., Quivy, J.P., Scilje, H.W., Nigg, E.A. & Almouzni, G. The histone chaperone Asf1 is dispensable for direct de novo histone deposition in *Xenopus* egg extracts. *Chromosoma* **116**, 487–496 (2007).
14. Polo, S.E. & Almouzni, G. Chromatin assembly: a basic recipe with various flavours. *Curr. Opin. Genet. Dev.* **16**, 104–111 (2006).
15. Harata, M. *et al.* The nuclear actin-related protein of *Saccharomyces cerevisiae*, Act3p/Arp4, interacts with core histones. *Mol. Biol. Cell* **10**, 2595–2605 (1999).
16. Luk, E. *et al.* Chz1, a nuclear chaperone for histone H2AZ. *Mol. Cell* **25**, 357–368 (2007).
17. Loyola, A. & Almouzni, G. Marking histone H3 variants: how, when and why? *Trends Biochem. Sci.* **32**, 425–433 (2007).
18. Tagami, H., Ray-Gallet, D., Almouzni, G. & Nakatani, Y. Histone H3.1 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell* **116**, 51–61 (2004).
19. Richardson, R.T. *et al.* Characterization of the histone H1-binding protein, NASP, as a cell cycle-regulated somatic protein. *J. Biol. Chem.* **275**, 30378–30386 (2000).
20. Shintomi, K. *et al.* Nucleosome assembly protein-1 is a linker histone chaperone in *Xenopus* eggs. *Proc. Natl. Acad. Sci. USA* **102**, 8210–8215 (2005).
21. Dutta, S. *et al.* The crystal structure of nucleoplasmin-core: implications for histone binding and nucleosome assembly. *Mol. Cell* **8**, 841–853 (2001).
22. Mousson, F., Ochsenbein, F. & Mann, C. The histone chaperone Asf1 at the crossroads of chromatin and DNA checkpoint pathways. *Chromosoma* **116**, 79–93 (2007).

23. Park, Y.J. & Luger, K. The structure of nucleosome assembly protein 1. *Proc. Natl. Acad. Sci. USA* **103**, 1248–1253 (2006).
24. Belotserkovskaya, R. *et al.* FACT facilitates transcription-dependent nucleosome alteration. *Science* **301**, 1090–1093 (2003).
25. Angelov, D. *et al.* Nucleolin is a histone chaperone with FACT-like activity and assists remodeling of nucleosomes. *EMBO J.* **25**, 1669–1679 (2006).
26. Daganzo, S.M. *et al.* Structure and function of the conserved core of histone deposition protein Asf1. *Curr. Biol.* **13**, 2148–2158 (2003).
27. Muto, S. *et al.* Relationship between the structure of SET/TAF-Iβ/INHAT and its histone chaperone activity. *Proc. Natl. Acad. Sci. USA* **104**, 4285–4290 (2007).
28. Umehara, T., Chimura, T., Ichikawa, N. & Horikoshi, M. Polyanionic stretch-deleted histone chaperone cia1/Asf1p is functional both *in vivo* and *in vitro*. *Genes Cells* **7**, 59–73 (2002).
29. Regnard, C. *et al.* Polyglutamylation of nucleosome assembly proteins. *J. Biol. Chem.* **275**, 15969–15976 (2000).
30. Namboodiri, V.M., Dutta, S., Akey, I.V., Head, J.F. & Akey, C.W. The crystal structure of *Drosophila* NLP-core provides insight into pentamer formation and histone binding. *Structure* **11**, 175–186 (2003).
31. Mousson, F. *et al.* Structural basis for the interaction of Asf1 with histone H3 and its functional implications. *Proc. Natl. Acad. Sci. USA* **102**, 5975–5980 (2005).
32. DeSilva, H., Lee, K. & Osley, M.A. Functional dissection of yeast Hir1p, a WD repeat-containing transcriptional corepressor. *Genetics* **148**, 657–667 (1998).
33. Kaufman, P.D., Kobayashi, R., Kessler, N. & Stillman, B. The p150 and p60 subunits of chromatin assembly factor I: a molecular link between newly synthesized histones and DNA replication. *Cell* **81**, 1105–1114 (1995).
34. Verreault, A., Kaufman, P.D., Kobayashi, R. & Stillman, B. Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. *Cell* **87**, 95–104 (1996).
35. Agez, M. *et al.* Structure of the histone chaperone ASF1 bound to the histone H3 C-terminal helix and functional insights. *Structure* **15**, 191–199 (2007).
36. Antczak, A.J., Tsubota, T., Kaufman, P.D. & Berger, J.M. Structure of the yeast histone H3–ASF1 interaction: implications for chaperone mechanism, species-specific interactions, and epigenetics. *BMC Struct. Biol.* **6**, 26 (2006).
37. English, C.M., Adkins, M.W., Carson, J.J., Churchill, M.E. & Tyler, J.K. Structural basis for the histone chaperone activity of Asf1. *Cell* **127**, 495–508 (2006).
38. Natsume, R. *et al.* Structure and function of the histone chaperone CIA/ASF1 complexed with histones H3 and H4. *Nature* **446**, 338–341 (2007).
39. English, C.M., Maluf, N.K., Tripet, B., Churchill, M.E. & Tyler, J.K. ASF1 binds to a heterodimer of histones H3 and H4: a two-step mechanism for the assembly of the H3–H4 heterotetramer on DNA. *Biochemistry* **44**, 13673–13682 (2005).
40. Schwabish, M.A. & Struhl, K. Asf1 mediates histone eviction and deposition during elongation by RNA polymerase II. *Mol. Cell* **22**, 415–422 (2006).
41. Adkins, M.W., Howar, S.R. & Tyler, J.K. Chromatin disassembly mediated by the histone chaperone Asf1 is essential for transcriptional activation of the yeast PHO5 and PHO8 genes. *Mol. Cell* **14**, 657–666 (2004).
42. Tamburini, B.A., Carson, J.J., Adkins, M.W. & Tyler, J.K. Functional conservation and specialization among eukaryotic anti-silencing function 1 histone chaperones. *Eukaryot. Cell* **4**, 1583–1590 (2005).
43. Tang, Y. *et al.* Structure of a human ASF1a-HIRA complex and insights into specificity of histone chaperone complex assembly. *Nat. Struct. Mol. Biol.* **13**, 921–929 (2006).
44. Sillje, H.H. & Nigg, E.A. Identification of human Asf1 chromatin assembly factors as substrates of Tousled-like kinases. *Curr. Biol.* **11**, 1068–1073 (2001).
45. Driscoll, R., Hudson, A. & Jackson, S.P. Yeast Rtt109 promotes genome stability by acetylating histone H3 on lysine 56. *Science* **315**, 649–652 (2007).
46. Han, J. *et al.* Rtt109 acetylates histone H3 lysine 56 and functions in DNA replication. *Science* **315**, 653–655 (2007).
47. Tsubota, T. *et al.* Histone H3-K56 acetylation is catalyzed by histone chaperone-dependent complexes. *Mol. Cell* **25**, 703–712 (2007).
48. Adkins, M.W., Carson, J.J., English, C.M., Ramey, C.J. & Tyler, J.K. The histone chaperone anti-silencing function 1 stimulates the acetylation of newly synthesized histone H3 in S-phase. *J. Biol. Chem.* **282**, 1334–1340 (2007).
49. Recht, J. *et al.* Histone chaperone Asf1 is required for histone H3 lysine 56 acetylation, a modification associated with S phase in mitosis and meiosis. *Proc. Natl. Acad. Sci. USA* **103**, 6988–6993 (2006).
50. Schneider, J., Bajwa, P., Johnson, F.C., Bhaumik, S.R. & Shilatifard, A. Rtt109 is required for proper H3K56 acetylation: a chromatin mark associated with the elongating RNA polymerase II. *J. Biol. Chem.* **281**, 37270–37274 (2006).
51. Xhemalce, B. *et al.* Regulation of histone H3 lysine 56 acetylation in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **282**, 15040–15047 (2007).
52. Morris, S.A. *et al.* Identification of histone H3 lysine 36 acetylation as a highly conserved histone modification. *J. Biol. Chem.* **282**, 7632–7640 (2007).
53. Lorain, S. *et al.* Core histones and HIRIP3, a novel histone-binding protein, directly interact with WD repeat protein HIRA. *Mol. Cell. Biol.* **18**, 5546–5556 (1998).
54. Sharp, J.A., Fouts, E.T., Krawitz, D.C. & Kaufman, P.D. Yeast histone deposition protein Asf1p requires Hir proteins and PCNA for heterochromatic silencing. *Curr. Biol.* **11**, 463–473 (2001).
55. Zhang, R. *et al.* Formation of MacroH2A-containing senescence-associated heterochromatin foci and senescence driven by ASF1a and HIRA. *Dev. Cell* **8**, 19–30 (2005).
56. Tyler, J.K. *et al.* Interaction between the *Drosophila* CAF-1 and ASF1 chromatin assembly factors. *Mol. Cell. Biol.* **21**, 6574–6584 (2001).
57. Sanematsu, F. *et al.* Asf1 is required for viability and chromatin assembly during DNA replication in vertebrate cells. *J. Biol. Chem.* **281**, 13817–13827 (2006).
58. Emili, A., Schieltz, D.M., Yates, J.R., III & Hartwell, L.H. Dynamic interaction of DNA damage checkpoint protein Rad53 with chromatin assembly factor Asf1. *Mol. Cell* **7**, 13–20 (2001).
59. Hu, F., Alcasabas, A.A. & Elledge, S.J. Asf1 links Rad53 to control of chromatin assembly. *Genes Dev.* **15**, 1061–1066 (2001).
60. Gunjan, A. & Verreault, A.A. Rad53 kinase-dependent surveillance mechanism that regulates histone protein levels in *S. cerevisiae*. *Cell* **115**, 537–549 (2003).
61. Groth, A. *et al.* Human Asf1 regulates the flow of S phase histones during replicational stress. *Mol. Cell* **17**, 301–311 (2005).
62. Polo, S.E. & Almouzni, G. Histone metabolic pathways and chromatin assembly factors as proliferation markers. *Cancer Lett.* **220**, 1–9 (2005).
63. Groth, A., Rocha, W., Verreault, A. & Almouzni, G. Chromatin challenges during DNA replication and repair. *Cell* **128**, 721–733 (2007).
64. Kulaeva, O.I., Gaykalova, D.A. & Studitsky, V.M. Transcription through chromatin by RNA polymerase II: histone displacement and exchange. *Mutat. Res.* **618**, 116–129 (2007).
65. Li, B., Carey, M. & Workman, J.L. The role of chromatin during transcription. *Cell* **128**, 707–719 (2007).
66. Workman, J.L. Nucleosome displacement in transcription. *Genes Dev.* **20**, 2009–2017 (2006).
67. Krogan, N.J. *et al.* RNA polymerase II elongation factors of *Saccharomyces cerevisiae*: a targeted proteomics approach. *Mol. Cell. Biol.* **22**, 6979–6992 (2002).
68. Bhaumik, S.R., Smith, E. & Shilatifard, A. Covalent modifications of histones during development and disease pathogenesis. *Nat. Struct. Mol. Biol.* **14**, 1008–1016 (2007).
69. Masumoto, H., Hawke, D., Kobayashi, R. & Verreault, A. A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. *Nature* **436**, 294–298 (2005).
70. Xu, F., Zhang, K. & Grunstein, M. Acetylation in histone H3 globular domain regulates gene expression in yeast. *Cell* **121**, 375–385 (2005).
71. Han, J., Zhou, H., Li, Z., Xu, R.M. & Zhang, Z. The Rtt109-Vps75 histone acetyltransferase complex acetylates non-nucleosomal histone H3. *J. Biol. Chem.* **282**, 14158–14164 (2007).
72. Duina, A.A. *et al.* Evidence that the localization of the elongation factor Spt16 across transcribed genes is dependent upon histone H3 integrity in *Saccharomyces cerevisiae*. *Genetics* **177**, 101–112 (2007).
73. Adkins, M.W. & Tyler, J.K. Transcriptional activators are dispensable for transcription in the absence of Spt6-mediated chromatin reassembly of promoter regions. *Mol. Cell* **21**, 405–416 (2006).
74. Kaplan, C.D., Laprade, L. & Winston, F. Transcription elongation factors repress transcription initiation from cryptic sites. *Science* **301**, 1096–1099 (2003).
75. Cairns, B.R. Chromatin remodeling: insights and intrigue from single-molecule studies. *Nat. Struct. Mol. Biol.* **14**, 989–996 (2007).
76. Saha, A., Wittmeyer, J. & Cairns, B.R. Chromatin remodelling: the industrial revolution of DNA around histones. *Nat. Rev. Mol. Cell Biol.* **7**, 437–447 (2006).
77. Raisner, R.M. *et al.* Histone variant H2A.Z marks the 5′ ends of both active and inactive genes in euchromatin. *Cell* **123**, 233–248 (2005).
78. Thiriet, C. & Hayes, J.J. Replication-independent core histone dynamics at transcriptionally active loci *in vivo*. *Genes Dev.* **19**, 677–682 (2005).
79. Levchenko, V., Jackson, B. & Jackson, V. Histone release during transcription: displacement of the two H2A–H2B dimers in the nucleosome is dependent on different levels of transcription-induced positive stress. *Biochemistry* **44**, 5357–5372 (2005).
80. Chen, H., Li, B. & Workman, J.L. A histone-binding protein, nucleoplasmin, stimulates transcription factor binding to nucleosomes and factor-induced nucleosome disassembly. *EMBO J.* **13**, 380–390 (1994).
81. Walter, P.P., Owen-Hughes, T.A., Cote, J. & Workman, J.L. Stimulation of transcription factor binding and histone displacement by nucleosome assembly protein 1 and nucleoplasmin requires disruption of the histone octamer. *Mol. Cell. Biol.* **15**, 6178–6187 (1995).
82. Orphanides, G., LeRoy, G., Chang, C.H., Luse, D.S. & Reinberg, D. FACT, a factor that facilitates transcript elongation through nucleosomes. *Cell* **92**, 105–116 (1998).
83. Orphanides, G., Wu, W.H., Lane, W.S., Hampsey, M. & Reinberg, D. The chromatin-specific transcription elongation factor FACT comprises human SPT16 and SSRP1 proteins. *Nature* **400**, 284–288 (1999).
84. Ito, T., Ikehara, T., Nakagawa, T., Kraus, W.L. & Muramatsu, M. p300-mediated acetylation facilitates the transfer of histone H2A–H2B dimers from nucleosomes to a histone chaperone. *Genes Dev.* **14**, 1899–1907 (2000).
85. Swaminathan, V., Kishore, A.H., Febitha, K.K. & Kundu, T.K. Human histone chaperone nucleophosmin enhances acetylation-dependent chromatin transcription. *Mol. Cell. Biol.* **25**, 7534–7545 (2005).
86. Zlatanova, J., Seebart, C. & Tomschik, M. Nap1: taking a closer look at a juggler protein of extraordinary skills. *FASEB J.* **21**, 1294–1310 (2007).
87. Korber, P. *et al.* The histone chaperone Asf1 increases the rate of histone eviction at the yeast PHO5 and PHO8 promoters. *J. Biol. Chem.* **281**, 5539–5545 (2006).
88. Owen-Hughes, T. & Workman, J.L. Remodeling the chromatin structure of a nucleosome array by transcription factor-targeted trans-displacement of histones. *EMBO J.* **15**, 4702–4712 (1996).
89. Jamai, A., Imoberdorf, R.M. & Strubin, M. Continuous histone H2B and transcription-dependent histone H3 exchange in yeast cells outside of replication. *Mol. Cell* **25**, 345–355 (2007).

90. Dion, M.F. *et al.* Dynamics of replication-independent histone turnover in budding yeast. *Science* **315**, 1405–1408 (2007).
91. Formosa, T. *et al.* Defects in SPT16 or POB3 (yFACT) in *Saccharomyces cerevisiae* cause dependence on the Hir/Hpc pathway: polymerase passage may degrade chromatin structure. *Genetics* **162**, 1557–1571 (2002).
92. Schermer, U.J., Korber, P. & Horz, W. Histones are incorporated *in trans* during reassembly of the yeast PHO5 promoter. *Mol. Cell* **19**, 279–285 (2005).
93. Endoh, M. *et al.* Human Spt6 stimulates transcription elongation by RNA polymerase II *in vitro*. *Mol. Cell. Biol.* **24**, 3324–3336 (2004).
94. Chimura, T., Kuzuhara, T. & Horikoshi, M. Identification and characterization of CIA/ASF1 as an interactor of bromodomains associated with TFIID. *Proc. Natl. Acad. Sci. USA* **99**, 9334–9339 (2002).
95. Simic, R. *et al.* Chromatin remodeling protein Chd1 interacts with transcription elongation factors and localizes to transcribed genes. *EMBO J.* **22**, 1846–1856 (2003).
96. Konev, A.Y. *et al.* CHD1 motor protein is required for deposition of histone variant H3.3 into chromatin in vivo. *Science* **317**, 1087–1090 (2007).
97. Brickner, D.G. *et al.* H2A.Z-mediatedlocalization of genes at the nuclear periphery confers epigenetic memory of previous transcriptional state. *PLoS Biol.* **5**, e81 (2007).
98. Leno, G.H., Mills, A.D., Philpott, A. & Laskey, R.A. Hyperphosphorylation of nucleoplasmin facilitates *Xenopus* sperm decondensation at fertilization. *J. Biol. Chem.* **271**, 7253–7256 (1996).
99. Downs, J.A., Nussenzweig, M.C. & Nussenzweig, A. Chromatin dynamics and the preservation of genetic information. *Nature* **447**, 951–958 (2007).
100. Bao, Y. & Shen, X. Chromatin remodeling in DNA double-strand break repair. *Curr. Opin. Genet. Dev.* **17**, 126–131 (2007).
101. Altaf, M., Saksouk, N. & Cote, J. Histone modifications in response to DNA damage. *Mutat. Res.* **618**, 81–90 (2007).
102. Osley, M.A., Tsukuda, T. & Nickoloff, J.A. ATP-dependent chromatin remodeling factors and DNA damage repair. *Mutat. Res.* **618**, 65–80 (2007).
103. Rodrigue, A. *et al.* Interplay between human DNA repair proteins at a unique double-strand break *in vivo*. *EMBO J.* **25**, 222–231 (2006).
104. Soutoglou, E. *et al.* Positional stability of single double-strand breaks in mammalian cells. *Nat. Cell Biol.* **9**, 675–682 (2007).
105. Berkovich, E., Monnat, R.J. Jr. & Kastan, M.B. Roles of ATM and NBS1 in chromatin structure modulation and DNA double-strand break repair. *Nat. Cell Biol.* **9**, 683–690 (2007).
106. Rogakou, E.P., Boon, C., Redon, C. & Bonner, W.M. Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J. Cell Biol.* **146**, 905–916 (1999).
107. Fillingham, J., Keogh, M.C. & Krogan, N.J. GammaH2AX and its role in DNA double-strand break repair. *Biochem. Cell Biol.* **84**, 568–577 (2006).
108. Bewersdorf, J., Bennett, B.T. & Knight, K.L. H2AX chromatin structures and their response to DNA damage revealed by 4Pi microscopy. *Proc. Natl. Acad. Sci. USA* **103**, 18137–18142 (2006).
109. Kent, N.A., Chambers, A.L. & Downs, J.A. Dual chromatin-remodelling roles for RSC during DNA double-strand break induction and repair at the yeast MAT locus. *J. Biol. Chem.* **282**, 27693–27701 (2007).
110. Kim, J.A., Kruhlak, M., Dotiwala, F., Nussenzweig, A. & Haber, J.E. Heterochromatin is refractory to gamma-H2AX modification in yeast and mammals. *J. Cell Biol.* **178**, 209–218 (2007).
111. Downs, J.A. *et al.* Binding of chromatin-modifying activities to phosphorylated histone H2A at DNA damage sites. *Mol. Cell* **16**, 979–990 (2004).
112. Morrison, A.J. *et al.* INO80 and gamma-H2AX interaction links ATP-dependent chromatin remodeling to DNA damage repair. *Cell* **119**, 767–775 (2004).
113. Shen, X., Ranallo, R., Choi, E. & Wu, C. Involvement of actin-related proteins in ATP-dependent chromatin remodeling. *Mol. Cell* **12**, 147–155 (2003).
114. van Attikum, H., Fritsch, O., Hohn, B. & Gasser, S.M. Recruitment of the INO80 complex by H2A phosphorylation links ATP-dependent chromatin remodeling with DNA double-strand break repair. *Cell* **119**, 777–788 (2004).
115. Qin, S. & Parthun, M.R. Recruitment of the type B histone acetyltransferase Hat1p to chromatin is linked to DNA double-strand breaks. *Mol. Cell. Biol.* **26**, 3649–3658 (2006).
116. Parthun, M.R., Widom, J. & Gottschling, D.E. The major cytoplasmic histone acetyltransferase in yeast: links to chromatin replication and histone metabolism. *Cell* **87**, 85–94 (1996).
117. Tamburini, B.A. & Tyler, J.K. Localized histone acetylation and deacetylation triggered by the homologous recombination pathway of double-strand DNA repair. *Mol. Cell. Biol.* **25**, 4903–4913 (2005).
118. Chowdhury, D. *et al.* gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. *Mol. Cell* **20**, 801–809 (2005).
119. Keogh, M.C. *et al.* A phosphatase complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery. *Nature* **439**, 497–501 (2006).
120. Cheung, W.L. *et al.* Phosphorylation of histone H4 serine 1 during DNA damage requires casein kinase II in *S. cerevisiae*. *Curr. Biol.* **15**, 656–660 (2005).
121. Papamichos-Chronakis, M., Krebs, J.E. & Peterson, C.L. Interplay between Ino80 and Swr1 chromatin remodeling enzymes regulates cell cycle checkpoint adaptation in response to DNA damage. *Genes Dev.* **20**, 2437–2449 (2006).
122. Squatrito, M., Gorrini, C. & Amati, B. Tip60 in DNA damage response and growth control: many tricks in one HAT. *Trends Cell Biol.* **16**, 433–442 (2006).
123. Shim, E.Y., Ma, J.L., Oum, J.H., Yanez, Y. & Lee, S.E. The yeast chromatin remodeler RSC complex facilitates end joining repair of DNA double-strand breaks. *Mol. Cell. Biol.* **25**, 3934–3944 (2005).
124. Chai, B., Huang, J., Cairns, B.R. & Laurent, B.C. Distinct roles for the RSC and Swi/Snf ATP-dependent chromatin remodelers in DNA double-strand break repair. *Genes Dev.* **19**, 1656–1661 (2005).
125. Polo, S.E., Roche, D. & Almouzni, G. New histone incorporation marks sites of UV repair in human cells. *Cell* **127**, 481–493 (2006).
126. Tyler, J.K. *et al.* The RCAF complex mediates chromatin assembly during DNA replication and repair. *Nature* **402**, 555–560 (1999).
127. Kuzuhara, T. & Horikoshi, M. A nuclear FK506-binding protein is a histone chaperone regulating rDNA silencing. *Nat. Struct. Mol. Biol.* **11**, 275–283 (2004).
128. Kleinschmidt, J.A., Fortkamp, E., Krohne, G., Zentgraf, H. & Franke, W.W. Co-existence of two different types of soluble histone complexes in nuclei of *Xenopus laevis* oocytes. *J. Biol. Chem.* **260**, 1166–1176 (1985).
129. Bortvin, A. & Winston, F. Evidence that Spt6p controls chromatin structure by a direct interaction with histones. *Science* **272**, 1473–1476 (1996).
130. Huang, S. *et al.* Rtt106p is a histone chaperone involved in heterochromatin-mediated silencing. *Proc. Natl. Acad. Sci. USA* **102**, 13410–13415 (2005).
131. Laskey, R.A., Honda, B.M., Mills, A.D. & Finch, J.T. Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. *Nature* **275**, 416–420 (1978).
132. Okuwaki, M., Matsumoto, K., Tsujimoto, M. & Nagata, K. Function of nucleophosmin/B23, a nucleolar acidic protein, as a histone chaperone. *FEBS Lett.* **506**, 272–276 (2001).
133. Rougeulle, C. & Avner, P. Cloning and characterization of a murine brain specific gene Bpx and its human homologue lying within the Xic candidate region. *Hum. Mol. Genet.* **5**, 41–49 (1996).
134. Okuwaki, M. & Nagata, K. Template activating factor-I remodels the chromatin structure and stimulates transcription from the chromatin template. *J. Biol. Chem.* **273**, 34511–34518 (1998).
135. Wang, G.S. *et al.* Transcriptional modification by a CASK-interacting nucleosome assembly protein. *Neuron* **42**, 113–128 (2004).
136. Selth, L. & Svejstrup, J.Q. Vps75, a new yeast member of the NAP histone chaperone family. *J. Biol. Chem.* **282**, 12358–12362 (2007).
137. Ai, X. & Parthun, M.R. The nuclear Hat1p/Hat2p complex: a molecular link between type B histone acetyltransferases and chromatin assembly. *Mol. Cell* **14**, 195–205 (2004).
138. Loyola, A., LeRoy, G., Wang, Y.H. & Reinberg, D. Reconstitution of recombinant chromatin establishes a requirement for histone-tail modifications during chromatin assembly and transcription. *Genes Dev.* **15**, 2837–2851 (2001).
139. Peterson, C.L., Zhao, Y. & Chait, B.T. Subunits of the yeast SWI/SNF complex are members of the actin-related protein (ARP) family. *J. Biol. Chem.* **273**, 23641–23644 (1998).
140. Ito, T., Bulger, M., Pazin, M.J., Kobayashi, R. & Kadonaga, J.T. ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. *Cell* **90**, 145–155 (1997).

Appendix #2:

HP1alpha as a prognostic marker in human cancer

(European patent)

Geneviève Almouzni & Leanne De Koning

Using early-stage breast cancer tissue samples, we showed that the expression levels of HP1α could constitute an interesting tool for the prognosis of patients suffering from this type of cancer (De Koning et al., *Heterochromatin Protein 1 alpha: a hallmark of cell proliferation relevant in clinical oncology*, EMBO Molecular Medecine, Vol 1 (issue 3), p178-191, 2009). According to our multivariate statistical analyses, HP1α might even predict disease outcome better than currently used prognostic markers, such as tumor grade, tumor size or the mitotic index. Therefore, we considered it interesting to make this tool available for potential industrial purposes. For industrial companies, it is crucial that the industrial property has been protected by the deposition of a patent. With help of the Department of Industrial Property at the Institut Curie, we therefore deposited a patent entitled "HP1alpha as a prognostic marker in human cancer". In this patent, our invention and claims concern the use of HP1α for the diagnosis or the prognosis of breast cancer and potentially other types of cancer, by assessing its expression at the protein or at the mRNA level. HP1α could thus be used to decide whether a subject affected with a cancer is susceptible to benefit from an adjuvant chemotherapy and/or radiotherapy, to predict clinical outcome and possibly to monitor the response to a treatment.

Appendix #3:

Is tumour aromatase expression prognostic for local control in young breast cancer patients after breast-conserving treatment?

Marc A Bollet, Alexia Savignoni, Leanne De Koning, Carine Tran Perennou, Catherine Barbaroux, Armelle Degeorges, Brigitte Sigal-Zafrani, Geneviève Almouzni, Paul Cottu, Rémy Salmon, Alain Fourquet, Patricia de Cremoux

Breast Cancer Research (in press)

This study, performed in tight collaboration with the Institut Curie hospital and the Department of Tumour Biology, aimed to determine what factors could be predictive for the relapse of young (< 40 years old) breast cancer patients after breast conserving surgery. Indeed, young age at the onset of breast cancer, even in the absence of any known hereditary factors, is an important risk factor for relapse after chirurgical removal (Bollet et al, 2007a; Oh et al, 2006b; Vrieling et al, 2003). The biological reasons for the increased recurrence are unknown and no prognostic factors have been identified so far. The main biological difference between these women and older patients is their pre-menopausal hormonal status, which could be at the origin of increased cell proliferation.

Therefore, this study focused on the expression levels of 17 candidate genes involved in hormone response and cell proliferation, in addition to the classical clinical and histopathological characteristics. The expression levels of the selected genes were analyzed by quantitative RT-PCR using frozen tissue samples from 53 young breast cancer patients for whom a follow-up of 10 years was available. We contributed to the study through the analysis of the expression levels of CAF-1 p60, a validated proliferation marker in breast cancer (Polo et al, 2004), and through our input in discussions and the writing.

Results

Although all analyzed proliferation markers, among which CAF-1 p60 and p150, correlated with each other, proliferation rates were not significantly associated with a higher risk of recurrence in these patients. This intriguing finding could be due to the biased selection of the samples: since all samples were of grade 3 and thus highly proliferative, the proliferation status was of course less discriminative among the samples. Alternatively, it is also possible that the disease outcome in young patients depends less on cell proliferation rates than it does in older patients.

Among the genes related to hormone response, three were significantly associated with an increased locoregional recurrence rate in univariate analysis: low aromatase expression, low estrogen receptor beta expression and high GATA3 expression.

GATA3 is a transcription factor, involved in human growth and differentiation, which is highly expressed in the luminal subtypes of breast cancer (reviewed in Kouros-Mehr et al, 2008). Expression of GATA3 is often associated with expression of estrogen receptor α and might thus depend on the presence of estrogens.

The cellular function of the estrogen receptor β remains poorly understood. Yet, its protective effect in breast cancer has been demonstrated before (reviewed in Green et al, 2008) and our study demonstrated that this might be particularly the case in young patients.

Aromatase is an enzyme that converts androgens into estrogen, which is often found upregulated in breast carcinomas. However, estrogens can repress aromatase transcription (Galmiche et al, 2006; Wang et al, 2008b). Thus, low aromatase mRNA levels in our study may be due to the high estrogen levels found in premenopausal women and therefore associate with poor outcome.

In multivariate analysis, only low expression of aromatase was significantly associated with increased locoregional recurrence.

In conclusion, the hormone status seems to be of major importance for the prognosis in young breast cancer patients. This is reflected by the expression levels of hormone-related genes (aromatase, estrogen receptor beta and GATA3), which could serve as additional prognostic markers in these young patients.

Is tumour aromatase expression prognostic for local control in young breast cancer patients after breast-conserving treatment?

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Abstract

This study was designed to determine whether the levels of tumour expression of 17 candidate genes directly or indirectly involved in hormone and growth factors signaling could be associated with locoregional control after breast-conserving treatment of early-stage breast cancer in young women. Gene expression was measured using RT-PCR in 53 young (<40 years) premenopausal patients. All treatments consisted of primary breast-conserving surgery followed by whole-breast radiotherapy (+/- regional lymph nodes) with or without systemic therapy (chemotherapy +/- hormone therapy). The median follow-up was 10 years. The 10-year locoregional recurrence rate was 70% (95% CI: 57%-87%). In univariate analysis, no clinicopathological prognostic factor was found to be significantly associated with locoregional control. Tumour expression of three genes was found to be significantly associated with an increased locoregional recurrence rate: low ERβ, low aromatase, high GATA3. In multivariate analysis, only the absence of aromatase was significantly associated with an increased locoregional recurrence rate (p=0.003, RR=0.49, 95% CI [0.29-0.82]). These results highlight the role of estrogen signalling pathways (mainly CYP19/aromatase, GATA3 and ERβ) in the risk of locoregional recurrence of breast cancer in young women, but need to be confirmed by larger prospective studies.

Key Words: breast cancer, aromatase, GATA3, breast-conserving treatment, prognostic factor, locoregional control.

Introduction

Breast-conserving therapy is the preferred treatment for patients with early-stage breast cancer (Temple *et al*, 2006). It offers equal local control and overall survival (Clarke *et al*, 2005) as well as superior psychosocial outcomes compared with modified radical mastectomy (Engel *et al*, 2004; Moyer, 1997). Locoregional recurrences can be traumatizing and even fatal despite aggressive therapies (Clarke *et al*, 2005). Young age is generally considered to be the most important risk factor for locoregional recurrence after breast-conserving treatments (Bollet et al, 2007b; Oh et al, 2006c; Vrieling et al, 2003). This higher risk, which is not yet understood despite numerous studies, could find its explanation in tumour biology.

The hormonal environment, with menopause as its archetype epitome, is the major physiological difference between younger and older patients. Estrogens are not only the main regulators of growth and differentiation in the normal mammary gland, but also play a major role in the onset and progression of breast cancer (Chen *et al*, 2008; Pike *et al*, 1993) (reviewed by Yager (Yager & Davidson, 2006)). Other signalling pathways, not directly related to estrogen receptors (ER), are also involved in the growth of epithelial tissues.

In pre-menopausal breast cancer patients, little is known about the expression levels of genes that are directly or indirectly involved in hormone (especially ER) and growth factors signalling pathways. The aim of this study, conducted in a series of women diagnosed with invasive breast cancers before the age of 40, was therefore to determine the relationship between locoregional relapse, classical biopathological factors and the levels of gene expression of 17 hormone receptors, growth factor receptors, or proliferation genes: ERα, ERβ, progesterone receptor (PR), nuclear receptor co-repressor (NCoR), nuclear receptor co-activator 3 (NCoA3/AIB1), aromatase (CYP19), GATA binding protein 3 (GATA3), human epidermal receptor (HER) 1 to 4, insulin-like growth factor 1-receptor (IGF1R), antigen identified by monoclonal antibody ki-67 (MKI67), Cyclin E1 (CCNE1), Cyclin E2 (CCNE1), S-phase kinase-associated protein 2 (SKP2) and the two subunits of chromatin assembly factor 1 (CAF-1 p150, CAF-1 p60). Quantitative reverse-transcriptase Polymerase Chain Reaction (RT-PCR) was chosen, as it is the most precise method to measure absolute levels of expression of selected target genes within a wide range, from very high to very low transcript levels (Larionov *et al*, 2005; Nolan *et al*, 2006).

Patients and Methods

Patients and tissue specimens

Between 1988 and 1999, 257 premenopausal women, younger than 40 years old, with no previous history of cancer were treated at the Institut Curie for early unilateral breast cancers. The present retrospective study was based on 53 of these 257 patients for whom frozen tumour tissue was available. Median age at diagnosis was 37 years (range: 23-40) with 30% of patients (16 patients) no older than 36. Median follow-up was 10 years (range: 2-18).

Patient and tumour characteristics are reported in Table 1. Clinical stage (Sobin & Wittekind, 2002) was either T1 or T2, N0 or N1.

All specimens were reviewed by the same pathologist (BSZ). Histological classification of the infiltrating carcinomas was reported according to the World Health Organisation criteria and histological grade was reported according to Ellis and Elston (Elston & Ellis, 1991). The median number of mitoses, calculated per 10 high power fields (Vincent-Salomon *et al*, 2004) was 13 (2-120). Hormone receptors (HR) were positive when nuclear staining for either estradiol receptors (ER) or progesterone receptors (PR) was observed in at least 10% of invasive cells by immunohistochemistry (Balaton *et al*, 1996). No pathological axillary lymph node involvement was observed in 31 patients (58%).

Treatments

Surgery consisted of breast-conserving procedures as first-line treatment in all cases. The quality of the surgical margins was classified as wide (>3mm) in 32 patients (65%), close (≤3mm) in 11 patients (22%), involved with ductal carcinoma in-situ in 2 patients (4%), involved with invasive carcinoma in 4 patients (8%) and unknown in 4 patients. Axillary lymph node dissection was performed in all patients.

Patients received post-tumourectomy radiotherapy with a median dose of 51Gy (range: 45-54) to the breast. A boost to the tumour bed was performed for 75% (40 patients) of women with a median dose of 16 Gy (range: 7-25). The median total dose to the tumour bed was 66 Gy (range: 50-75). Supraclavicular irradiation was performed in

51% (27 patients) of women. Internal mammary irradiation was performed in 72% (38 patients) of women. In the case of lymph node involvement, the internal mammary chain and the supraclavicular area were both irradiated. In the absence of lymph node involvement, irradiation of the internal mammary chain, with or without the supraclavicular area, was indicated for centrally located tumours with histopathological features of aggressiveness. Axillary irradiation was added in the presence of extensive axillary involvement or in the absence of axillary lymph node dissection.

No protocol to boost all young patients with negative surgical margins was available at that time and some patients reported in this series were accrued in the EORTC boost trial that randomised between boost and no boost from 1989 to 1996 (Antonini *et al*, 2006; Bartelink *et al*, 2001; Vrieling *et al*, 2003). In the case of positive surgical margins, a radiotherapy boost of generally 20-28 Gy was added to the whole-breast irradiation. For patients not participating in the EORTC randomised trial, a boost of 10-16 Gy was added in the case of aggressive histopathological features (unsatisfactory margins, high histopathological grade, high proliferation index, absence of hormone receptors).

The reasons for absence of re-excision were not always specified, but when they were specified the reasons were the patient's choice not to undergo a new surgical procedure that could have been mastectomy.

No systemic therapy was administered in 16 patients (30%). Chemotherapy only was given to 23 patients (43%) consisting of anthracycline-based combination chemotherapy in most cases (usually 6 cycles of 5-fluorouracil, doxorubicin, cyclophosphamide). A combination of chemotherapy and hormone therapy (tamoxifen for 5 years) was administered to 14 patients (26%).

RNA isolation, RT-PCR

Total RNA was extracted from frozen tumour samples and first strand cDNA synthesis was performed as previously described from 1µg of total RNA using Superscript II RT (Invitrogen) (de Cremoux *et al*, 2004). ERα, ERß, PR, HER1 to HER4, MKi67, Cyclin E1 and E2, GATA3, IGF1-R, NCor, NCoA3, CYP19 (aromatase gene), SKP2 and CAF-1 p150, CAF-1 p60 transcripts were quantified using real-time quantitative reverse transcription-PCR assays. The nucleotide and probe sequences and the conditions of PCR have been described previously for ERα, ERß, PR, HER1 to HER4,

MKi67, Cyclin E1 and E2, NCor, NCoA3, CYP19 (de Cremoux *et al*, 2004; de Cremoux *et al*, 2007). GATA3, IGF1R were quantified by Assays-on-Demand from Applied Biosystems (Applied Biosystems Inc., Forster City, CA). The nucleotide and probe sequences SKP2 and CAF-1 were chosen with the help of Primer express software (Applied Biosystems Inc., Forster City, CA,). TBP (TATA box Binding Protein) was used as the endogenous reference gene for acute quantification of transcripts. Searches were conducted in dbEST, htgs and nr databases (http://blast.ncbi.nlm.nih.gov) to confirm the total gene specificity of nucleotide sequences. Primers were placed at the junction between two exons. All PCR were performed in duplicate using ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems) and the Core reagent kit (Eurogentec). A 5 µL diluted sample of cDNA (12.5ng) was added to 20 µL of the PCR mix. The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min, and 45 cycles at 95°C for 15 s and either 60°C or 65°C depending of the target for 1 min.

Results were expressed as N-fold differences in target gene expression relative to a reference gene defined as "N target" (Arbitrary Units) and was determined as follows: $N\ target = E_{target}^{(Ct\ calibrator\ -\ Ct\ sample)} / E_{reference\ gene}^{(Ct\ calibrator\ -\ Ct\ sample)}$, where E is the efficiency of PCR measured using the slope of the calibration curve, and Ct is the cycle threshold.

Statistical analysis

Survival rates, defined from the date of surgery, were estimated using the Kaplan-Meier estimate - groups were compared using the Log rank test. Local relapses were defined as the occurrence of breast carcinoma (either invasive or ductal carcinoma in situ) in the treated breast. Locoregional relapses were defined as either a local relapse or a recurrence in the ipsilateral lymph node areas (axillary, internal mammary, supraclavicular). Contralateral breast cancers could be either ductal in situ or infiltrating carcinoma. Distant disease was defined as disease occurring elsewhere than in the contralateral breast or locoregional site. Disease-free was defined as the absence of locoregional and/or distant relapses. Survival rates and relative risks (RR) are presented with their 95% confidence intervals (CI). Annual risks were calculated and plotted. The RT-PCR series of 53 patients was compared with the whole series of all consecutive patients treated over the same period (1988-1999) at the Institut Curie (257 pts) after exclusion of the RT-PCR patients. Fisher's exact test or Chi-square test

were used to compare percentages, as appropriate. Univariate analyses were performed to identify prognostic factors of outcome and estimate crude relative risks (RR). The influence of each factor, adjusted to the others, was assessed in a multivariate analysis by the Cox proportional hazards model (Cox & Oakes, 1984). A stepwise modelling algorithm was used, with a limit of significance of 0.10 for entering and 0.05 for removing risk factors. The limit of significance was 0.05. Analyses were performed using R software, 2.5.0.

Results

The comparison of our series of patients with unselected, consecutive patients is summarised in Table 1. The patients analysed by RT-PCR had tumours of higher clinical stage, higher histological grade and more lymphovascular invasion, and more often received chemotherapy and a total dose to the tumour bed higher than 60 Gy. All other patient, tumour and treatment characteristics were similar.

Fifteen locoregional recurrences occurred (13 only local, 2 local and regional). Five-year and 10-year locoregional control rates were 82% (95% CI: 72%-93%) and 70% (95% CI: 57%-87%), respectively. All local recurrences occurred in the same quadrant as the primary tumour. Treatment of the local recurrence included salvage mastectomy with or without systemic therapy in 11 cases (73%). Three patients (6%) developed contralateral breast cancer. Fifteen patients developed distant metastases. Five-year and 10-year distant disease-free survival rates were 82% (95% CI: 72%-93%) and 69% (95% CI: 56%-85%), respectively. Five-year and 10-year overall survival rates were 85% (95% CI: 76%-95%) and 78% (95% CI: 65%-92%), respectively. No patient developed a second cancer.

Because regional recurrence was always accompanied by local recurrence, risk factors for local and locoregional recurrence were the same (data not shown). In univariate analysis, no clinical/pathological prognostic factor was found to be significantly associated with decreased locoregional control (Table 2). In univariate analysis, three gene expression levels were found to be significantly associated with an increased locoregional recurrence rate: low estrogen receptor beta, low aromatase, high

GATA3 (Table 3). Two other factors were only associated with a trend ($p<0.10$): low HER1 and SKP2. Multivariate analysis was performed (Table 4), taking the factors associated ($p<0.10$) with locoregional recurrence into account in a Cox model , i.e. 5 RT-PCR features (Estrogen receptor beta, HER1, GATA3, SKP2 and aromatase). Only low tumour expression of aromatase was significantly associated with an increased locoregional recurrence rate ($p=0.003$, Relative Risk=0.49, 95% CI [0.29-0.82]). GATA-3 was associated with a trend towards an increased locoregional recurrence rate ($p=0.06$, Relative Risk=1.49, 95% CI [0.02-2.39].

Discussion

This study was based on a series of 53 young (< 40 years old) premenopausal women with a long (10 years) follow-up treated at the Institut Curie for invasive breast cancer by primary breast-conserving surgery. Given the specificity of the hormonal environment in young patients, genes directly or indirectly involved in hormone and growth factor signalling pathways were analysed. In addition to the usual clinical and histopathological features, the levels of expression of 17 candidate genes for an association with locoregional control were also examined.

To verify that these findings could be extrapolated, we compared the patients' and tumours' characteristics of the 53 patients included in this study with the 257 other patients treated over the same period (1988-1999) at the Institut Curie who met the same inclusion criteria (age, medical history, therapeutic sequence) (Table 1). This comparison showed that this RT-PCR series consisted of patients with tumours with a more advanced clinical stage, higher histological grade and more lymphovascular invasion and who therefore more frequently received chemotherapy and a total dose to the tumour bed higher than 60 Gy. All other patient, tumour and treatment characteristics were comparable. It could be hypothesised that these differences were due, at the time of this study (1988-1999), to technical parameters: tissue samples for frozen storage would be easier to obtain from larger tumours.

In this series of 53 patients, neither clinical nor pathological features were associated with increased locoregional recurrence rates. Very young age, in particular, was not significantly associated with increased locoregional recurrences, in contrast

with a previous study of unselected young patients from the Institut Curie (Bollet et al, 2007b).

RNA expressions of 17 target genes were studied here by RT-PCR for their association with locoregional control. They are involved in hormone signalling, either directly (ERα, ERβ, PR, NCoA3/AIB1, NCoR, GATA3, CYP19/aromatase, SKP2) or indirectly (HER family, IGF1-R) or in proliferation (MKi67, Cyclins E and CAF-1). Classical hormone receptors (namely ERα, PR) have been extensively explored in breast cancers (Yager & Davidson, 2006). In contrast with previously published studies (Holli & Isola, 1997), a high frequency of young women in this series presented positive hormone receptors (ERα and PR in 79% and 81% of cases by immunohistochemistry, and in 75% and 82% of cases by RT-PCR).

Among the genes activated in response to ER activation (NCoA3/AIB1, NCoR, SKP2, GATA3, CYP19 /aromatase), only the absence of CYP19/aromatase was significantly associated with an increased locoregional recurrence rate (p=0.003, Relative Risk=0.49 95% CI [0.29-0.82]). Given the small size of this series, it is also noteworthy that the level of GATA-3 was associated with a trend towards an increased locoregional recurrence rate (p=0.06, Relative Risk =1.49 [0.02-2.39]). CYP19/aromatase plays a critical role in breast cancer development by converting androgen into estrogens. CYP19/aromatase mRNA and protein have previously been detected in both tumour stroma and parenchymal cells in breast cancer tissue (Miki *et al*, 2007). Previous data have demonstrated that CYP19/aromatase mRNA is correlated with CYP19/aromatase enzymatic activity in cultured breast tumour fibroblasts (Santner *et al*, 1997). The importance of *in situ* estrogen production has been demonstrated in breast carcinoma (Yue *et al*, 1998), where it is higher than in normal breast tissue (Chen *et al*, 1999; Chetrite *et al*, 2000). Inhibition of the aromatase pathway is considered to be clinically useful to reduce progression of breast tumours in postmenopausal women (Smith & Dowsett, 2003). An apparently paradoxical finding was that a low level of CYP19/aromatase transcripts in this population of young, premenopausal patients was significantly associated with an increased locoregional recurrence rate. However, preclinical studies have demonstrated that estrogens or estrogenic compounds repress the transcriptional control of CYP19/aromatase

(Galmiche et al, 2006; Wang et al, 2008b). It can be hypothesized that, in premenopausal women, who already present high plasma estradiol levels, the level of aromatase transcripts may be inversely correlated with the plasma estradiol level (Pauley *et al*, 2000). Low tumour expression of CYP19/aromatase would reflect a high level of circulating estrogen and would therefore be associated with poor outcome. This finding is supported by a study by Zhang et al (Zhang et al, 2003b) in a series of 162 invasive ductal breast carcinomas that showed that aromatase mRNA levels were lower in patients younger than 50 years, with tumours larger than 2 cm and with axillary lymph node involvement (Zhang et al, 2003b).

However, the association between tumour aromatase expression and outcome remains controversial; no consistent correlation between aromatase immunoreactivity, activity or mRNA level and known clinicopathological factors or outcome has been conclusively reported (Evans et al, 1993; Miller et al, 1982; Salhab et al, 2006; Sasano et al, 1996; Silva et al, 1989; Zhang et al, 2003b). Some authors have even reported, in univariate analysis, a correlation between high tumour aromatase activity and poor outcome (Salhab *et al*, 2006; Silva *et al*, 1989).

The association between tumour aromatase activity or expression and estrogen receptor is also very controversial, as some authors have reported a positive correlation (Miller et al, 1982; Salhab et al, 2006; Zhang et al, 2003b), while others have reported no correlation (Silva *et al*, 1989), or even an inverse correlation (Sasano *et al*, 1996). In the present series, no correlation was observed between aromatase expression and either age, hormone receptors or histological grade (data not shown).

GATA3 is a transcription factor involved in human growth and differentiation. Gene expression profiling has shown that GATA3 is highly expressed in luminal A and B subtypes of cancer and closely related to ERα (Bertucci *et al*, 2000; Sorlie *et al*, 2001; Sorlie *et al*, 2003). This study, the first to our knowledge to use quantitative RT-PCR in a population of young patients, showed a trend towards an association between a high level of GATA3 expression with higher locoregional recurrence rates. The favourable outcome in very young patients with ER-positive breast cancers is a controversial issue. Aebi *et al* reported a series of 3,500 premenopausal women treated in four randomised trials from the International Breast Cancer Study Group (IBCSG) (Aebi *et al*, 2000). They

found that younger patients with ER-positive tumours had a significantly poorer disease-free survival than younger patients with ER-negative tumours.

In addition, conflicting data have been published regarding the independent prognostic value of GATA3 in unselected breast cancer. Some studies have found GATA3 to be associated with good prognosis (Mehra *et al*, 2005; Oh *et al*, 2006a). The study by Voduc *et al* found no independent prognostic value of GATA3 in 3,119 breast cancer patients by immunohistochemistry on tissue microarrays (Voduc *et al*, 2008).

ERβ expression in breast (normal and tumour) and the relationship between ERβ and other clinicopathological features and its role in hormone therapy have been extensively investigated (recently reviewed by Zhao et al (Zhao *et al*, 2008)). A consensus seems to have been reached regarding the protective role of ERβ against breast cancer development. No clinically validated cut-off has been defined for ERβ; transcript levels are generally lower in tumour tissue than in normal tissue. The loss of ERβ expression by promoter methylation, frequently observed in breast tumours, has led to the hypothesis that ERβ is a possible tumour suppressor gene (Garinis *et al*, 2002). The ERβ transcript level in breast cancers has been analysed in a recent study by Anders *et al*. (Anders *et al*, 2008b). Using clinically annotated microarray data from 200 early-stage breast cancers in women, they observed that the ERβ transcript level was statistically lower in young women (< 45 years) than in older women (> 65 years, p=0.02). As expected from the above data, the present study confirmed the protective effect of ERβ in terms of locoregional control.

In younger women with breast cancer, a higher incidence of growth factor receptors, namely HER2, has been observed both in terms of protein expression, associated with a more aggressive phenotype (Agrup *et al*, 2000; Hartley *et al*, 2006) and HER2 gene expression with no predictive value for DFS (Anders *et al*, 2008b).

Interestingly, the HER family and IGF1R have been shown to be intertwined with the estrogen-mediated signalling pathway (Hamelers & Steenbergh, 2003).

Hormone receptors and growth factor receptors act as mitogens, promoting cell proliferation in normal tissue and in breast carcinomas. There is strong evidence that IGF1, HER and estrogen-mediated signalling are closely connected (Hayashi *et al*, 2003). HER1 (or EGFR) has been described as both a prognostic marker and a predictor of

hormone therapy resistance in breast cancer (Nicholson *et al*, 2003). However, downregulation of EGFR has never been explored. The present study showed a trend towards an impact of low EGFR expression on locoregional recurrence in young women. No other effect was observed for any of the other growth factor receptors.

Proliferation markers, included in the definition of histological grading (Elston & Ellis, 1991), are also prognostic factors associated with poor outcome and high locoregional recurrence rates (Elkhuizen *et al*, 1999; Vrieling *et al*, 2003), and predictive factors of the response to chemotherapy (Andre *et al*, 2005; Vincent-Salomon *et al*, 2004). The levels of gene expression of two subunits of CAF-1 (p150 and p60) (Polo *et al*, 2004) and other proliferation markers (the classical KI67, Cyclins E1 and E2 and SKP2) were well correlated in the present study (data not shown) but were not associated with a higher risk of locoregional recurrence. One explanation could be a lack of statistical power, as most patients (68%) in this series had grade 3 tumours.

The search for a signature associated with local recurrence for premenopausal women treated by breast-conserving therapies using high throughput gene expression analyses is ongoing (Kreike *et al*, 2006; Niméus *et al*, 2006; Nuyten *et al*, 2006) and will permit to validate and extent our results.

Conclusion

The present results highlight the role of estrogen signalling pathways, mainly CYP19/aromatase, GATA3 and ERβ in the risk of recurrence in young women with breast cancer. These results need to be confirmed in a larger prospective study. One hypothesis would be that the higher the level of circulatory estrogen, the higher the risk of locoregional recurrence, while low tumour expression of aromatase and high tumour expression of GATA3 would only reflect the high plasma estrogen levels. However, recent data also give credit to the fact that breast cancer arising in young women is a distinct biological entity driven by specific oncogenic pathways (Anders *et al*, 2008a; Anders *et al*, 2008b). This could imply that what is true for women younger than 40 years does not apply to older women.

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Table 1: Patient characteristics of the rt-PCR series (53 patients) in comparison to the whole population of consecutive patients with the same selection criteria treated over the same period (1988-1999) at the Institut Curie (257 patients)

	RT-PCR series		Whole series		
Total	N	%	N	%	p
Family history of breast cancer (MD = 0 and 1)*					0.77
Without	42	79	198	77	
With	11	21	58	23	
Age (MD = 0 and 0)*					
Median (min-max) in years	37 (23-40)		37 (23-40)		0.71
≤ 35 years old	37	70	176	68	0.85
> 35 years old	16	30	81	32	
Clinical T stage (MD = 0 and 0)*					0.0496
cT0-1	31	58	186	72	
cT2	22	42	71	28	
Clinical N stage (MD = 0 and 0)*					0.70
N0	52	98	246	96	
N1	1	2	11	4	
Type of invasive carcinoma (MD = 0 and 0)*					0.18$
Ductal	46	87	223	87	
Lobular	6	11	16	6	
Other	1	2	18	7	
Histological grade (MD = 0 and 1)*					0.03£
1-2	14	26	110	43	
3	36	68	120	47	
Unclassifiable	3	6	26	10	
Estrogen receptor (ER) (MD= 6 and 37)*					0.59
ER-	10	21	55	25	
ER+	37	79	165	75	
Progesterone receptor (PR) (MD = 6 and 39)*					0.84
PR-	9	19	39	18	
PR+	38	81	179	82	
Hormone receptors (HR) (MD = 6 and 37)*					0.87
HR-	7	15	35	16	
HR+	40	85	185	84	
Lymphovascular involvement (MD= 3 and 43)*					0.0004
Absent	33	66	188	88	
Present	17	34	26	12	
Histological T stage (MD = 5 and 12)*					0.89
pT1	34	71	176	72	
pT2	14	29	69	28	
Histological N stage (MD = 6 and 34)*					0.06
pN0	31	58	182	73	
pN1-3	16	42	41	27	

Surgical Margins (MD= 4 and 10)*					0.10
Satisfactory (≥ 3mm)	32	65	129	52	
Unsatisfactory	17	35	118	48	
Systemic therapy (MD = 0 and 0)*					0.0001
None	16	30	151	59	
Hormone therapy (HT) only	0	0	101	39	
Chemotherapy +/- HT	37	70	5	2	
Total RT dose (MD = and 0)*					
Median (min-max) in Gy	66 (50-75)		64 (0-80)		0.14
< 60 Gy	13	25	101	39	0.043
≥ 60 Gy	40	75	156	61	

MD missing data (both in the rt-PCR and in the whole series)
$ 0.37 when the comparison excluded other histological types than ductal or lobular
£ 0.05 when the comparison included all histological grades
Total RT dose: total radiotherapy dose to the tumour bed (whole breast radiotherapy dose + boost dose)

Table 2: Univariate analysis of clinical and histopathological prognostic factors for locoregional control (LRC)

	rt-PCR series		
	10-year LRC (%) [95%CI]	p	RR [95% CI]
Family history of breast cancer		1.0	
No	70 [56-87]		1
Yes	NA		1 [0.22-4.59]
Age (continuous variable)		0.38	0.94 [0.83-1.07]
Age (dummy variable)		0.57	
> 35	73 [59-90]		1
≤35	60 [32-100]		1.37 [0.47-4.0]
Clinical tumour stage		0.71	
cT1	70 [53-91]		1
cT2	74 [56-96]		0.81 [0.28-2.39]
Histological T stage		0.55	
pT1	70 [54-91]		1
pT2	70 [49-100]		0.71 [0.24-2.13]
Surgical Margin		0.70	
≥ 3mm	61 [43-86]		1
< 3mm	81 [64-100]		0.81 [0.27-2.37]
Lymphovascular invasion		0.78	
Absent	71 [56-91]		1
Present	71 [50-100]		1.18 [0.36-3.87]
Histological N stage		0.54	
pN0	63 [47-84]		1
pN1	78 [53-100]		0.49 [0.13-1.77]
pN2	80 [52-100]		0.77 [0.17-3.52]
Histological type		0.14	
Ductal	75 [63-90]		1
Lobular	33 [7-100]		2.61 [0.72-9.53]
Estrogen receptors (ER)		0.14	
ER-	90 [73-100]		1
ER+	63 [47-85]		4.28 [0.55-33.27]
Progesterone receptors (PR)		0.84	
PR-	71 [43-100]		1
PR+	69 [53-88]		1.18 [0.26-5.27]
Hormone receptors (HR)		0.48	
ER- and PR-	86 [63-100]		1
ER+ or PR+	67 [52-86]		2.1 [0.06-3.72]
Histopathological index		0.59	
Grade 1-2	64 [35-100]		1

Grade 3	73 [59-91]		0.70 [0.39-5.19]
Systemic therapy		0.35	
None	54 [33-86]		1
ChT	85 [70-100]		0.47 [0.15-1.49]
ChT + HT	43 [11-100]		0.48 [0.12-1.90]
Total RT dose *		0.27	
≥60 Gy	76 [59-97]		1
<60 Gy	50 [28-88]		1.8 [0.63-5.15]

NA Not applicable
* total dose of radiotherapy to the tumour bed (whole breast dose + boost dose)
CI: Confidence interval
£ RR is relative risk for locoregional recurrence

Table 3: Univariate analysis of gene expression prognostic factors for locoregional recurrences

Gene expression by quantitative rt-PCR	P	HR (RR £) [95% CI]
Estrogen Receptor alpha	0.15	1.22 [0.90-1.66]
Estrogen Receptor beta	0.04	0.61 [0.39-0.95]
Progesterone Receptor	0.32	1.13 [0.88-1.45]
Human Epidermal Receptor 1	0.10	0.65 [0.37-1.13]
Human Epidermal Receptor 2	0.49	0.89 [0.64-1.25]
Human Epidermal Receptor 3	0.87	1.06 [0.55-2.02]
Human Epidermal Receptor 4	0.14	1.22 [0.91-1.63]
MKI 67	0.56	0.89 [0.61-1.30]
Cyclin E1	0.43	0.81 [0.48-1.36]
Cyclin E2	0.18	0.75 [0.49-1.14]
GATA 3	0.04	1.61 [0.95-2.73]
Insulin Growth Factor 1 Receptor	0.13	1.41 [0.93-2.14]
Nuclear Receptor CoRepressor	0.82	0.90 [0.38-2.15]
Nuclear Receptor CoActivator A3 (NCoA3/AIB1)	0.14	0.51 [0.21-1.22]
CYP19 (Aromatase)	0.003	0.48 [0.28-0.80]
Skp2	0.10	0.52 [0.23-1.17]
CAF-1 p150	1.00	1.00 [0.48-2.12]
CAF-1 p60	0.94	1.00 [0.97-1.03]

£ RR is relative risk for locoregional recurrence.
CI: Confidence Interval

Table 4: Multivariate analysis of prognostic factors for locoregional recurrences

	P	HR £ [95% CI]
HER1 (RT-PCR)	0.72	
GATA3 (RT-PCR)	0.06	1.49 [0.92 – 2.39]
Estrogen receptor beta (RT-PCR)	0.54	
SKP2 (RT-PCR)	0.18	
Aromatase (RT-PCR)	0.003	0.49 [0.29-0.82]

£ RR is relative risk for locoregional recurrence
CI: Confidence Interval