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HSF1 promotes TERRA transcription and telomere protection upon heat shock

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Abstract

"HSF1 promotes TERRA transcription and telomere protection upon heat stress"

In response to metabolic or environmental stress, cells rapidly activate powerful defense mechanisms to prevent the formation and accumulation of toxic protein aggregates. The main orchestrator of this cellular response is HSF1 (Heat Shock Factor 1), a transcription factor involved in the up-regulation of protein-coding genes with protective roles. However, it is now becoming clear, that HSF1 function extends beyond what was previously predicted and that HSF1 can contribute to pericentromeric heterochromatin remodeling and activation as well as to efficiently support malignancy. In this study, we identify subtelomeric DNA as a new genomic target of HSF1 upon heat shock (HS). We show that HSF1 binding to subtelomeric regions plays an essential role in the upregulation of TERRA lncRNAs. We also bring solid evidence that under HS, HSF1 contributes to preserve telomere integrity by significantly limiting telomeric DNA damage accumulation. Altogether, our findings therefore reveal a new direct and essential function of HSF1 in transcription activation of TERRA and in telomere protection upon stress in human cancer cell lines. This work provides new insights into how telomeres are preserved under stressful heat shock conditions and allow us to propose a model where HSF1 may exert its protective function at telomeres via the expression of TERRA lncRNAs. Based on our results and given the important role of HSF1 in tumor development, defining the role of HSF1 with regard to telomere stability in tumor development already emerges as a promising challenge.

Résumé

« HSF1 promeut la transcription des ARNs non-codants télomériques TERRA et participe à la protection des télomères dans les cellules soumises à un stress thermique »

En réponse à un stress métabolique ou environnemental, l'activation instantanée de voies moléculaires puissantes permet aux cellules de prévenir la formation et l'accumulation d'agrégats protéiques toxiques. HSF1 (Heat Shock Factor 1) est le facteur de transcription majeur capable d'orchestrer cette réponse cellulaire et d'induire la synthèse de protéines au rôle protecteur nommées chaperonnes. Cependant, il apparaît clairement aujourd'hui que les fonctions initialement attribuées au facteur HSF1 dépassent son rôle inducteur de protéines chaperonnes. En effet, HSF1 joue un rôle essentiel dans l'activation et le remodelage de régions répétées appartenant à l'hétérochromatine péricentromérique. Au cours de mon travail de thèse, nous avons identifié pour la première fois l'hétérochromatine télomérique comme une nouvelle cible génomique d'HSF1 dans les cellules stressées. Nous avons démontré, que la liaison directe et spécifique d'HSF1 aux régions subtélomériques induisait la surexpression de longs ARNs non codants, connus sous le nom de TERRA, et issus de ces régions. Nous avons également mis en évidence le rôle d'HSF1 dans le maintien de l'intégrité télomérique dans les cellules stressées. Ainsi, le nouveau lien que nous établissons entre un facteur promoteur de tumeurs et des régions importantes pour la survie cellulaire et la stabilité du génome ouvre des perspectives nouvelles et extrêmement prometteuses en cancérologie.

Abbreviations list

53BP1: 53 Binding protein 1

aa: Amino acids

ALT: Alternative Lengthening of Telomeres

ATM: Ataxia Tlangiectasia

Mutated serine/threonine protein kinase

ATR: Ataxia Telangiectasia and Rad3-

related protein

ATRX: Alpha thalassemia mental

Retardation syndrome X linked

BER: Base Excision Repair

CEN: Centromere

ChIP: Chromatin ImmunoPrecipitation

CpG: 5'-Cytosine-Phosphase-Guanine-3'

CST: Ctc1, Stn1, and Ten1 containing

complex

CT: Centromere

CTC1: Conserved Telomere maintenance

Component 1

CTCF: CTC-binding Factor

CTD: Carboxyl-Terminal-Domain

Daxx: Death domain-associated protein

DBD: DNA Binding Domain

DDR: DNA Damage Response

D-loop: Displacement-loop

DNA: DeoxyriboNucleic Acid

DNAPol: DNA Polymerase

DNMTs: DNA Methyl Transferases

DSB: Double Strand Break

dsDNA: double-stranded DNA

ECTR: Extra-Chromosomal Repeats

ERCC1: Excision Repair Cross-

Complementation group1

ESET: ERG-associated protein with SET

domain

EtBr: Ethidium Bromide

FISH: Fluorescence In Situ Hybridization

fL: Femtoliters (10^-15L)

H2A.X-P: Histone variant H2A.X

Phosphorylation

H3 or H4: Histone 3 or histone 4

H3.3: Histone variant 3.3

H3K9Ac: Histone 3 Lysine 9 Acetylation

H3K9me3: Histone 3 Lysine 9 tri-

Methylation

H4K20me3: Histone 4 Lysine 9 tri-

methylation

HAT: Histone Methyl-Transferase

HDAC: Histone DeACetylase

HDM: Histone DeMethylase

HMT/ HMTase: Histone Methyl-

Transferase

hnRNPA1: Heterogeneous nuclear

ribonucleoproteins A1

HP1: Heterochromatin Protein-1

HR: Homologous Recombination

HS: Heat Shock

HSE: Heat Shock Element

HSF: Heat Shock Factors

HSF1: Heat Shock Factor 1

HSP: Heat Shock Proteins

HSR: Heat Shock Response

HSR-1: Heat Shock RNA 1

ICF: Immunodeficiency, Centromere instability, Facial anomalies

IF: Immuno Fluorescence

IgF1R: insulin-like growth factor 1 receptor

IgG: Immunoglobulin G

IGS: InterGenic Spacer

IP: ImmunoPrecipitation

iPS: induced Pluripotent Stem cells

ISG15: Interferon-Stimulated Gene 15

IT: Input

ITSs: Interstitial Telomeric Sequences

KD: Knock Down

Ku70/80 (protein): 'Ku' is derived from the surname of the Japanese patient in which the protein was discovered

lncRNA: long non-coding RNA

LSD1: Lysine Demethylase 1

m7G: 7-Methyl Guanosine

MEFs: Mouse Embryonic Fibroblasts

MLL: Mixed Lineage Leukemia

MRE11: Meiotic REcombination 11

NBS1: Nijmegen Breakage Syndrome 1

NHEJ: Non Homologous End Joining

OAS3: 2'-5'-OligoAdenylate Synthetase 3

ORC: Origin Recognition Complex

P53: Tumor Protein 53

PARP1-2: Poly (ADP-Ribose) Polymerase 1-2

PCT: PeriCenTromere

PDSM: Phosphorylation-dependent Sumoylation Motif

PIP1: POT1 Interacting-Protein

pL: Picoliter (10^-12 L)

POT1: Protection Of Telomeres

pRb: Protein Retinoblastoma

PTM: Post Translational Modifications

RAD21: Radiosensitive-cohesin complex component

RAD50: Radiosensitive-DSB repair protein

RAD51: Radiosensitive-recombinase RecA homologue

RAP1: Ras-proximate-1 or Ras-related protein 1

Rb: Retinoblastoma

RD: Regulatory Domain

RNAPII: RNA Polymerase II

RPA: Replication Protein A

SANT or STN1 domain: Swi3, Ada2, N-Cor, and TFIIIB

SatIII: Satellite III non-coding RNA

SIRT1 : Sirtuin 1

ssDNA: single-stranded DNA

STAT1 : Signal Transducer And Activator Of Transcription 1

SUV39H1/2: SUppressor of Variegation 3-9 homologue

SUV4-20: SUppressor of Variegation 4-20 homologue

TAD: TrAnsactivating Domain

TelRNA: Telomeric RNA

TEN1: TElomeric pathways with STn1

TERC: Telomerase-associated RNA template

TERRA: TElomere Repeats containing RNA

(h)TERT: (human) TElomerase Reverse Transcriptase

TET: Ten-Eleven Translocation

TIF: Telomere dysfunction Induced Foci

TIN2: TRF1-Interacting protein

TMM: Telomere Maintenance Mechanisms

TPE: Telomere Position Effect

TPP1: TIN2-POT1 organizing protein

TRF1/2: Telomeric Repeat binding Factor 1 and 2

T-SCE: Telomere Sister Chromatid Exchange

VCP: Valosin-Containing Protein

WT: Wild-Type

XPF: Xeroderma Pigmentosum Group F-Complementing Protein

INTRODUCTION

Chapter I| Stress and HSF1

I.   Cellular stress and heat shock response (HSR)

Historical discovery of the HSR

The term "Stress" used today as a unifying concept to understand the interaction of organic life with the environment, was coined by the physiologist Hans Seyle, in the forties. As a medical student Seyle observed that patients suffering from different diseases often share identical signs and symptoms. These observations led him later to the discovery and publication of his seminal work "A Syndrome Produced by Diverse Nocuous Agents", in Nature (Seyle 1936). His experiments on large cohorts of rats showed that if the organism is severely damaged by acute nonspecific toxic agents such as exposure to cold, surgical injury, excessive muscular exercise, or intoxications with sublethal doses of diverse drugs, a typical syndrome appears independently of the damaging agent nature or the pharmacological type of the drug employed. Seyle's writings describe stress to be the expression of a general alarm of the organism when suddenly confronted with a critical situation and the whole syndrome as a generalized effort of the organism to adapt.

In the forties, while at the organism level knowledge about the way responses to stress are controlled by hormones was evolving (Charmandari et al. 2005), at the cellular level, stress responses remained unexplored. The scientific community had to wait for nearly 20 years, in 1962, when the Italian scientist Ferruccio Ritossa and colleagues gave the first clear demonstration of environmentally induced changes in genes expression (Ritossa 1962). Ritossa was studying chromosomal "puffs" transcription sites in salivary glands of drosophila larvae. An accidental shifting of the incubator's temperature, led him to the discovery of a heat dependent-rapid and important changes in the pattern of chromosome puffing implicating new genes expression. This cellular response to stress was later introduced by Ritossa as the "Heat Shock Response" (HSR) that he later found to be triggered in other drosophila tissues and not only by HS but also upon exposure to chemicals like Salicylate and 2-4 Dinitrophenol (Ritossa 1996; Myohara & Okada 1988).

Heat shock (HS) was found to result in a rapid induction of new protein species, while the majority of different proteins made before the shock was inhibited, sometimes drastically (Pauli et al. 1992; Sistonen et al. 1994). This was seen in all the different tissues which were examined: salivary glands, brain, Malpighian tubes and wing imaginal discs. Tissières' lab with others, clearly demonstrated that mRNAs produced at heat shock puff sites were translated into, what they called, Heat Stress Proteins (HSPs) (Pauli et al. 1992; Ritossa 1962; Ritossa 1996). Nearly a decade later, HSPs function was elucidated. Indeed many HSPs were shown to function as molecular chaperones, preventing the formation of nonspecific protein aggregates and assisting proteins in the acquisition of their native structures, helping the cell to cope with the induced proteotoxic burden (Bose et al. 1996; Bukau & Horwich 1998). Today HSPs specific synthesis is considered a prominent feature of cells undergoing proteotoxic stress. Together those findings allowed scientists to shed some light on the way stress response was managed at the cellular level and placed HSPs as front row actors in that survival process.

I. 1.   Triggers of the HSR

A variety of stress conditions, including environmental, physicochemical, and physiological factors, are able to induce the Heat Shock response (HSR) as reviewed by Morimoto (Morimoto et al. 1990; Morimoto 1998). Organisms are constantly challenged by ever-changing variables in their environment, including fluctuating nutrient levels, osmotic imbalance, exposure to toxic organic or inorganic molecules and non-optimal temperatures (**Figure 1**). Strikingly, a temperature increase of just a few degrees can rapidly trigger the HSR survival pathway. This reactivity of the cell could be explained by the fact that non-optimal temperature can rapidly induce protein unfolding, unspecific interactions and aggregation. Indeed, protein conformation needs to be flexible to perform their functions in the cell, conferring proteins with a relative instability and sensitivity to environmental cues like heat. Hence, an important accumulation of unfolded/damaged proteins, as a result of stress, can be considered as a signal to the cell to start counter measures. This is even true for organisms living at extreme temperatures like archaea (Rohlin et al. 2005; Richter et al. 2010).
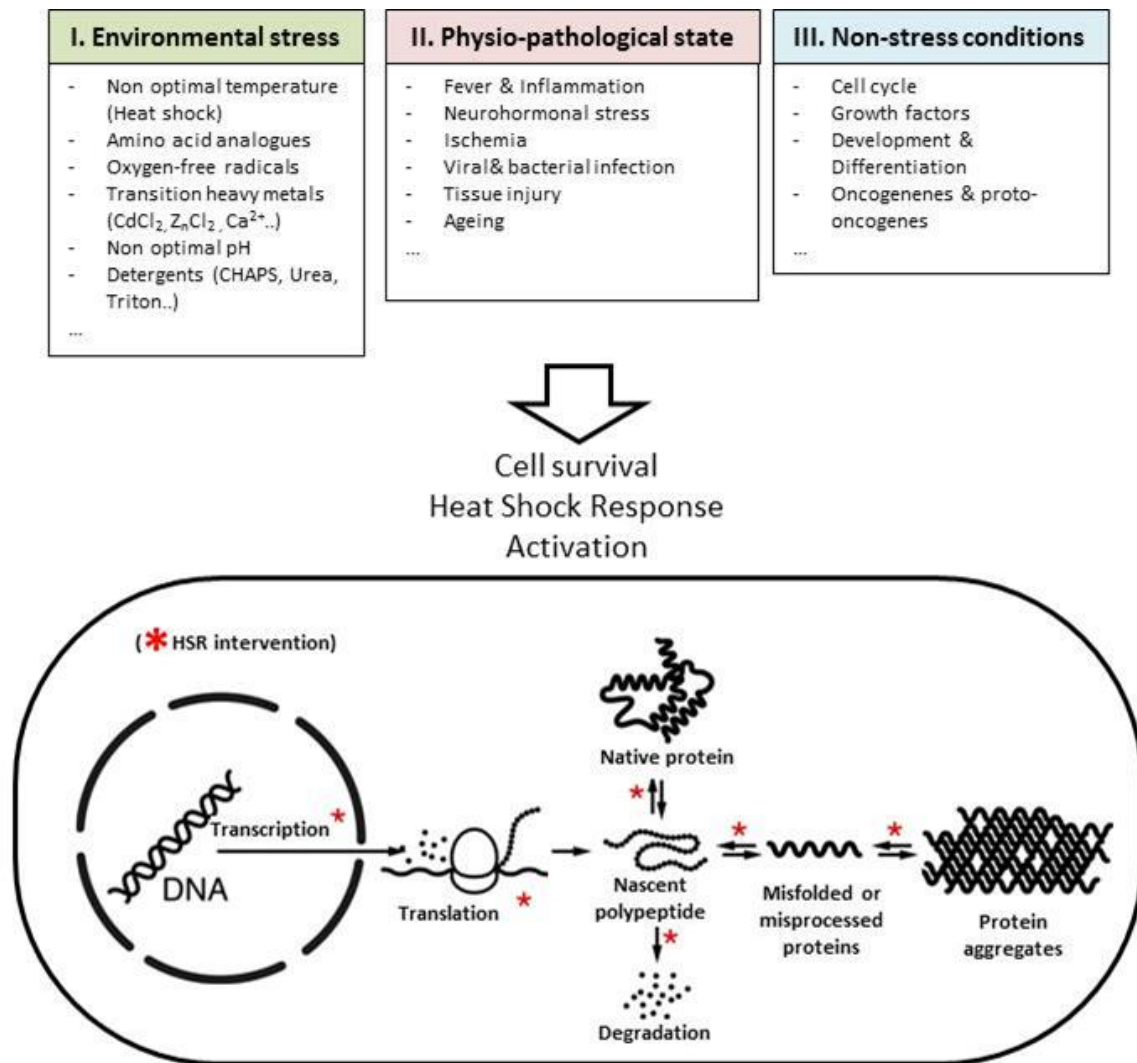
Figure 1| Triggers of the cellular heat shock response (HSR) and role in proteostasis. The HSR is triggered by a plethora of stress signals, including (I) environmental stresses, (II) pathophysiology & disease states, and (III) non-stress conditions. The HSR directly acts on the regulation of protein homeostasis through the upregulation of diverse molecular chaperones. The proteostasis model depicts the 'life of protein' and includes synthesis, folding, processing, degradation, and aggregation of proteins. HSR pathway intervention within the process is denoted with red asterisks (*). The protective role of HSPs is a measure of their capacity to assist in the repair of protein damage. ***Adapted from: Morimoto & Tissières 1990, Morimoto 1998, Sonja 2016***

Besides HS, a great part of the proteotoxic stressors currently used in labs to trigger the cellular HSR are bio-chemical agents such as metal-ions, solvents, detergents, toxic chemicals, heavy metals, amino acid analogs, and various small pharmacologically active molecules. An important step in the field was the observation that many of the known stress proteins were highly expressed in cells and tissues representing a broad distribution of human diseases including ischemia, oxidant injury, cardiac hypertrophy, fever, inflammation, metabolic diseases, neurodegenerative diseases, cancer and cell and tissue damage (Jolly & Morimoto 2000; Richter et al. 2010). In addition, during the natural process of aging, protein

damages accumulation also contributes to the collapse of protein homeostasis and to the HSR activation. These physio-pathological conditions are able to induce the HSR in order to limit and prevent further protein damages by regulating the expression of molecular chaperones and other components of the proteostasis network (Morimoto et al. 1990; Morimoto 1998; Shi et al. 1998).

The continuously evolving list of triggers, from different categories (environmental, physiological, and pathological), all responsible for the deployment of a same evolutionary conserved and efficient HSR pathway, provides us with useful information enabling to complete our understanding of HSR biology and its relevance to diseases of protein conformation.

I. 2.   Deleterious effects of cellular stress

Many of the morphological and phenotypical effects of heat shock can be explained by the aggregation of proteins and an imbalance of protein homeostasis in general. However extensive studies of the molecular impact of HS as well as other stressors demonstrated deleterious effects were multiple and impact the cell internal organization as well as major nuclear processes.

HS has deleterious effects on the cellular organization beyond the unfolding of individual proteins (Richter et al. 2010). Especially in eukaryotes, one of the major damages observed in response to stress conditions are defects of the cytoskeleton. Mild HS leads to the reorganization of actin filaments into stress fibers, while severe HS results in the aggregation of vimentin or other filament-forming proteins, leading to the collapse of intermediary, actin, and tubulin networks (Welch & Suhan 1985; Toivola 2011).

Along with cytoskeleton modifications, the loss of the correct localization of organelles and a breakdown of intracellular transport processes are observed. The Golgi system and the endoplasmic reticulum become fragmented under stress conditions and the number of mitochondria and lysosomes decreases (Welch & Suhan 1985).

Cellular membranes can also be severely affected by HS. Changes in membrane morphology were observed together with changes in the ratio of protein to lipids and a higher fluidity of the membranes (Hofmann 2009). Thus, membrane permeability is enhanced and leads to a drop in cytosolic pH and changes in ion homeostasis (Voellmy & Boellmann 2007).

Nuclear processes such as RNA splicing were shown to be particularly sensitive to heat (Vogel et al. 1995). Recently published transcriptome-wide RNA sequencing analysis of mammalian fibroblast under mild or severe HS treatments, brought evidence for widespread inhibition of splicing, affecting over 1,700 genes, particularly in severe heat shock (Shalgi et al. 2014). Nucleoli, the sites of ribosome assembly, swell, and large granular depositions composed of incorrectly processed ribosomal RNAs and aggregating ribosomal proteins become visible (Welch & Suhan 1985; Boulon et al. 2010).

HS was also shown to affect DNA replication and repair mechanisms in mammalian cancer and primary cell lines. Different DNA damage repair machineries are specifically repressed upon HS like, BER, HR, and NHEJ. A part from inhibition, HS itself is a DNA-damaging factor (Velichko, N. V Petrova, et al. 2012; Velichko et al. 2013). The mechanism by which HS induces double stranded breaks (DSBs) formation remains unclear. Several hypotheses are likely to explain DSBs formation upon HS: the production of reactive oxygen species, an increase in retroelement activity, and the inhibition/damaging of the DNA repair system as well as the consequent slowing down or blocking of endogenous, spontaneously forming DSBs repair (Velichko et al. 2013).

Recent studies demonstrated a dual effect of heat shock on DNA replication and genome integrity. Velichko and colleagues pointed out that "duality" and dug into mechanisms rendering S-phase cells hypersensitive to HS-induced DNA damage. They show that in asynchronous cell population non-S-phase cells (G1 or G2) transiently accumulate numerous H2A.X-P-marked DSBs under HS (Velichko, N. V Petrova, et al. 2012). In contrast, S-phase cells undergo a short DNA replication arrest accompanied by the generation of top1-dependent single stranded DNA breaks (SSBs) upon HS. Moreover they were able to demonstrate that unrepaired SSBs accumulation during replication pausing led to difficult-

to-repair DSBs, finally resulting in a p21-dependent cellular senescence-related G2 arrest (Velichko et al. 2015). These recent findings of highly sensitive S-phase cells to SSBs-inducting agents and in contrast a strong resistance of non-S-phase cells could be an interesting key in the current challenge of arresting highly proliferating cancer cells (**Figure 2**).

Depending on the duration and severity of the HS, the accumulation of defects can result in cell death. Importantly, if HS is not lethal, it may lead to the tolerance of more severe and otherwise fatal stresses. The extent of cellular damages that can be caused by HS requires a consequent and efficient response from the cell in order to survive.
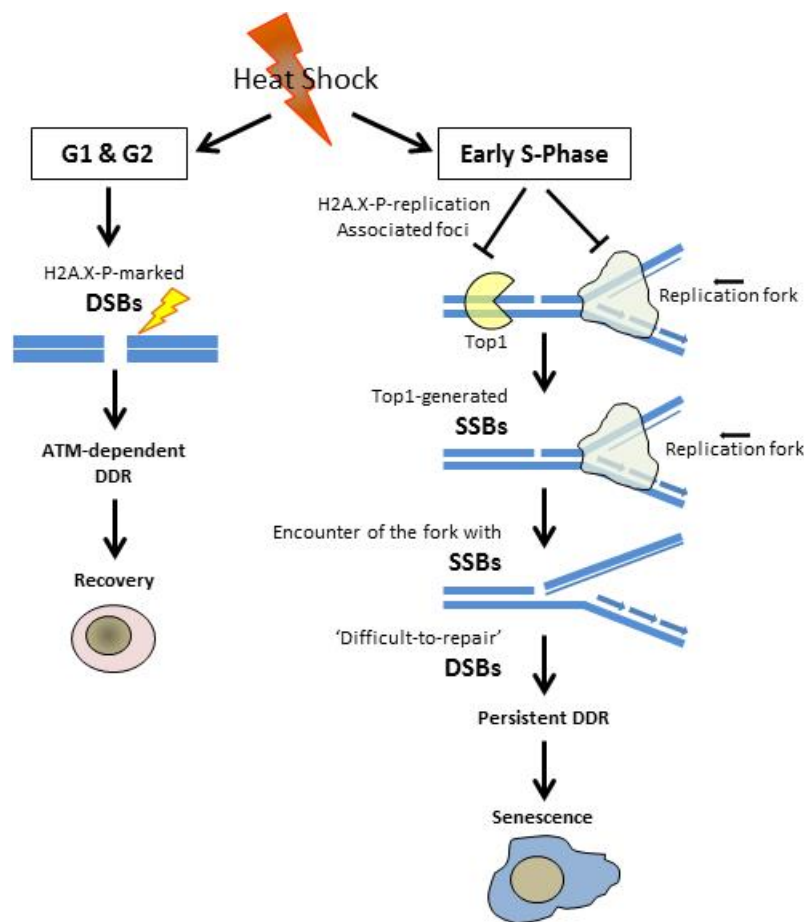
Figure 2| Dual effect of heat shock on DNA replication and genome integrity. Model suggested by Velichko et al (2012, 2015) illustrating the way HS impacts genome integrity. Non-S-phase G1 and G2 phase cells may be subjected to DSBs upon HS exposure, triggering a rapid ATM-dependent DNA damage repair (DDR) pathway that will assure cell survival and thermotolerance. In contrast S-phase cells are hypersensitive to HS-induced SSBs. During DNA replication the encounter of DNA replication forks with topoisomerase I-generated SSBs results in the generation of persistent 'difficult to repair' DSBs, at the origin of heat stress-induced cellular senescence in early S-phase cells. *Adapted from: Velichko et al_2015_NAR.*

I. 3.   Overview of the HSR

The HSR is characterized by an important remodeling of gene transcription pattern and of the cell's epigenetic landscape, which aims to focus the cellular energy and resources on stress recovery. While several groups of genes are induced upon the HSR, others were shown to be importantly down regulated. The transcriptional remodeling was shown to be accompanied by a general slowdown or blockade of various post-transcriptional processes, including splicing and translation. Finally, modifications of mRNAs half-life as well as the induction of several non-coding RNAs were additionally found to play an integral part of the cellular HSR pathway (Mahat et al. 2016; Miozzo et al. 2015).

a/ Heat shock proteins-coding genes activation

Initially, evidence for genes remodeling under HS were mainly focused on *HSPs* genes transcriptional upregulation. *HSP*s strong induction in response to moderate stress conditions is the one of the major keys for cell survival. HSPs function as molecular chaperones; they control protein translation, folding, degradation and in parallel they operate to eliminate damaged/misfolded/aggregated proteins in order to maintain protein homeostasis (Palotai et al. 2008; Kim et al. 2013). The massive need for chaperones upon stress reflects the fact that they are required in stoichiometric ratios relative to the unfolded "client" proteins (Kiefhaber et al. 1991). Interestingly, ''crossprotection'' is possible: HSPs induced by one type of stress provide protection against other stresses (Lindquist 1986). However, it is now well established that, in addition to HSPs, the transcriptional program triggered upon HS modulates numerous protein coding genes.

b/ Modulation of other protein coding genes

Recent genome wide RNA sequencing study consolidated the idea HSPs is only the tip of the iceberg when it comes to genome regulation by the HSR pathway. Mahat et al. revealed that the extent of the HSR is much more pervasive than previously appreciated, with significant upregulation of 10% and downregulation of 55% of all active genes. This regulation is extremely rapid, inducing changes in transcriptional patterns in as little as a minute and a half in mouse embryonic fibroblasts. Notably, in addition to HSPs coding genes, HS-regulated genes were shown to be implicated in various cellular functions such as,

apoptosis, metabolism, cell cycle, mRNA processing and cytoskeletal genes regulation (Mahat et al. 2016).

c/ Upregulation of non-coding genes

Interestingly, the HSR was also shown to modulate non coding regions of the genome. The human and mouse short interspersed elements (SINEs) Alu and B2 retrotransposons (Walters et al. 2009; Pandey et al. 2011) were shown to be upregulated during HS and play a role in global RNAPII transcription thus facilitating gene silencing upon stress. In addition, nucleolar ncRNA molecules derived from large intergenic spacer region (IGS) of the rDNA (Audas et al. 2012), were found to be upregulated and to play a central role in the immobilization of proteins within the nucleolus, thus becoming pivotal elements for the regulation of molecular networks in response to stress. The telomeric non coding RNAs TERRA (Martínez-Guitarte et al. 2008; Blasco & Schoeftner 2008; Eymery et al. 2009) and the pericentric non coding SatIII (Eymery et al. 2009) were also found to be upregulated in various human cell lines as well as other model organisms upon HS. However, the exact function of TERRA and pericentric SatII ncRNAs upregulation upon HS is still unclear.

Together this collection of new data showing a clear correlation between the HSR and the upregulation of non-coding regions of the mammalian genome, adds a supplementary and still rarely explored piece to the mechanism put in place by the cell in order to resist stress insult. Although the exact function of ncRNAs during the HSR is still unclear, one may assume cells can more rapidly respond to stress via these ncRNA quickly induced and potentially capable of regulating major molecular processes.

d/ Epigenetic modifications

Fritah and colleagues contributed to establish that HS rapidly causes global and drastic changes in histone epigenetic marks within the nucleus of HeLa cells. They showed an HS-induced global histone deacetylation and different kinetics of histone reacetylation upon recovery. For example, H4K16 and H3K9 both show rapid de-acetylation upon HS and respectively present reacetylation during the late and early recovery phases (Fritah et al. 2009).

Global deacetylation during HS could be explained by histone deacetylases HDAC1 and 2 activity enhancement since their mutual silencing in HeLa cells abrogated histone deacetylation. Importantly, global histone deacetylation within the nucleus upon HS was suggested to correlate with the general shutdown of transcription. In addition, phosphorylation and methylation histone marks are also altered during the HSR. A reduction in the histone marks H3pS10 and H3K20me3 was observed, whereas an increase in H3K9me2 was detected during the recovery period (Fritah et al. 2009).

Moreover the HSR triggered by HS or other proteotoxic stressors was also shown to modulate the epigenetic pattern of constitutive heterochromatin regions. Indeed upon the HSR, transcription of pericentric Sat III ncRNAs is induced thanks to a HSR-dependent conversion of this heterochromatin structure regions into a euchromatin-like one (Eymery et al. 2010; Biamonti & Vourc'h 2010). At this specific inaccessible locus, the opening of chromatin in response to HS is accompanied by the loss of the heterochromatin protein HP1 and by the recruitment of histone acetylases (HATs), including the transcriptional co-activator protein CBP (Jolly et al. 2004). Recent unpublished data from our lab support histone acetylation will, in turn, direct the recruitment of Bromodomain and Extra-Terminal (BET) proteins BRD2, BRD3, BRD4, which are required for satellite III transcription by RNAP II (Col & Hoghoughi 2016 unpublished data). Much remains to be understood concerning the HSR-dependent activation of these sequences and implicated epigenetic co-factors are likely to be identified soon. To conclude, in order to access and modulate gene expression upon the HSR, cells undergo major epigenetic changes using and coordinating various molecular actors.

Time and technology allowed researchers to gain deeper insights into the complexity of this conserved molecular pathway and to understand that the power of the HSR is its capacity to overturn the cells transcriptome hence to transiently control the cells proteome in response to stress. Past and future discoveries of key molecular actors implicated in the HSR pathway are important milestones in our progression of the field of research.

II.   Heat Shock Factor 1 (HSF1)

II. 1.   Key actor of the cellular HSR

The molecular actors implicated in the HSR pathway, their precise function, partners and conservation were, and still are, highly studied. Very rapidly in the field, the discovery of common DNA motifs in *HSP*s gene promoters led researchers to realize that HSPs expression under stress stimuli was controlled by an evolutionary conserved DNA binding factors proteins, named Heat Shock Factors (Gene & Pelham 1982; Wu et al. 1986; Sistonen et al. 1994). Studies in bacteria including Escherichia coli show that *HSP*s genes are controlled by the heat shock promoter-specific transcription factor, σ32, a subunit of RNA polymerase. σ32 expression is turned on when the bacteria are exposed to heat, σ32 specific binding to RNAP reduces its affinity for nonspecific DNA while increasing specificity for promoters, allowing transcription to initiate at correct sites (Arsène et al. 2000).

In mammals, the principal factor implicated in the molecular HSR was determined to be HSF1 (Heat Stress Factor 1). HSF1 belongs to the mammalian HSF family that counts four different members, HSF1 to 4. Interestingly, each of them possess unique and overlapping functions, a tissue specific-expression pattern and is subjected to multiple post translational modifications. Consistent with this, HSF2 is activated during embryogenesis, spermatogenesis and erythroid differentiation, HSF3 functions as a high temperature activator (in avian), and HSF4 has properties of a negative regulator of heat shock gene expression (Amici et al. 1992; Sistonen et al. 1994; Sarge et al. 1993; Nakai et al. 1997). Finally, HSF1 responds to the classical inducer of the heat shock response. Indeed, among the four mammalian HSFs, HSF1 was highlighted as the master regulator of cells response to stress, (see review: Morimoto 1998; Pirkkala et al. 2001). For instance, studies using mice lacking the HSF1 gene are shown to be unable to elevate HSPs levels in response to thermal insult and display reduced survival after challenge with the bacterial toxin lipopolysaccharide. Furthermore, fibroblasts derived from HSF1−/− mice show no stress-induced transcription of *HSP*s genes and succumb to heat-induced apoptosis, demonstrating that the function of HSF1 cannot be compensated by other HSFs in mammals (McMillan et al. 1998; Morimoto 1998).

Although HSF1 was attributed the functions of HSR major orchestrator, it is noteworthy that recent genome wide transcriptomic analysis in mouse allowed gaining new insights into the HSR regulation pathway (Mahat et al. 2016). Mahat et al. confirm the powerful transcriptome remodeling of hundreds upregulated and thousands downregulated genes during heat shock. Although they confirm HSF1 to be critical for induction of HSPs, other chaperones, and over 200 additional genes during HS, they state that the majority of genes are modulated independently from HSF1 and HSF2. Interestingly, their results strongly suggest SRF (Serum Response factor) as a novel regulator of cytoskeletal genes during HSR. The discovery of new essential transcription factors implicated in the cell response to stress is likely to develop our knowledge and perception of cellular survival mechanisms.

II. 2. Structure

HSF1 protein is composed of five distinguishable functional domains (**Figure 3**). The DNA-binding domain (DBD) is located at the N terminus, whereas the transactivation domain (TAD) resides in the C terminus. Trimerization-dependent activation of HSF1 occurs through an intermolecular interaction of leucine-zipper-like heptad repeat domains (HR-A/B) between HSF1 monomers. Spontaneous trimerization under normal conditions is prevented by another heptad repeat region (HR-C), which facilitates intramolecular interactions between HR-A/B and HR-C domains. A centrally located part of HSF1 called the regulatory domain (RD) in heavily modified by phosphorylation and contains HSF1 phosphorylation-dependent sumoylation motif (PDSM) (Anckar & Sistonen 2011; Budzynski et al. 2015). .

a/ DNA Binding Domain (DBD)

Among the identified HSFs functional domains, the DNA-binding domain (DBD) was shown to be the best preserved domain in evolution and belongs to the family of winged helix-turn-helix DBDs (Damberger et al. 1994; Harrison et al. 1994; Vuister et al. 1994; Littlefield & Nelson 1999). Once the DBD sequence boundaries were mapped in mammals (Wiederrecht et al. 1988), this fragment was shown to be capable of binding HSE sequence specifically (Flick et al. 1994). Because initially HSFs DBD sequences comparisons failed to identify extensive homology to any known DNA-binding motifs, it came as a general surprise when the resolved HSF DBD crystal structure (Damberger et al. 1994; Harrison et al. 1994;

Vuister et al. 1994) was found to be very close to the well-known family of helix-turn-helix DNA-binding motifs.

However, HSF differentiates for other members of the family, in the function of the flexible 'wing' found between the two β-strands following the helix-turn-helix motif. While usually used to contact DNA (Littlefield & Nelson 1999), the HSF structure of the 'wing' does not contact the DNA but instead, it participates in forming the dimer interface between two DBDs bound to the DNA favoring the formation of cooperative interactions between DBDs both within a single trimer and between multiple trimers bound at adjacent HSEs, suggesting HSF has gained the ability to fine-tune the expression of its target genes.
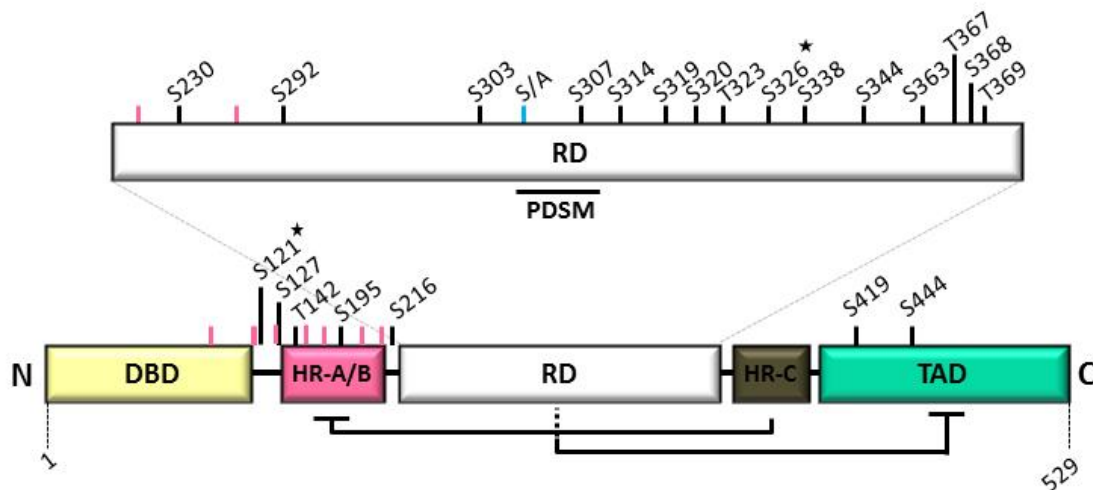
Figure 3| Structural domains of the human heat shock factor 1 (HSF1). Scheme representing, HSF1 functional domains and known sites of post-translational modifications (PTMs). HSF1 DNA binding domain (DBD) encompasses the N-terminal ~100 amino acids and is the best-preserved region within the HSF family and among species. Binding of HSF1 to DNA requires a trimerization step. HSF1 trimerization occurs through interactions between HR-A/B regions and is negatively regulated by intramolecular interactions between the HR-A/B and HR-C domains. The *trans*-activating capacity of HSF1 resides within a C-terminal *trans*-activation domain (TAD), which in turn is negatively regulated by the centrally located regulatory domain (RD). HSF1 protein harbors numerous PTMs sites and its regulatory domain is the most heavily modified. Identified sites for acetylation (in pink), phosphorylation (black) and sumoylation (blue) of HSF1 are indicated, as well as the phosphorylation-dependent sumoylation motif (PDSM). HSF1 S326 (∗) phosphorylation was directly associated to HSF1 activated form. HSF1 S121 (∗) on the other hand was described as a repressive mark of HSF1 under metabolic stress such as glucose deprivation. ***Adapted from: Anckar & Sistonen_2011_Annual.Rev.Biochem, Budzynski et al_2015_MCB.***

b/ Trimerization Domain (HR-A/B)

HSFs trimerization domain (also HR-A and -B), was found to be located on the carboxyterminal side of the DNA-binding domain (Perisic et al. 1989; Littlefield & Nelson

1999), marked by three arrays of hydrophobic heptad repeats several of which are characteristic of helical coiled-coil structures, commonly known as leucine zippers. This region is well conserved among the animal HSFs and poorly conserved in plant and yeast (Wu 1995). HSF multimerization was shown to be essential for a high affinity DNA binding in eukaryotes. Mammalian and drosophila HSFs were shown to be maintained in a latent, monomeric state until the onset of heat stress, when monomers are converted quantitatively into trimers. Various experiments like non-conservative substitutions or deletion of hydrophobic residues in the HR-A/B of vertebrates HSF lead to constitutive trimerization and DNA binding of the mutant protein, indicating that the trimerization region may also participate in maintaining the latent monomeric structure (Westwood & Wu 1993; Sarge et al. 1993; Rabindran et al. 1993; Sistonen et al. 1994).

c/ Regulatory domain (RD)

HSF1 regulatory domain (RD) located at the center of the protein was shown to prevent HSF1 activation in the absence of protein damage, this through the TAD inhibition. Importantly, several studies including a very recent biochemical analysis of HSF1 show that the RD carries a self-sufficient capacity of sensing heat (Green et al. 1995; Newton et al. 1996; Hentze et al. 2016). Moreover, among the different functional domains of HSF1 the RD is the most heavily subjected to PTMs. Under stress the RD can be subjected to modifications such as, hyperphosphorylation, sumoylation, and acetylation. RD repressive ability was suggested to be modulated by its posttranslational signature (Anckar & Sistonen 2011). A recent study support this idea since, they demonstrate that under stress conditions HSF1 mutant harboring a completely dephosphorylated RD will induce higher transcriptional *HSP*s activation compared to WT (Budzyński et al. 2015).

d/ Spontaneous trimerization domain HR-C

In the absence of stress, spontaneous trimerization of HSF1 is suppressed by an additional hydrophobic heptad repeat region, HR-C, located upstream of the RD. The HR-C domain is thought to fold back and interact with the HR-A/B domain to keep HSF1 in an inactive state as introduction of mutations of the HR-C domain allows constitutive HSF1 trimerization and DNA-binding activity (Sorger & Nelson 1989; Wu 1995). In agreement with this, HSFs (yeast HSF1, mammalian HSF4) which contain the HR-A/B domain but lack the HR-

C domain are constitutively trimeric at normal growth temperatures (Rabindran et al. 1993; Nakai et al. 1997). The mechanism by which stress favors HSF1 trimerization and weakens HR-C repression capacity was suggested to imply stress-induced biochemical changes of those specific domains (Hentze et al. 2016).

e/ Transactivation domain (TAD)

HSF1 C-terminal (150 aa) part, globally called the transactivation domain (TAD), is in fact the association of 2 different adjacent modules — Activation Domain AD1 and 2, which are rich in hydrophobic and acidic residues (Newton et al. 1996). HSF1 transactivation is controlled by the RD, which is able to repress both AD1 and 2 and render them heat inducible. HSF1 TAD regulates the magnitude of HSF1 activation and facilitates transcriptional activation of its target genes. Indeed, the HSF1 TAD1 interacts directly with TFIID TATA box-binding protein-associated factor (TAF-9) *in vitro* (Choi et al. 2000), and mutagenesis of the hydrophobic residues markedly impaired HSF1 trans-activating capacity (Newton et al. 1996). *In vitro* transcription assays, TAD1 and TAD2 are able to individually stimulate both transcriptional initiation and elongation. Interestingly, HSF1's TAD was shown to be responsible for the recruitment of BRG1, the ATPase subunit of the chromatin-remodeler SWI/SNF complex, essential for heat inducible chromatin remodeling of *HSP*s genes (Brown et al. 1998; Sullivan et al. 2001; Corey et al. 2003).

II. 3.   Regulation of HSF1

Although the mechanisms involved in HSF1 activation are not fully understood, researchers stipulated there must be several regulation pathways of HSF1. An important point supporting this hypothesis is the great diversity of stimuli capable of inducing the HSR as well as the variety of HSF family members. Data accumulating throughout the years not only proved them right but uncovered an unexpected complexity and diversity for HSF1 regulatory pathways. Indeed, HSF1 activation was shown to be a multistep pathway.

First during HS and other stimuli, a conversion of inert HSF1 monomers undergo conformational change to form trimers that bind to specific DNA sequences. Secondly, transcriptional activation of HSF1 target genes is induced. Interestingly, HSF1 transcriptional

activity was shown to be uncoupled from the protein's trimerization and DNA binding capacity (Cotto et al. 1996) hence, revealing another layer of complexity regarding HSF1 regulation, including post translational modifications of the protein. Basal activity of HSF1 is also regulated in unstressed conditions.

Discovering HSF1 importance in the HSR then raised the question by which efficient mechanisms protein unfolding leads to HSF1 activation? Different labs in the past decade contributed to answer to that fundamental question, uncovering new mechanisms and unsuspected molecular actors. The following paragraphs will describe the molecular mechanisms of activation and repression of HSF1. (**Figure 4**)
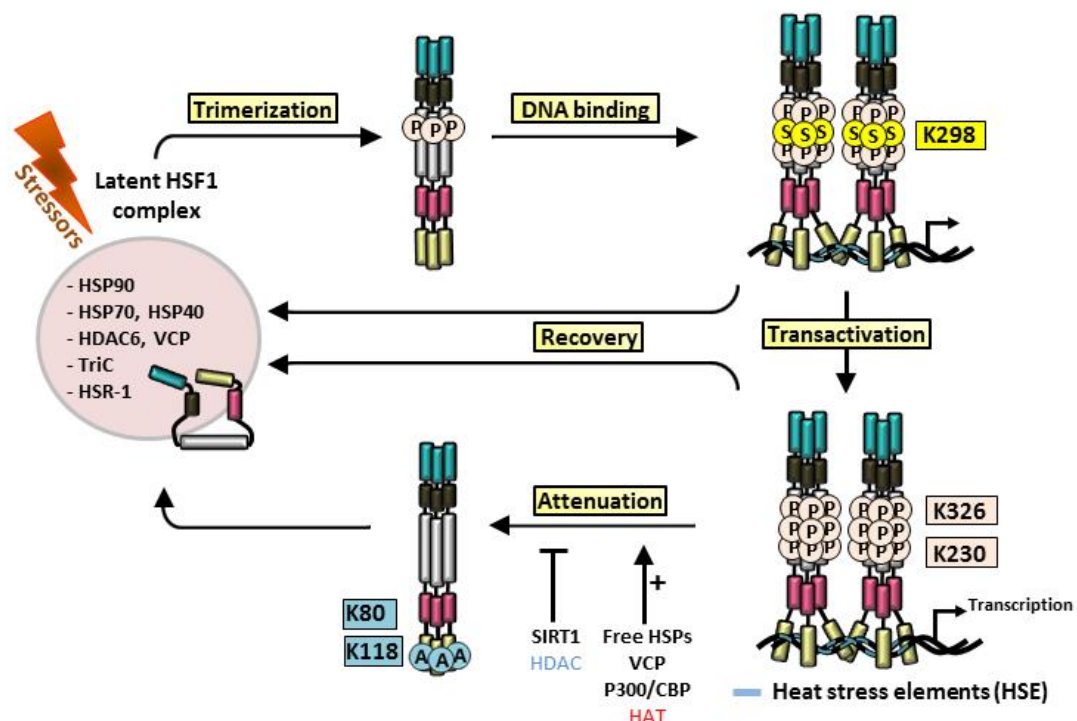
Figure 4| HSF1 activation-attenuation cycle. Simplified resume of known steps implicated in HSF1 activity induction and attenuation cycle. HSF1 latent monomer is complexed to HSPs and other repressive partners including chaperones HSP90, HSP70, HDACs and Chaperonin TriC. Upon stress, HSF1 monomers are allowed to trimerize and to bind DNA through the recognition of HSEs motifs by the TAD. This preliminary step is accompanied by PTMs such as hyperphosphorylation of the RD. Specific phosphorylation and sumoylation events are involved in regulating the transactivation capacity of HSF1. Illustrated, the K298 sumoylation (S) and K326, K230 phosphorylation (P) respectively shown to play a repressive and positive effects. HSF1 induces a transcriptional program including a major upregulation of HSPs. Transcriptional activity of HSF1 is abrogated during the attenuation phase. Attenuation involves two regulatory steps. First, a negative feedback from "unemployed" HSPs, repress the transactivation of DNA-bound HSF1. Secondly, inhibition of HSF1 DNA binding occurs through K80 and K118 acetylation (A) in the DBD of HSF1. The acetylation-dependent attenuation phase of HSF1 was shown to be regulated by the HAT P300/CBP and repressed by the HDAC sirtuin SIRT1. ***Adapted from: Vihervaara & Sistonen_2014_Cell Science***

a/ Activation

- **Temperature**

Since the accidental use of heat induced-stress in D. melanogaster that led to the discovery of Heat shock Proteins and the HSR (1962), heat shock remained one of the favorite models to activate HSF1 and study the heat shock response. Duration and amplitude of the applied HS have to be optimized depending on the cell lines used or the type of tissue in order to simultaneously obtain a measurable HSR and prevent lethality. Moreover, the modulation of the HS conditions directly impacts recovery kinetics of cells. Heat-induced protein damage was shown to be the origin of HSF1 activation under heat insult, suggesting an indirect activation of HSF1 by heat (Lindquist 1986). These findings allowed understanding the function of several proteins implicated in proteostasis, including chaperones, in HSF1 activity regulation (developed below). However, a recent publication consolidates the idea HSF1 protein carries a self-sufficient capacity of sensing heat. Hentze et al. show that two regions of the HSF1 protein that changed shape dramatically when the temperature increased. The RD of HSF1 unfolds, while the region involved in making the trimer (HR-C) becomes more stable thus favoring HSF1 trimeric state. Therefore, HSF1 can directly sense and respond to changes in temperature (Green et al. 1995; Newton et al. 1996; Hentze et al. 2016).

Nevertheless, existing evidence suggest HSF1 activation is not strictly dictated by the absolute temperature, but by a combination of increased temperature and a particular cellular context (Clos et al. 1993; Batulan et al. 2003; Gothard et al. 2003). Indeed, several independent studies of HSF1 heterologous expression between human and drosophila cells revealed HSF1 activation threshold was surprisingly "adapting" to the host cells (Clos et al. 1993). Consistent with this, mammalian cells originated from different tissues show striking differences in HSF1 activation threshold, for example motor neurons will activate HSF1 only after hours of exposure to 42°C HS, while T-lymphocytes are able to induce HSF1 activation starting from almost physiological temperatures (1h, 38°C-39°C) (Batulan et al. 2003; Gothard et al. 2003). This evidence encouraged others to explore auxiliary cellular factors that could regulate HSF1 activation.

- **Implication of Post Translational Modifications (PTMs)**

HSF1 protein uncovers a variety of PTMs sites including acetylation, phosphorylation and sumoylation, and like other transcription factors HSF1 associated-PTMs were shown to contribute to the orchestration of HSF1 protein functions. HSF1 phosphorylation contribution to HSF1 activation was extensively studied. The human HSF1 protein contains various phosphorylation sites including 22 on serine and 4 on threonine (Xu et al. 2012; Anckar & Sistonen 2011). About 70% of HSF1 phosphorylation sites are located to its regulatory domain (RD) (Guettouche et al. 2005) (**Figure 3** and **4**). Although HSF1 is constitutively phosphorylated under non stress conditions as it is the case for S303 and S230 (Kline & Morimoto 1997; Chu et al. 1996), both the yeast HSF and the mammalian HSF1 were shown to undergo hyperphosphorylation event in response to stress stimuli, such as HS and heavy metal exposure (Phosphorylation et al. 1988; Sarge et al. 1993; Budzyński et al. 2015). Indeed, a visible 'shift' in HSF1 molecular mass can be observed upon stress and was shown to be mostly due to phosphorylation, as demonstrated by a decrease in molecular size upon phosphatase treatment (Budzyński et al. 2015).

While in yeast HSF is constitutively bound to DNA and its phosphorylation has been suggested to stimulate transactivation (Phosphorylation et al. 1988) this is not the case in higher eukaryotes, where HSF1 DNA binding occurs in response to stress, and the positive regulation of HSF1 activation by stress-induced phosphorylation or hyperphosphorylation is currently discussed in the field (Mivechi et al. 1994; Guettouche et al. 2005; Budzyński et al. 2015). At the moment, only S326 and S230 were reported to substantially contribute to HSF1 transcriptional activity and are widely used as markers for activated HSF1 (Holmberg et al. 2001; Guettouche et al. 2005; Vihervaara & Sistonen 2014; Dai et al. 2015). A new study by Zijian et al. demonstrates the major MEK/ERK signaling pathway, frequently deregulated in cancer, in HSF1 direct activation via S326 phosphorylation (Tang et al. 2015).

HSF1 was also shown to undergo acetylation, adding another layer of complexity to HSF1 regulatory process (Anckar & Sistonen 2011). Mass spectrometry analyses of HSF1 revealed that numerous lysine (at least 9) were targeted by acetyl groups and in contrast to HSF1 phosphorylation, occurring mostly in the RD, acetylation localize to domains implicated in other fundamental properties of HSF1 activity, such as DNA recognition, oligomerization,

and subcellular localization. HSF1 is not acetylated under normal conditions and stress induced acetylation of HSF1 seems to occur independently from phosphorylation and in later stages of the HSR including the recovery period (Anckar & Sistonen 2011). Different functions were attributed to HSF1 acetylation upon stress induction. For example, K116 and K118 are located in the flexible linker region that connects the HSF1 DBD to the HR-A/B domain, a region affecting HSF1 trimer formation (Liu & Thiele 1999). Moreover, the acetylation on K208 and K224 was shown to be critical for nuclear localization signal, and mutation of either lysine leads to a cytoplasmic accumulation of HSF1 suggesting a role in HSF1 nuclear export (Vujanac et al. 2005).

- **ncRNA**

It is noteworthy, that in 2006 a new and surprising molecular actor was suggested to be implicated in a positive regulation of HSF1 activation in human and rodent cells. The constitutive expression of a previously unknown 600 nt, ncRNA called HSR1 (Heat Stress RNA 1) was shown to be necessary for an *in vivo* DNA binding HSF1 and HSPs production upon hyperthermia and cell survival following lethal heat-shock challenge (Shamovsky et al. 2006). However, a major controversy was created with the recent publication demonstrating the exogenous origin of the HSR1, suggesting it was derived from a bacterial genome fragment either by horizontal gene transfer or by bacterial infection of the cells (Choi 2015). Thus, acknowledging HSR1 as a potential RNA thermometer in eukaryotes should be revised.

b/ Repression

- **Chaperones**

The first HSF1 regulatory pathways exploited were autoregulation feedback mechanisms implicating chaperones and chaperone-like proteins. In the nineties, two labs were able to bring *in vivo* evidence for HSF1 association to different chaperones through its transactivation domain. Five years later, HSP90 and HSP70 chaperones have emerged for their roles as direct repressors of HSF1 activation (Baler et al. 1992; Abravaya et al. 1992; Zou et al. 1998; Shi et al. 1998). HSP90 and HSP90-immunophilin-p23 complexes were suggested to play a repressive role on HSF1 oligomerization and activation through its sequestration in a dynamic heterocomplex prior and through HS (Guo et al. 2001). In

addition, HSP70 and the co-chaperone HSP40 HS dependent-upregulation were suggested to act as a negative feedback loop on HSF1 transactivation capacity (Shi et al. 1998; Voellmy & Boellmann 2007). The chaperone titration model elegantly explains the inactivation of HSF1 in the presence of unemployed chaperones, and its dramatic activation if chaperones are busy due to the presence of unfolded proteins (**Figure 4**)

Other negative feed-back loop mechanisms were described to control HSF1 activity upon stress. The proteins HDAC6 (cytoplasmic Histone DeACetylase 6) together with p97/VCP (Valosin-Containing Protein) were found to be associated to HSF1 in unstressed mammal cells and described as stress sensors. In their publications Boyault et al. demonstrated HDAC6 specific binding to poly-ubiquitinylated misfolded proteins upon MG132-induced HSR as a critic event for triggering HSF1 dissociation form its inactive protein complex (Boyault et al. 2007); while VCP/p97 was found to play an important role in reassembling the inactivating complex during recovery. Together these two new molecular actors contribute to a tight control of the duration of HSF1 activation and thus HSPs production (Pernet et al. 2014).

- **Chaperonin complex named TRIC/CCT**

A repressive and conserved control mechanism of HSF1 upon stress was recently described. The bacterial GroE/L protein folding machinery (Guisbert et al. 2004) and its eukaryotic functional analogue TriC/CCT cytosolic chaperonin complex (Neef et al. 2014) were respectively showed to bind, σ32 and HSF1 directly and to be implicated in their activation control mechanisms. Next, TriC was showed to directly interact with HSF1 *in vitro* and repress HSF1-dependent gene activation *in vivo* human cells. Interestingly parallel studies in *C. elegans* inducing RNAi dependent-inhibition of TriC induced a tissue specific activation of HSF and HS-inducible *HSP70* reporter gene (Guisbert et al. 2013) supporting the conservation of this repressive pathway.

- Post Translational Modifications (PTMs)

Several phosphorylation sites were assimilated to HSF1 repression mechanisms. For example the S121 phosphorylation, targeting HSF1's transactivation domain (TAD), was shown be responsible for HSP90 binding thus contributing to the formation of the repressive

HSF1 complex in the absence of stress. Interestingly the MAPK, central metabolic sensor, was described to directly phosphorylate HSF1 on S121 thus repressing the HSR under metabolic insult such as glucose deprivation (Dai et al. 2015). Finally, HSF1 S303 and S307 phosphorylation were associated with HSF1 nuclear export hence supporting the attenuation phase and repression of HSF1 (Kline & Morimoto 1997; Xu et al. 2012).

Phosphorylation-dependent sumoylation of HSF1 was identified and related to HSF1 activity regulation. SUMO proteins are transiently and covalently bound to specific lysine residues of multiple cellular proteins (Anckar & Sistonen 2007). The only HSF1 sumoylation site (K298) described to date was shown to be dependent of serine S303 phosphorylation and play a repressive role on HSF1. Hence, under stress, S303 induced phosphorylation triggers Ubc9 (SUMO E2 conjugating enzyme) dependent sumoylation of the adjacent lysine K298 (Hietakangas et al. 2003). Both monoacids are located into HSF1 RD domain and more precisely into what was identified as the phosphorylation-dependent sumoylation target motif. SUMO proteins are well-established repressors of transcription (Geiss-friedlander & Melchior 2007), and accordingly, phosphorylation-dependent sumoylation of HSF1 leads to repression of HSF1 transcriptional activity in reporter gene assays and on endogenous target gene promoters (Hietakangas et al. 2006). In cells exposed to a mild heat shock, HSF1 sumoylation is sustained and can be detected even after prolonged heat shock treatments. In contrast, cells exposed to more severe heat shock temperatures display a more transient HSF1 sumoylation, suggesting that the persistence of SUMO on HSF1 functions as a stress-sensitive barrier that restrains HSF1 activity upon moderate stress. Importantly, HSF1 DNA binding under stress seems to occur independently from sumoylation (Hietakangas et al. 2003). Thus, from what is known about sumoylation functions, mechanisms allowing HSF1 sumoylation to lead to repression of HSF1 activity can be based on previously described SUMO function as a mediator of protein-protein contacts (Geiss-friedlander & Melchior 2007). Yet, the speculated SUMO motif-containing transcriptional corepressors of HSF1 are to be identified. Finally, the removal of HSF1 from chromatin during the HSR attenuation phase was shown to be facilitated at least by the acetylation of K80 and K118, an amino acid residue that directly contacts target DNA (Westerheide et al. 2009; Raychaudhuri et al. 2014). The enzymes responsible for HSF1 acetylation and deacetylation were identified to be respectively the HAT P300/CBP or GCN (for K80) and the HDAC sirtuin1 (SIRT1) proteins.

III. HSF1 targets and functions in the HSR

HSF1 protein has been defined for decades by its ability to coordinate chaperone protein expression and to enhance survival in the face of stress stimuli. The next paragraphs will try to give a large overview of the research investigating HSF1 functions, which are clearly broader and deeper than initially imagined (see also **Figure 9**).

III. 1. HSE: Heat Shock Elements

The members of the heat shock factors family are regulators of transcription. HSFs act by binding to repeating arrays of the 5-bp Heat Shock Elements (HSE) sequences nGAAn, present in multiple copies upstream of target genes (Gene & Pelham 1982). The first evidence for HSF binding to HSE upstream of HSPs production was obtained *in vitro* using drosophila heat shocked-nuclear extracts (Wu 1984). In the DNA-bound form of HSF, each DNA-binding domain (DBD) recognizes the HSE in the major groove of the double helix (Wu 1995). HSF1 DNA binding motifs can be found in gene promoters as well as in distal regions (Mendillo et al. 2012) (**Figure 5**).
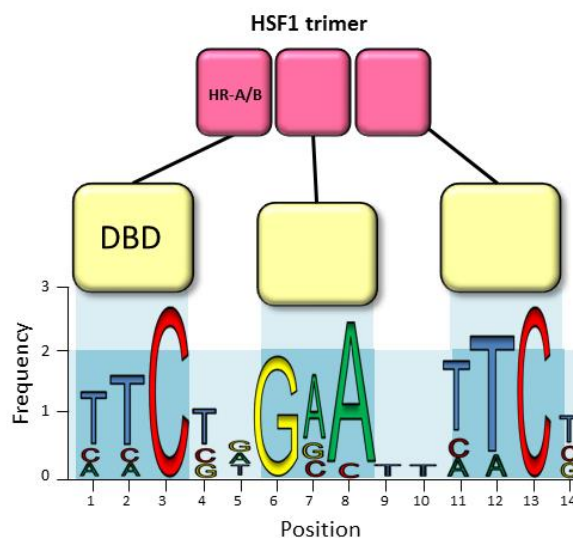
Figure 5| Heat shock element (HSE), HSF1 binding sites. Simplified representation of the human HSE consensus sequence bound by the three DBDs of an HSF1 trimer. Letter height is proportional to the frequency of the corresponding nucleotide. At the target loci, HSF1 binds to cis-acting elements that are composed of inverted nGAAn pentamers and are collectively called heat shock elements. In the DNA-bound form of HSF, each DNA-binding domain (DBD) recognizes an HSE in the major groove of the double helix. The number and exact nucleotide sequence of nGAAn pentamers vary at distinct target loci and contribute to the affinity of HSF1 to the DNA. However, as shown here, guanines and guanines exact spacing is strikingly conserved among HSE and is thought to be a key determinant for recognition by HSFs and transcriptional activation. *Adapted from: Trinklein_2004_Mol Biol cell; Vihervaara & Sistonen_2014_Cell Science.*

The type of HSEs that can be found in the proximal promoter regions of *HSP*s genes is highly conserved and composed of at least three contiguous inverted repeats: nTTCnnGAAnnTTCn (Perisic et al. 1989; Xiao & Lis 1988). The promoters of HSF target genes can also contain more than one HSE, thereby allowing the simultaneous binding of multiple HSFs. The binding of an HSF to an HSE occurs in a cooperative manner, whereby binding of HSF trimer facilitates binding of the next one (Xiao et al. 1991; Littlefield & Nelson 1999).

More recently, Trinklein and colleagues used chromatin immunoprecipitation to enrich sequences bound by HSF1 in heat-shocked human cells to define the HSE consensus sequence. They confirmed the original finding of Xiao and Lis, who identified guanines as the most conserved nucleotides in HSEs. Although there are variations in these HSEs, the spacing and position of the guanines are invariable (Xiao & Lis 1988; Perisic et al. 1989; Xiao et al. 1991; Trinklein et al. 2004). Therefore, both the nucleotides and the exact spacing of the repeated units are considered as key determinants for recognition by HSFs and transcriptional activation.

III. 2. Chaperones

The highly conserved transcriptional activation of HSPs upon proteotoxic stress was shown to be orchestrated by HSF1 in mammals (Wu 1984; Wu et al. 1986; Sistonen et al. 1994). Historically, the study of HS gene transcription has been marked by the discovery that the RNA polymerase Pol II (RNAPII) is preloaded and transcriptionally engaged in the promoter-proximal region of the Drosophila *HSP70* gene prior to HS but paused (Rougvie & Lis 1988; Rasmussen & Lis 1993). This "starting bloc" positioning of the RNAPII provides the cell with a highly reactive transcriptional induction of chaperones upon HS. In addition, heat stress factors from yeast to human play an essential role in *HSPs* transcription through direct recruitment of chromatin remodeling partners to *HSPs* genes. The constitutively active mammalian HSF1 was shown to initiate *HSP70* transcription and promote nucleosomes displacement on the coding region of the *HSP70* gene during the elongation process (Sullivan et al. 2001) (**Figure 6**).
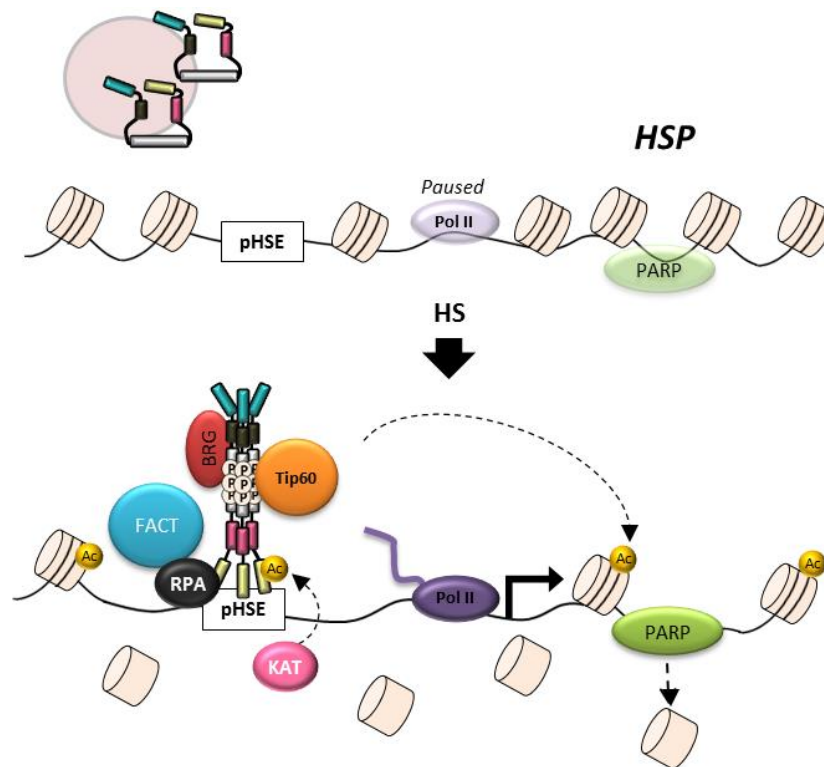
Figure 6| Transcriptional regulation and factor occupancy of a typical *HSP* gene under non-stress and HS conditions. Before HS, the *HSP70* gene contains a transcriptionally engaged and paused RNA Pol II phosphorylated on the CTD Ser5 residue, downstream of the transcription start site. In addition, inactive PARP (Poly (ADP-ribose) polymerase) is located in the vicinity of the transcription start site. Upon HS, activated trimeric HSF1 is recruited to proximal heat stress elements (pHSE) located in *HSP* promoter region; with the assistance of the single-stranded DNA-binding protein replication protein A (RPA), the chromatin-remodeling enzyme Brahma-related gene 1 (BRG1), and the histone chaperone facilitates chromatin transcription (FACT). HSF1 also attracts the HAT Tip60 complex. Tip60-induced histone acetylation triggers the activation and spreading of PARP, which provokes nucleosome loss along the gene. On the other hand, lysine acetyltransferases (KAT) acetylates the DBD and weaken the DBD-DNA interaction during the recovery phase. Altogether factors occupying *HSP* genes collaborate to quickly create a transcriptional compartment in response to stress. ***Adapted from: Miozzo et al_2016_JMB, Fujimoto et al_2012_Mol Cell, Nakai_2016 Nat Struc & Mol Biol.***

HSF1 exerts its transcriptional functions via interaction with several partners such as, BRG1 transcription factor (chromatin remodeling complex SWI/SNF) and the histone acetylating complex Tip60 (Sullivan et al. 2001). HSF1-dependent recruitment of Tip60 to *HSP*s genes results in histone acetylation (H2A5, H4), PARP activation and spreading along the gene which creates and maintains the transcriptional compartment (Jolly et al. 2004). More globally, among HSF1 identified partners found to contribute to *HSP*s induction, the replication protein RPA, which binds and stabilizes single-strand DNA regions during DNA replication and repair (Wold 1997). One mechanism by which the HSF1/RPA1 complex could gain access to and open nucleosomal DNA is by recruiting the histone chaperone FACT,

which displaces the histone H2A–H2B dimer (Fujimoto et al. 2012) These studies reveal the role of the mammalian HSF1 in recruiting and activating chromatin-remodeling enzymes and co-factors on specific genes and in stress situations.

HSF1 induces not only the classical but also non-classical HSPs groups involved in various processes in the normal state and in response to heat shock. The predominant class of molecular chaperones, comprises five major and broadly conserved families-HSP100s, HSP90s, HSP70s, HSP60s, and small heat shock protein (Richter et al. 2010) Several other heat-inducible molecular chaperones, like HSP33 (Kumsta and Jakob, 2009), are known.

Chaperones proteins HSP70s and HSP90s are the most highly conserved and studied for their role in the HSR. Under physiological conditions, HSP70s are involved in the de novo folding of proteins; while under stress they prevent and can even refold aggregated proteins (Mayer & Bukau 2005). The activity of HSP70s is regulated by cofactors. The largest class of Hsp70 cofactors is the group of HSP40 J-domain-containing proteins (Kampinga & Craig 2011). They bind the nonnative proteins and deliver it to HSP70. HSP90 is present at very high concentrations in the cytosol of bacteria and eukaryotic cells under physiological conditions, and it is further upregulated under stress (Baler et al. 1992). This chaperone does not bind unfolded proteins, but rather nativelike proteins (Bose et al. 1996). It constitutes one of the most sophisticated chaperone machinery known in eukaryotes, working together with a large cohort of co-chaperones that associate in a defined order during the chaperone cycle (Pearl & Prodromou 2006; Taipale et al. 2010). Whether the substrate spectrum of HSP90 changes under stress conditions is an important open issue.

The key observation that guided scientists to the discovery of new HSF1 targets genes, beyond *HSP*s genes promoters, was the stress-induced HSF1 localization at specific nuclear foci visibly excluded from *HSP*s genomic foci (Jolly et al. 1997).

III. 3.   Heterochromatin

Surprisingly HSF1 was shown to localize and transcriptionally activate regions of genome classified as stable and transcriptionally inert constitutive pericentric heterochromatin.

a/ Constitutive heterochromatin

The eukaryotic genome is categorized into two major functional states euchromatin and heterochromatin (Passarge 1979 and chapter II.1). Euchromatin corresponds to a rather open and transcriptionally active conformation, while heterochromatin designates a condensed and transcriptionally inert conformation. Heterochromatin has been further classified into facultative and constitutive form. Facultative heterochromatin refers to a type that may form at various chromosomal regions, which usually contain genes that must be kept silent upon developmental cues. In contrast, constitutive heterochromatin is believed to occur at the same genomic regions in every cell type and these regions usually do not contain protein coding genes. In most organisms, constitutive heterochromatin concentrates at pericentric, telomeric, and ribosomal regions, as well as at different loci along the chromosome. In human, centromeres consist mainly of alpha satellites and pericentromeres of chromosome specific satellite repeats, including satellites I, II and III (Saksouk et al. 2015) (**Figure 7**). Historically, pericentric heterochromatin has been viewed as an unvarying and static structure.
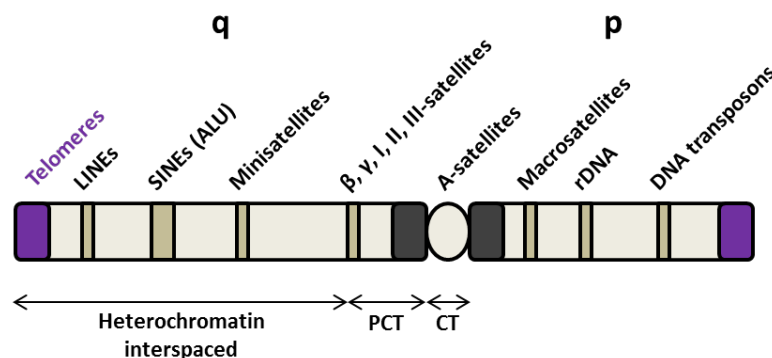
Figure 7| Human constitutive heterochromatin is found at centromeric (CT), pericentromeric (PCT), telomeric, and ribosomal regions, as well as at different loci along the chromosome. Constitutive heterochromatin constitutes the majority of the human genome (> 50%) and is characterized by gene poor-repetitive DNA, mid and late replication and compact chromatin structure.

However, this view is changing and in fact there are increasing evidence that pericentric satellite repeats are present in a multitude of organisms, in various biological contexts, and, possibly, in a controlled strand-specific manner. These data suggest that the regulation and the formation of constitutive heterochromatin domains may be more dynamic than anticipated.

b/ Nuclear stress bodies (nSBs)

The HSR triggered by HS or other proteotoxic stressors results in transcriptional upregulation of constitutive heterochromatin regions located at pericentromeres coding for SatII and III non coding RNAs (Eymery et al. 2010).

In situ experiments allowed determining HSF1 presence both in the cytoplasm and in nucleus of unstressed cells, while the activated form is localized to the nucleus (Sarge et al. 1993). HSF1 nuclear accumulation under stress was shown to involve inhibition of the constitutive nucleoplasmique shuttling of HSF1 active form (Vujanac et al. 2005). Coincident with the stress-induced activation of HSF1 and the induction of heat shock gene transcription, HSF1 localizes within the nucleus to transiently form, large irregularly shaped granules distinct from other nuclear bodies that were termed nuclear stress bodies (nSBs) (Sarge et al. 1993; Cotto et al. 1997; Jolly et al. 1997). Intriguingly, nSBs were observed in large variety of human and primate cells only, suggesting a characteristic evolutionary difference between hominidae and other mammals such as rodents (**Figure 8 A**) (Jolly et al. 1997; Denegri et al. 2001; Biamonti 2004). Biological characterization revealed nSBs are large structures, ranging in size from 0.3 to 3μm, and are usually located close to the nucleoli or to the nuclear envelope. The number of nSBs detected in primary and transformed human cells correlates with the ploidy of the cells, consistent with HSF1 granules having a chromosomal target (**Figure 8 B**) (Jolly et al. 1997; Cotto et al. 1997; Denegri et al. 2001).

c/ Activation of pericentric satIII ncRNA

Fluorescence *in situ* hybridization experiments tempting to map the genomic localization of HSF1 granules under HS show it does not correspond to *HSP*s genes loci but to pericentric repetitive regions of chromosomes (1, 2, 9 , X, Y) and mostly the 9q12 (Jolly et al. 1997; Denegri et al. 2001). Further molecular characterization proved, nSBs are initiated

through a direct interaction between HSF1 and unexpected large pericentric (Sat III) heterochromatic blocks and correspond to active transcription sites for noncoding satellite III RNAs (Metz et al. 2004; Rizzi et al. 2004; Eymery et al. 2010).

Although frequently used as hallmark of HSR activation in human cells, the exact functions of SatIII ncRNA and nSBs are still unknown. Yet, different studies characterizing nSBs dynamics and composition allow to hypothesis concerning their probable function. NSBs were described to be very dynamic structure and to have highly packed nucleoprotein content. Proteins like the acetylase p300/CBP, RNAPII and various splicing factors were found to localize into nSBs upon stress stimuli.
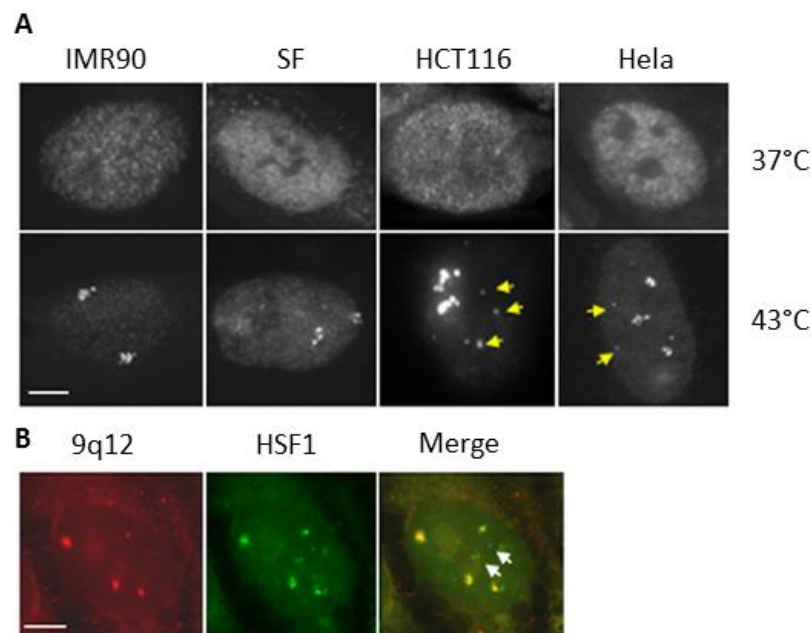
Figure 8| Nuclear Stress Bodies (nSBs) formation. Coincident with the stress-induced activation of HSF1 and the induction of HSPs in human and primates, HSF1 concentrates within the nucleus to transiently form nuclear stress bodies (nSBs). **A.** Immunodetection of HSF1 distribution in different human cell lines after heat shock. HSF1 was detected in two normal primary cultures, fetal IMR90 cells and normal skin fibroblasts (SF), and two tumor cell lines, HeLa and HCT116 cells, before (upper panel) or after (lower panel) HS. At 37 °C, HSF1 is diffusely distributed in the nucleus in all four cell lines. After heat shock, HSF1 concentrates into two foci in the nucleus of normal cells and tumor HCT116 cells, while HeLa hyperploid cells display three to four large foci. In addition, several smaller foci are also present in tumor cells (arrows). **B**. Codetection of HSF1 by immunofluorescence (green) and the pericentromeric 9q12 locus by DNA FISH (red) in HeLa cells exposed to HS. The four large HSF1 foci colocalize with the four copies of the 9q12 locus. Bar: 5 μm. *Adapted from: Eymery et al _2010_Exp cell Res.*

Our lab recently reported that under HS HSF1 recruits major cellular acetyltransferases, GCN5, Tip60 and p300 to pericentric heterochromatin leading to a targeted hyperacetylation that in turn, directs the recruitment of Bromodomain and Extra-

Terminal (BET) proteins BRD2, BRD3, BRD4, which are required for satellite III transcription by RNAP II (Col & Hoghoughi 2016, submitted data) (**Figure 9**). Moreover, Sat III ncRNAs were shown, intriguingly, to remain close to their sites of transcription (Chiodi et al. 2004). Thus, nascent Sat III RNA transcripts are proposed to act as seeds to assemble nSBs, which could then function as a molecular sponges for transcription and splicing factors contributing to global shut down of transcription (Fritah et al. 2009) and splicing alteration (Denegri et al. 2001; Metz et al. 2004; Chiodi et al. 2004). Alternatively, these transcripts were suggested to play roles in heterochromatin assembly and maintenance, or to affect the organization of the cell nucleus in response to stress (Metz et al. 2004; Biamonti & Vourc'h 2010).
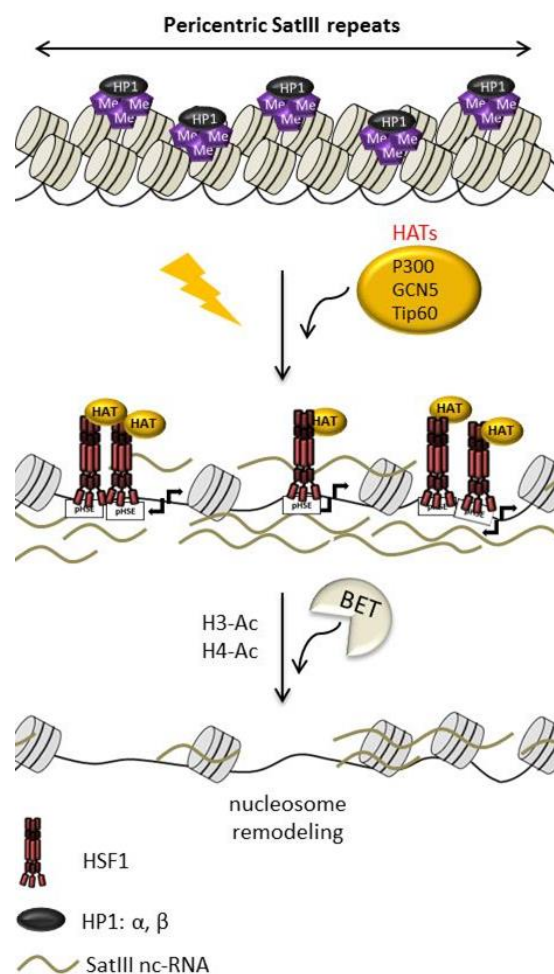
Figure 9| Schematic illustration of HSF1-dependent transcription and chromatin remodeling at pericentric SatIII repeats, in human. SatIII non-coding RNAs are barely detectable under physiological conditions while they are massively transcribed under HSF1-activating stresses. This peculiar stress response of highly compacted chromatin regions is thought to occur in a two-step process. First, the 9q12 locus is decompacted through the loss of epigenetic repressive marks (H3K9me3, HP1), HSF1-dependent histone acetylases recruitment (HAT) to heterochromatin resulting in massive histone H3 and H4 acetylation hence chromatin decompaction and SatIII transcription. Next, the substantial histone acetylation attracts bromodomain (BET) proteins leading to nucleosomal remodeling.

IV. Other functions of HSF1

IV. 1. Fertility and development

In addition to protecting cells against proteotoxic stress, accumulating evidence demonstrate a role for HSF1 in many physiological functions, especially during developmental processes. HSF1 (and HSF2) have been attributed regulatory functions in oogenesis, spermatogenesis and brain development (Mezger, Rallu, et al. 1994; Mezger, Renard, et al. 1994; Xiao et al. 1999; Akerfelt et al. 2010). Specifically during female gametogenesis, both factors were shown to play vital roles in a cell type and stage specific manner.

Concentrating on HSF1 functions, interesting differences can be observed among eukaryotes. Unlike yeast HSF, the Drosophila protein is dispensable for general growth or viability under normal conditions yet it is required for functional oogenesis and larval development (Jedlicka et al. 1997) In mouse, hsf1 depletion did not prevent newborns from attaining adulthood but a clear phenotype can be observed. Adult mice lacking hsf1 present significant growth retardation, female infertility or prenatal lethality, high expression of TNFα (Tumor Necrosis Factor α) and incapacity of triggering the HSR. The phenotype of Hsf1-KO mice also demonstrates the involvement of HSF1 in placenta formation, placode development and in the immune system, further strengthening the evidence for a protective function of HSF1 in development and survival (Xiao et al. 1999; Metchat et al. 2009; Akerfelt et al. 2010).

HSF1 specific function in testis and spermatogenesis is still unclear. Several studies show that HSF1 protein expression was limited to spermatocytes and round spermatids; while hsf1-KO mice present minor fertility defects. The upregulated active HSF1 form induces spermatogenesis blockage and spermatozoa lethality. HSF1 and HSF2 ChIP seq studies revealed more than 700 target genes in mice testis comprising mostly sex chromosomal multi-copy genes spermatogenesis specific transcript (Metchat et al. 2009; Akerfelt et al. 2010). Given that the sex chromatin mostly remains silent after meiosis, HSF1 and HSF2 are currently the only known transcriptional regulators during post-meiotic repression. These results, together with the earlier findings that HSF2 can also form

heterotrimers with HSF1 in testis (Sandqvist et al. 2009), strongly suggest that HSF1 and HSF2 act in a heterocomplex and fine-tune transcription of their common target genes during the maturation of male germ cells. Very interestingly, Probst and colleagues show that during murine early development an activation of major Sat ncRNA (functionally equivalent to the human Sat III) occurs in the male pronucleus (Probst et al. 2010). The role of HSF1 in mammalian testis tissues is not yet established and should be a key to clarify its molecular function in the process of spermatogenesis (**Figure 10**).
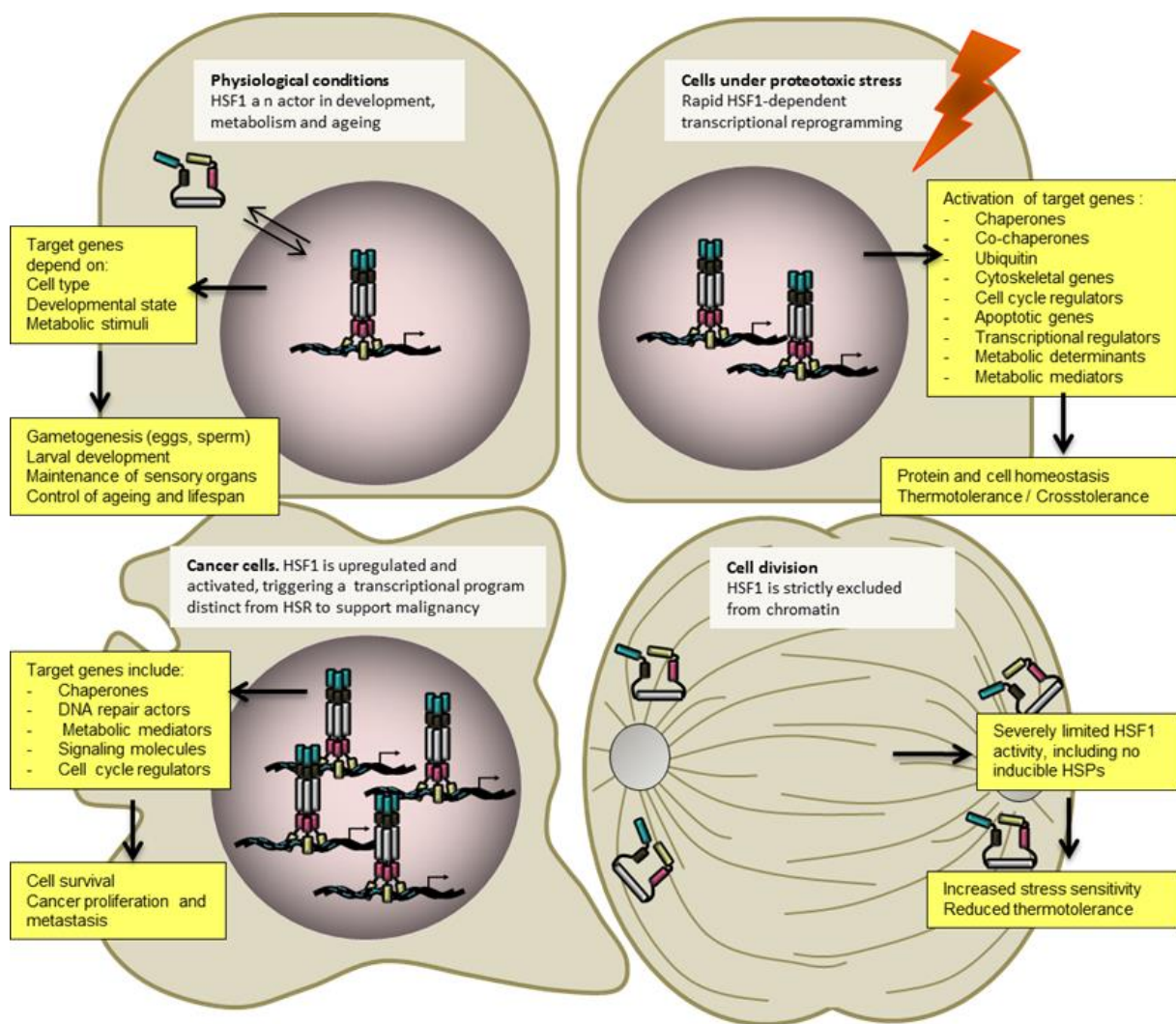
Figure 10| Overview of HSF1 functions in diverse cellular processes. An overview of HSF1 highlighted functions, including activated target genes categories and functional consequences for the cell. HSF1 coordinates stress-induced transcription and directs versatile physiological processes in eukaryotes. The pivotal role of HSF1 in cellular homeostasis is mediated mainly through its strong effect in transactivating heat stress protein genes including chaperones. HSF1 is capable of reprogramming transcription more extensively than previously assumed; it is also involved in a multitude of processes in stressed and non-stressed cells. The importance of HSF1 in fundamental physiological events, including metabolism, gametogenesis and aging, has become apparent and its significance in pathologies, such as cancer progression, is now evident. ***Adapted from: Vihervaara et al_2014_Compagnie of Biologists.***

IV. 2. Ageing and cancer

Through its capacity to fight proteotoxicity at the cell level, HSF1 was suggested to contribute to cellular ageing, and numerous pathophysiological conditions, associated with impaired protein quality control (Powers et al. 2009; Anckar & Sistonen 2011). Diseases involving problems in protein homeostasis, or "proteostasis", include cystic fibrosis, Alzheimer's, Parkinson's, and Huntington's disease (**Figure 10**).

- **Ageing**

A wide range of different model systems and experimental strategies have been used to investigate molecular basis of ageing. The insulin and insulin-like growth factor 1 receptor (IgF1R) signaling pathway, has emerged as a key pathway. The first studies investigating the functional relationship between HSFs and the IgF1R signaling pathway were carried in *C. elegans* carrying mutations in different components of the IgF1R-mediated pathway. Together those studies agree to say HSF1 and HSPs positively act on lifespan by maintaining proteostasis thus prolonging the health of the organism (Hsu et al. 2003; Morley & Morimoto 2004; Ben-Zvi et al. 2009). Limited food intake or caloric restriction is another process that is associated with an enhancement of lifespan and age-related diseases. The stress and caloric restriction-associated deacetylase Sirtuin-1 (SIRT1) was shown to maintain HSF1 active form by directly acetylating its DBD (Bishop & Guarente 2007; Anckar & Sistonen 2011). During ageing, the DNA-binding activity of HSF1 and the amount of SIRT1 were shown to be reduced. Consequently, a decrease in SIRT1 levels was shown to inhibit HSF1 DNA-binding activity in a cell based model of ageing and senescence (Westerheide et al. 2009). Taken together these results suggest that with IgF1R and SIRT1, HSF1 acts as regulatory hub in a network linking cell nutrition, stress and lifespan.

- **Cancer**

Moreover, elevated levels of HSF1 have been detected in several types of human cancers, such as breast cancer, colon, lung and prostate cancer (Tang et al. 2005; Khaleque et al. 2008; Mendillo et al. 2012). Importantly, HSF1 deficient mice exhibit a lower incidence of tumors and increased survival compared to their WT counterparts in a chemical skin carcinogenesis model and in a genetic model expressing p53 oncogenic mutation (Dai et al.

2007). Similar results have been obtained in human cancer cells lines, in which HSF1 was depleted using an RNA interference strategy (Dai, Whitesell, Arlin B Rogers, et al. 2007). HSF1 expression is likely to be crucial for stress phenotype of cancer cells, described in many cancer cells due to their high intrinsic level of proteotoxic and oxidative stress, frequent spontaneous DNA damage and aneuploidy (Whitesell & Lindquist 2009). In 2012, Lindquist and colleagues published a study piercing one of the biggest mysteries surrounding the field of HSF1 implications in cancer. They showed that during tumorigenesis of human breast cells, HSF1 is capable of driving a transcriptional program specific to malignant cells and that do not have much in common with the heat –induced transcriptome. This HSF1 cancer program was shown to be active in breast, colon and lung tumors isolated directly from human patients and to be strongly associated with metastasis and death. Cancer-specific genes in this program are implicated in cell-cycle regulation, signaling, metabolism, adhesion and translation, *HSP*s genes are also part of this program (Mendillo et al. 2012).

Given the unique role of HSF1 in proteome stability, enhanced activity of this principal regulator was clearly proposed to be a potent modifier of tumorigenesis and, therefore, a potential target for cancer therapeutics (Whitesell & Lindquist 2009). Many small molecule regulators of HSF1 are actively being searched for (Anckar & Sistonen 2011; Arneaud & Douglas 2016).

Under metabolic stress, such as glucose deprivation, the complex MAPK (mitogen-activated protein kinases) is mobilized by the cell. MAPK is a stress and metabolic sensor shown to be critic for the maintenance of cellular energy homeostasis. Interestingly, activated MAPK phosphorylates HSF1 on serine residues (S121) and repress its transcriptional activation *in vivo* upon metabolic stress induction (Chu et al. 1996; Dai et al. 2015). Indeed, recently metformin (metabolic stressor) was found to trigger HSF1 inhibition. Thus, these findings uncover a novel interplay between the metabolic and proteotoxic stress sensor HSF1 that profoundly impacts stress resistance, proteostasis, and malignant growth (**Figure 11**).
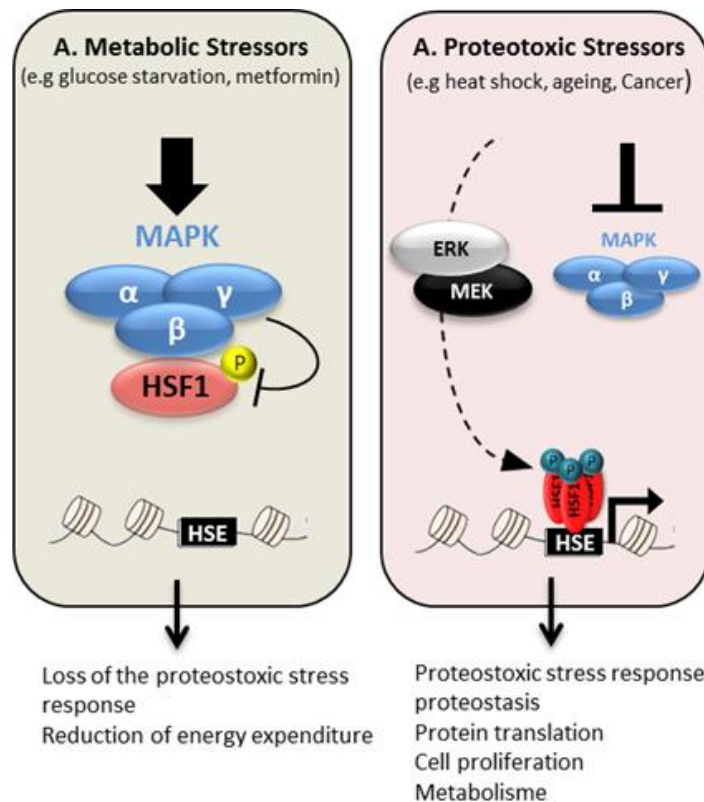
Figure 11| Metabolic stress-activated AMPK induces HSF1 repression and proteostasis disruption. A. In response to metabolic stress, the tumor-suppressive LKB1 signaling could inactivate HSF1 through AMPK-mediated Ser121 (P) phosphorylation. AMPK, a pivotal sensor of energy depletion, critically regulates the metabolic stress response. Through mobilization of AMPK, metabolic stressors, including metformin and nutrient deprivation, inactivate HSF1. **B.** Under proteotoxic stress occurring under HS, lifespan and cancer, AMPK is inhibited and HSF1 can promote cell proteostasis, growth and survival. Moreover HSF1 was identified as a new substrate for MEK. MEK physically interacts with and phosphorylates HSF1 at Ser326 (P). Thus revealing that the RAS/MAP kinase pathway regulates proteostasis in normal cells and that it can be targeted to promote proteomic instability and amyloidogenesis in cancer cells. ***Adapted from Zijian et al_2015_Cell, Dai et al_2015_EMBO.***

Chapter II| Telomeres and TERRA

I.   Telomeres

This second chapter will begin with a broad definition of chromatin and heterochromatin followed by a large view on the current knowledge concerning telomeres and the non-coding telomeric RNA (TelRNA). Telomeric chromatin state characteristics, telomere functions and maintenance mechanisms will be presented and finally an overview on telomeres transcription regulation and functions will be proposed.

I. 1.   Chromatin: general introduction

Each cell of an organism contains within its nucleus 2 meters of genomic DNA negatively charged that produces electrostatic repulsion between adjacent DNA regions. Therefore, it would be difficult for a long DNA molecule alone to fold into a small 10µm diameter nucleus (a volume of only~100 fL to 1 pL) (Bloomfield 1996; Hirano et al. 2012). To overcome this problem, 147bp-DNA is wrapped around a basic protein complex known as a core histone octamer, which consists of the histone proteins H2A, H2B, H3, and H4, to form a positively charged nucleosome. Each nucleosome particle is connected by linker DNA (20–80 bp) to form repetitive motifs of~200 bp, commonly referred to as the 10nm "beads on a string" fiber (**Figure 12**) (Olins & Olins 2003). Other histone and non-histone factors contribute to maintain and compact the DNA such as linker histone H1, cations, and other positively charged molecules.

Histones are no longer considered to be simple 'DNA packaging' proteins; they are recognized as being regulators of chromatin dynamics. Both DNA and histones are subject to covalent modifications that alter chromatin structure and regulate its accessibility to specific actors such as transcription factors or chromatin remodelers (Bannister & Kouzarides 2011). In vertebrates, a major DNA modification is methylation (**Figure 12**). Extensively studied, DNA methylation was shown to occur on cytosine present in CpG dinucleotides. Although the mammalian genome CpG dinucleotides content is relatively poor, existing short CpG-rich DNA stretches (also called CpG islands) mostly located at gene promoters were found to play

important role in genes activity (Thomson et al. 2010). It is important to note that aberrant changes in DNA methylation were among the first event to be recognized in cancer (Feinberg & Vogelstein 1983). DNA methylation is catalyzed by specific molecular actors termed DNA methyltransferase (DNMTs). DNMT3a and DNMT3b are known for their role in de novo methylation of non-methylated CpGs while DNMT1 ensures methylation of newly synthetized DNA strands (Jin & Robertson 2013). Mechanisms of DNA demethylation imply passive and active processes including active demethylation by the enzymes ten-eleven translocation (TET) family (Kohli & Zhang 2013).
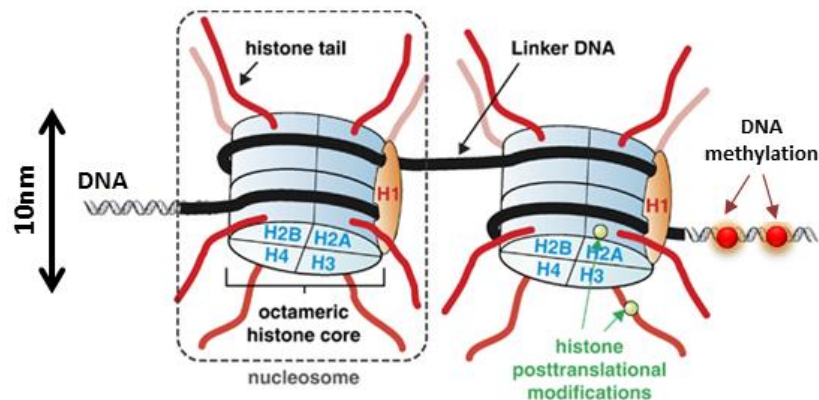
Figure 12| Chromatin organization. In the nucleus DNA is bound and wrapped around histones and non-histones nuclear proteins to form chromatin. Chromatin's basic unit is the nucleosome, spaced by "linker DNA", and which is composed of two copies of the four major core histones (H2A, H2B, H3 and H4) and is wrapped by 147bp of 2nm DNA. The structure of the nucleosome core particle is remarkably conserved among species. Linker histone H1 is positioned on top of the nucleosome core particles stabilizing the higher order 10nm chromatin fiber. Both DNA and histones are subject to covalent modifications. DNA CpG dinucleotides methylation is the most extensively studied epigenetic modification. Histones undergo a variety of post-translational modifications, on their N-terminal tails, but also in their globular core region. *Adapted from: Füllgrabe et al_2011_Oncogene.*

Unlike DNA methylation which is considered to be rather stable, histone undergo various modifications, such as acetylation, methylation, phosphorylation, ubiquitylation and glycosylation and others, all of which are carried out by histone-modifying enzyme complexes in a dynamic manner (Khorasanizadeh 2004). These modifications occur primarily within the histone amino-terminal tails extend from the surface of the nucleosome as well as on the globular core region (Cosgrove et al. 2004) (**Figure 12**). Among the specialized molecular actors implicated in histone modifications, can be cited: histone acetyltransferases (HATs), which acetylate the histone tails and induce chromatin decondensation; histone deacetylases (HDACs), which remove the acetyl groups and promote a tighter binding of histones to DNA; histone methyltransferases (HMTs), which

promote or inhibit transcription depending on the target histone residue; and histone demethylases (HDMs), which counteract the HMTs (Allis et al. 2007).

The combination between the state of DNA methylation and the type of histone modifications at a precise genomic locus define different states of chromatin: euchromatin and heterochromatin. Euchromatin is characterized by a more "open" configuration and is associated with transcriptionally active regions, while heterochromatin is usually associated with gene silencing (Bannister & Kouzarides 2011). Euchromatin is characterized by unmethylated DNA and acetylated histone marks (hyper acetylation of histone H3 and H4). Particularly the presence of acetylated H3K9 (lysine 9 of histone H3) is considered to be a mark of active chromatin preventing the methylation of this residue and thereby found enriched at regions surrounding transcriptional start sites (Füllgrabe et al. 2011). Importantly, H3 and H4 deacetylation at gene promoters was shown to be associated with cancer, tumor progression and poor prognosis (Füllgrabe et al. 2011).

On the other hand, heterochromatin associated DNA is heavily methylated and histones are hypoacetylated. Two different kind of heterochromatin can be distinguished, facultative and constitutive heterochromatin. While facultative heterochromatin corresponds to genomic regions expressed almost exclusively during development and differentiation constitutive heterochromatin was thought to be constantly silenced.

Constitutive heterochromatin is associated with specific genomic loci (telomeres, centromeres, pericentromeres) and an epigenetic signature symbolized by specific repressive histone marks like H3K9 (lysine 9 at histone 3) and H4K20 (lysine 20 at histone 4) di- and tri-methylation (**Figure 13**; see also **Chapter I Figure 7**). These modifications are mostly established by Suv39H1/2 (suppressor of variegation 3-9 homologue) and SUV4-20 HMTs (Bannister & Kouzarides 2011; Füllgrabe et al. 2011). H3K9me3 were found be molecular docking sites for all forms of the mammalian heterochromatin protein 1 (HP1) α, β and γ, which in turn, recruits Suv39, thereby participating in heterochromatin formation and spreading (Bannister et al. 2001; Lachner et al. 2001).
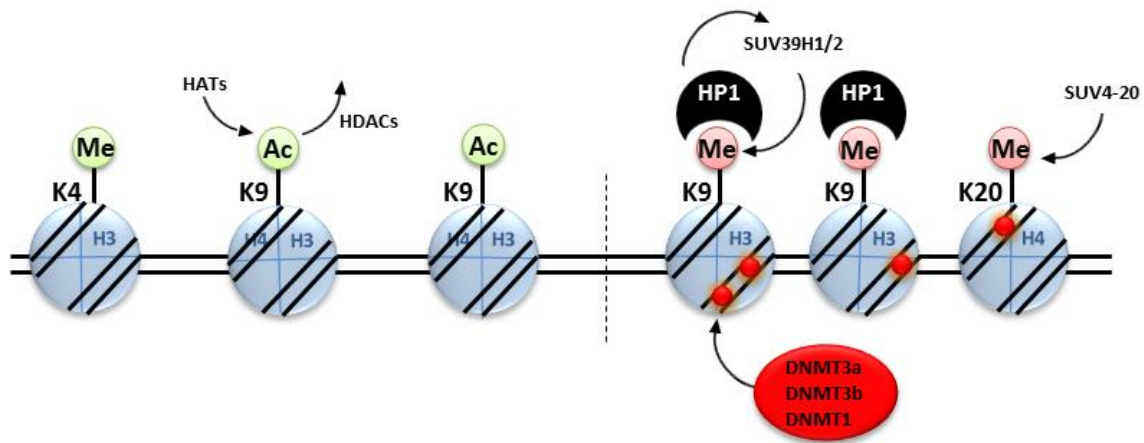
Figure 13| Distinctive distribution patterns of the epigenetic markers in euchromatin and heterochromatin. In loosely packed euchromatin, left, transcription is active, and commonly associated with H3 and H4 Lysine 9 acetylation, H3K4 methylation and unmethylated DNA. Histone acetylases (HATs) and deacetylases (HDACs) dynamically act to put in place and remove histone acetylation. In contrast, tightly packed heterochromatin is less permissive to transcription. Constitutive heterochromatin is usually enriched in epigenetic marks like hypermethylated DNA, H3K9me3 and H4K20me3 respectively deposited by the enzymes SUV39H1/2, SUV4-20 and DNMT3a/b/1. H3K9me3 is a docking site for the heterochromatin protein 1 (HP1) that consecutively contributes to heterochromatin formation by recruiting SUV39.

Each core histone (except for H4) presents histone variants, which have also been associated with chromatin state. In contrast to core histones, only expressed during S-Phase and inserted to chromatin after DNA replication, variants are expressed at low levels, in a DNA replication independent manner (Skene & Henikoff 2013). They are subjected to various modifications that impact various cellular processes such as, development, gene expression and silencing, DNA damage repair and many others (Skene & Henikoff 2013).

The most common variant for H2A are H2A.X, H2A.Z and macroH2A. More particularly, H2A.X contains a serine residue in its C-terminal part, which is subject to rapid phosphorylation in response to the detection of a double strand break (DSB), producing a modified histone termed γH2A.X (or H2A.X-P), and spreading up to 1-2 Mb from the damaged site. The kinases responsible for H2A.X phosphorylation are the ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) in mammals. Although H2A.X phosphorylation is not essential for the detection or repair of DSBs, it facilitates the assembly and activity of DNA repair complexes at the DNA damage site (Skene & Henikoff 2013) (**Figure 14**).
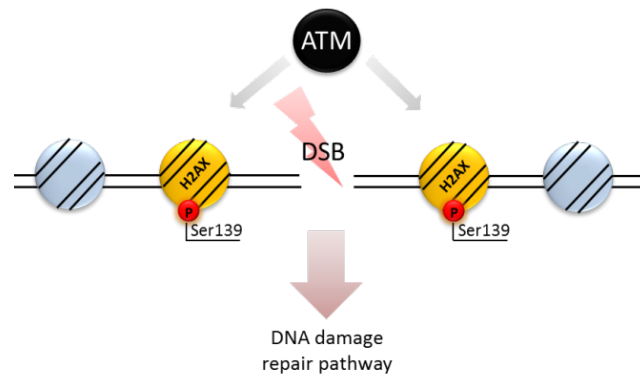
Figure 14| Gamma-H2A.X signaling in DNA double strand break (DSB) induced DNA damage response. When a DSB takes place, the PI3-kinase related kinase ATM (Ataxia Telangiectasia Mutated) is recruited and activated. ATM phosphorylates histone variant H2AX on its C-terminal Ser139 residue. This modification is called γ-H2AX and is spread within minutes to thousands of H2AX proteins that are in proximity to the damage site. This phosphorylation of H2AX on Ser139 is crucial to activating the DNA damage response pathway, a complex molecular mechanism to detect and repair DNA damage.

In our study we will also focus on histone H3. For H3 five variants have been described in mammals: CENP-A, H3t, H3.X, H3.Y and H3.3. Particularly, the variant H3.3 that differs from H3 by only 5aa (amino acids) is nevertheless clearly distinct from H3. Hence, H3.3 was shown to be associated with euchromatin, but has also been found to be enriched at pericentromeres and telomeres where it is deposited by the ATRX/DAXX complex (Szenker et al. 2011). ATRX/Daxx is a histone chaperone chromatin remodeling complex implicated in variant H3.3 deposition at several genomic regions including telomeric and pericentromeric repeats where it plays a repressive role (Skene & Henikoff 2013; Goldberg et al. 2010).

I. 2.   Chromatin at telomeres

Telomeres are protective nucleoprotein complexes that cap the end of linear chromosomes and play a key role in preserving genomic stability. The importance of telomere chromatin integrity in genome replication and stability was recognized by the 2009 Noble prize attributed to Elizabeth Blackburn, Jack Szostak and Carol Greider (Blackburn & Challoner 1984; Corey 2010).

a/ Genomic sequences

Repetitive telomeric DNA sequence and their organization are highly conserved among organisms and between chromosomes of the same species (Moyzis et al. 1988; Sfeir 2012).

Telomeric specific sequences are made of tandem G-rich repeated. In vertebrates, telomeric sequences consist of TTAGGG repeats (Moyzis et al. 1988). The number of repeats per telomere varies widely among species; while in human telomeres present a mean length of 5-15 Kb they can be as long as 100 kb in rodents or of about ~350–500 bp in *S. cerevisiae* (Palm & de Lange 2008) (**Figure 15**).

Organism	Telomere repeats sequence motif	Telomeric tract length	Overhang length
H. sapiens	TTAGGG	5-15 Kb	30-500 nt
M. musculus	TTAGGG	20-100 Kb	?
S. pombe	$GGTTACA_{0-1}C_{0-1}G_{0-6}$	5 Kb	< 50 nt
S. cerevisiae	TG_{1-3}	300 bp	14 nt
T. brucei	GGGTTA	2-26 Kb	10-40 nt
T. thermophila	TTGGGG	120-420 bp	14-21 nt
C. elegans	TTAGGC	4-9 Kb	> 30nt
A. thaliana	TTTAGGG	2-5 Kb	20-30 nt
Oxytricha	TTTTGGGG	20bp	16nt

Figure 15| Diversity of telomeric DNA sequences. Some of the known telomere nucleotide sequences are listed in the table above. Telomere repeated motifs as well as the mean length of the telomeric tract are indicated. *Adapted from Sfeir et all_2012_Cell Science at a glance.*

Telomeric specific repeats can be found located at the very extremity of chromosomes as well as on adjacent so called "subtelomeric" regions (See **Figure 16**). Subtelomeres contain chromosome specific sequences in addition to the specific repetitive telomeric motifs sequences and spread up to ~500 Kb towards centromeres. The more distal 2 Kb region of subtelomeres consist of telomeric repeat variants and unique DNA sequences (Allshire et al. 1986). Telomeric characteristic sequences can also be found located inside chromosomes forming the so-called interstitial telomeric sequences (ITSs). Analysis of flanking sequences suggests that ITSs were inserted and maintained into the genome during DNA double-strand breaks repair events, which occurred in the course of evolution. Short stretches of telomeric hexamers distributed at internal sites of the chromosomes ITSs were shown to be implicated in chromosomal stability (Ruiz-Herrera et al. 2008). Another key

feature of the telomere end in all organisms is that not only most of the telomeric GT-rich repeats are composed of double-stranded DNA, but in addition they present a 3' single-stranded DNA commonly referred to as G-tail or G-rich overhang (Makarov et al. 1997; McElligott & Wellinger 1997) (**Figure 15**).

Mammalian G-rich overhangs are of about 30-500 nucleotides long (Chai et al. 2005) and are generated by the "end replication problem" of linear chromosomes (described in paragraph **I. 3. e/**).

b/ Heterochromatin status

Telomeres major contribution to genome stability mostly relies on their capacity to protect chromosomes ends from being recognized as double-strand DNA breaks (DSBs). Telomeres have evolved into complex nucleoprotein structures where nucleosome occupancy, epigenetic modifications, DNA methylation and specific proteins binding all together assure a dynamic yet protective shield to natural ends of eukaryotic chromosomes.

- **Chromatin compaction and telomere histones modifications**

With the exception of some lower organisms with short telomeres (budding yeast and several protozoa), the major part telomeres is folded into nucleosomes which are regularly spaced with intervals of about ~160 bp, thus ~20–40 bp shorter than in bulk chromatin (Tommerup et al. 1994; Galati et al. 2012). This particularity was shown to be highly efficient against DDR machinery recruitment to telomeres (Bandaria et al. 2016). Telomere specifically-bound proteins were shown to modulate its chromatin compaction (Bandaria et al. 2016). Yet it remains unclear whether and to what extent posttranslational modifications of DNA and histones at telomeres and subtelomeric regions are primarily responsible for hypercondensation of telomeric chromatin.

In mammals, extensive studies have been carried out in mouse to characterize the epigenetic marks associated with telomeres and subtelomeres (Blasco 2007). Both regions were shown to be enriched in heterochromatin marks, namely H3K9me3 and H4K20me3, and to be hypoacetylated on histones H3 and H4 (**Figure 16**).
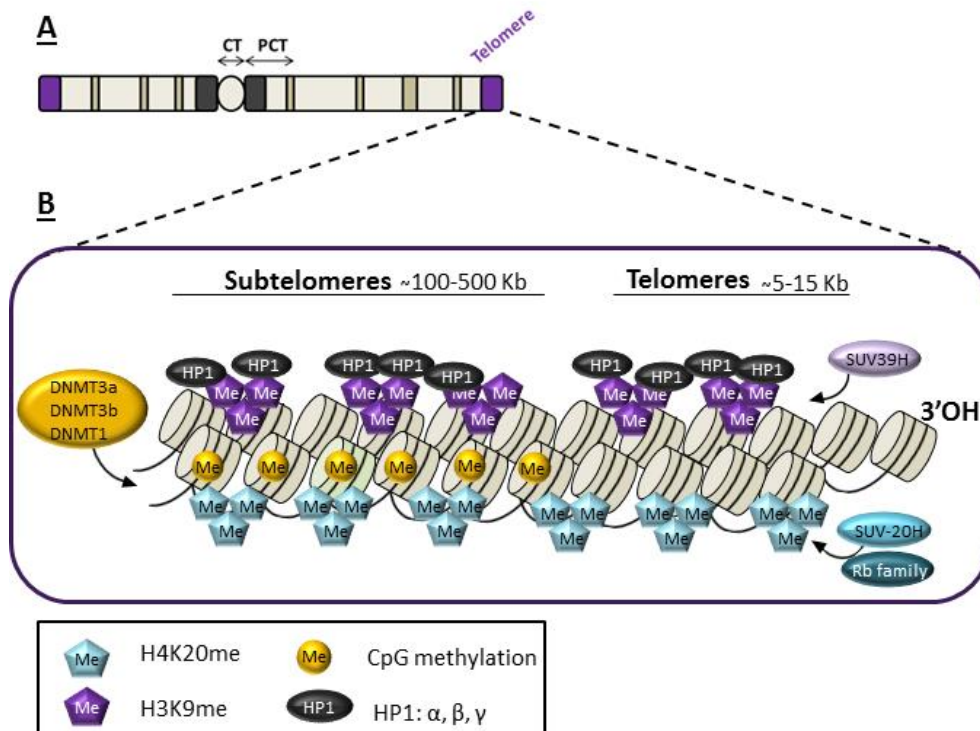
Figure 16| Telomeres, natural end of eukaryotic chromosomes A. Schematic representation of human chromosome, indicated centromere (CT), pericentromeres (PCT) and Telomeric regions. Human telomeres contain the repeat TTAGGG, which may be reiterated in tandem for up to 15 Kb. **B.** Both telomeric and distal subtelomeric chromatin (In man and mouse) regions are enriched in trimethylated H3K9 and H4K20, and HP1 α, β, γ isoforms. The two histone modifications are carried out by the SUV39H and SUV4-20H (in collaboration with Retinoblastoma (Rb) proteins) HMTases, respectively. In addition, subtelomeric DNA is heavily methylated by the DNMT1, NMT3a and DNMT3b enzymes. Both histone trimethylation and DNA methylation have been shown to independently act as negative regulators of telomere length and telomere recombination. ***Adapted from: Blasco_2007_Focus on Epigenetics.***

In addition, subtelomeric DNA was found to be heavily methylated. Similarly to D. melanogaster and *S. pombe*, normal cells of vertebrate, telomeres are enriched for binding of HP1) isoforms: HP1α, HP1β and HP1γ via the H3K9 and H4K20 modifications that are carried out by the HMTases – SUV39H and SUV4-20H, respectively (García-Cao et al. 2003; Blasco et al. 2005). In addition to these histone heterochromatic marks, telomeric repeats also contain di-methylated H3K79, mediated by the histone methyl transferase Dot1L (Carchilan et al. 2007). Dot1L protein plays a major role in meiotic checkpoint control and is also important for the di-methylation of H3K9, acting in association with additional H3K9-specific HMTases, such as ESET (ERG-associated protein with SET domain) (Carchilan et al. 2007). Consistent with H3 and H4 hypoacetylation at telomeres, lack of the histone deacetylase SIRT6 results in elevated H3K9-acetylation levels at human telomeres and can lead to telomere dysfunction (Blasco 2007; Blasco & Schoeftner 2009). Interestingly

mammalian subtelomeric regions are heavily methylated in contrast to *S. cerevisiae* and D. melanogaster, which lack or display low levels of DNA methylation (Gonzalo et al. 2006). The DNA metyl transferases responsible for the mammalian de novo methylation patterns at telomeres, are the DNMT3a and DNMT3b; and DNA methylation is maintained by DNMT1, which copies parental-strand methylation onto the de novo synthesized daughter strand after DNA replication (**Figure 16**).

Although the epigenetic state of human telomeres has yet to be fully elucidated (Galati et al. 2013; Galati et al. 2012), it has become apparent that epigenetic regulation of the telomeric chromatin template critically impacts telomere function and telomere-length homeostasis in several organisms ranging from yeast to human.

Single or combined loss of the previously cited histone heterochromatic marks, HMTase or HP1 was shown to result in substantial telomere elongation (Benetti, Blasco, et al. 2007; Benetti, Gonzalo, et al. 2007; Arnoult et al. 2012) and impairs heterochromatin and genome stability. Indeed several studies corroborate this and prove disturbing the telomeric epigenetic signature leads to deleterious effects such as telomere elongation, fusion and recombination. In particular, cells that lack the SUV39H1 and SUV39H2 HMTases show decreased levels of H3K9 trimethylation at telomeres, concomitant with aberrant telomere elongation. A similar deregulation of telomere length is seen in cells that lack all three members of the retinoblastoma family and show decreased levels of H4K20 trimethylation at telomeres (Gonzalo et al. 2006; Benetti, Blasco, et al. 2007; Michishita et al. 2009). In line with these data, it was also demonstrated the knockout of human SIRT6 leads to hyperacetylation of telomeric H3K9 and H3K56, resulting in severe consequences on chromosome stability such as telomere fusion, premature senescence and abrogation of the telomere position effect (TPE) (Michishita et al. 2009; Tennen et al. 2012). More recently, the availability of histone variant H3.3, known to maintain transcriptional memory at active chromatin, was shown to be essential for maintenance of a heterochromatic state through H3.3K9 trimethylation and ATRX/DAXX (chaperone complex) recruitment to telomeres, and thereby for proper telomere function. Together, these data bring evidence that appropriate levels of histone methylation and acetylation at heterochromatic marks mediate normal telomere function and stability.

■   **DNA CpG methylation**

The discovery of methylated G-rich DNA stretches within at least 20 subtelomeric TERRA-promoter regions, in human cells under physiological conditions, suggested TERRA expression may be regulated by DNA methylation (Nergadze et al. 2009). First evidence supporting this idea were found in cells from patients suffering from ICF (Immunodeficiency, Centromere instability , Facial anomalies) syndrome, caused by loss of DNMT3B, that display low subtelomeric DNA methylation, together with increased TERRA levels (Yehezkel et al. 2008; Deng et al. 2009). Moreover, DNMT1 or DNMT3ab deficiency in mouse cells induces a loss of DNA heterochromatic marks and causes a dramatic telomere elongation, which is driven by increased homologous recombination events between telomeric sister chromatids (Gonzalo et al. 2006) and increased abundance of histone repressive marks at telomeres (Benetti, Blasco, et al. 2007). However, the mechanisms by which DNMT are recruited to subtelomeres remains unclear. Loss of SUV39h HMTases does not affect subtelomeric DNA methylation (Fuks et al. 2003; Lehnertz et al. 2003; Benetti, Blasco, et al. 2007). This suggests the existence of an alternative pathway of DNMT recruitment to subtelomeres.

I. 3.   Telomere capping

Telomeres are essential for chromosome stability. They are capped to protect them from breakage and to prevent their recognition as DNA double strand breaks. The first characterized telomere-capping pathway in vertebrates involves shelterin, a complex that bridges the duplex and the 3'overhang parts of telomeres. A second complex was recently discovered in budding yeast called the CST complex. These 2 complexes bound and stabilized the telomeric nucleoprotein structure

a/ The T-loop structure

First, telomeres have the capacity to switch between a linear and a loop structure. This unique feature at chromosome ends was called "T-loop", based on the invasion of the 3' G-rich overhang into upstream telomeric DNA (C-rich strand). This also results in a second small so-called D-loop (Displacement-loop) structure at the invasion site (Oeseburg et al.

2010) (**Figure 17**). This first structure basis is regulated and maintained in all eukaryotes by at least two telomere binding protein complexes: shelterin and CST.
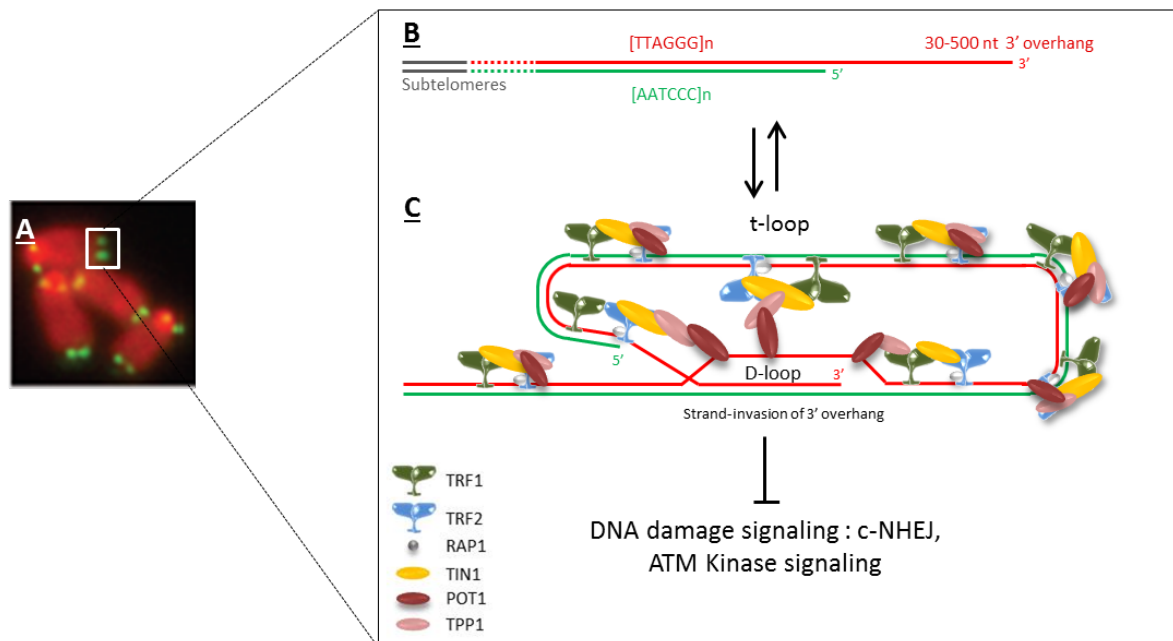
Figure 17| Telomeric nucleoproteins-complex. **A.** Fluorescence In Situ Hybridization (FISH) with telomere probe showing telomeric (TTAGGG) repeats localization at the extremity of sister chromatids. **B.** Simplified view of vertebrate chromosomes end in an array of repeats that varies in length. Proximal to the telomeric repeats is subtelomeric repetitive elements. The telomere terminus contains a long G-strand overhang. The 3' end sequence is not precisely defined whereas the 5' end of human chromosomes nearly always features the sequence ATC at the 5' end. **C.** Schematic illustration of the dynamic interchange between 2 different structural states (linear ↔ t-loop) depending on telomere length and cell cycle progression. Long telomeres adopt a closed structure that protects chromosome ends from DDR and NHEJ. The way telomere specific protein complex (shelterin) might be positioned on telomeric DNA and D-loop are represented. TRF1 and TRF2 DNA interaction is highlighted as well as the binding of POT1 to the single-stranded TTAGGG repeats. Although one of the shelterin complexes may have the proposed six-protein structure, telomeres contain numerous copies of the complex bound along the ds TTAGGG repeat array and it is not clear whether all (or even most) shelterin are present in such a complex. ***Adapted from : Palm et al_2008_Annu.Rev.Genet.***

b/ Shelterin complex

The specialized protein complexes known as the telosome or shelterin is always present in all organisms, and participate to telomere length regulation and telomere capping (Blackburn 2001). Single or combined deletion of the shelterin complex members show they are vital since, they enable cells to distinguish their natural chromosome ends from DNA breaks, by repressing DNA repair reactions, and by regulating telomerase-based telomere maintenance (Palm & de Lange 2008).

The components of shelterin specifically localize to telomeres; are abundant at telomeres throughout the cell cycle; and except for TRFs (Telomeric Repeat Factors) (Simonet et al. 2011) there is no evidence for them to function elsewhere in the nucleus. The specificity of shelterin for telomeric DNA is due to the recognition of TTAGGG repeats by three of its components: Telomeric Repeat binding Factors 1 and 2 (TRF1 and TRF2) bind the duplex part of telomeres, whereas Protection Of Telomeres 1 (POT1) can bind the single strand TTAGGG repeats present at the 3' overhang and at the D loop of the t-loop configuration. TRF1 and TRF2 represent a platform for the association for the rest of the complex. They recruit: the TRF2- and TRF1-Interacting Nuclear protein 2 (TIN2), Rap1 (the human ortholog of the yeast Repressor/Activator Protein 1), TPP1 (formerly known as TINT1, PTOP, or PIP1), and POT1. Shelterin complex variants can form in cells lacking either TRF1 or TRF2/Rap1 at telomeres but their specific functions are not yet known (Liu et al. 2004; Palm & de Lange 2008).

TRF1 and TRF2 act as architectural factors, changing the higher-order structure of telomeric DNA. TRF2 has the ability to form t-loop-like structures when provided with a model telomere substrate (Stansel et al. 2001). *In vivo* evidence for TRF2-dependent RTEL1 (Regulator of Telomere Elongation Helicase 1) expression and t-loop unwinding during S-phase was recently described (Sarek et al. 2015) clearly demonstrating TRF2 importance for t-loop regulation *in vivo*. To date, TRF2 and TRF1 are the predominant mediators responsible for the maintenance of protein interactions within the shelterin complex. It is noteworthy that mammalian telomeres contain a large number of other proteins that make important contributions to the maintenance and protection of chromosome ends. TRF1 and TRF2 both contribute to non-shelterin protein recruitment to telomeres through specific functional domains (Palm & de Lange 2008). Most of the non-shelterin proteins recruited to telomeres are involved in DNA processing such as: DNA repair, DNA damage signaling DNA replication or chromatin structure (Palm & de Lange 2008).

c/ CST complex

The CST is a trimeric complex composed of Ctc1, Stn1, and Ten1 in higher eukaryotes. CST localizes specifically to the single-stranded telomeric DNA, including the telomeric overhang where it is involved in chromosome end capping and telomere length regulation

(Rice & Skordalakes 2016). Although initially thought to be unique to yeast, it is now evident that the CST complex is present in a diverse range of organisms, including human, where it contributes to genome maintenance. The CST accomplishes these tasks via telomere capping and by regulating telomerase and DNA polymerase alpha-primase (implicated in initiation of DNA synthesis in eukaryotic replication) access to telomeres, and a process closely coordinated with the shelterin complex in most organisms. In contrast to shelterin complex, recent studies have shown that the human CST complex may have additional functions beyond the telomeres. Studies have shown that the CST complex rescues genome-wide (telomeric and non-telomeric) replication fork stalling during conditions of replication stress by facilitating dormant origin firing (Rice & Skordalakes 2016). It is worth noting that in vertebrates the capping properties of the vertebrate CST may be dispensable *in vivo* due to the presence of shelterin, which also caps the ends of chromosomes (De Lange 2005).

d/ Vital functions of telomere capping

However, if telomeres are not protected by shelterin, they are recognized as DSBs and processed accordingly by DNA repair pathways. In mammalian cells, DSBs are primarily repaired by nonhomologous end joining (NHEJ) and homology directed repair (HDR), two pathways that threaten the integrity of chromosome ends (**Figure 18**).

Because telomere erosion occurs in most human somatic cells, 50 to 100 base pairs per cell division, this can eventually cause telomeres too short to bind enough shelterin for optimal telomere protection. As a result, the short telomeres activate a DNA damage signal that induces cell cycle arrest, as well as senescence or apoptosis. Moreover, the repair of the dysfunctional telomeres by various forms of NHEJ results in end-to-end fused dicentric chromosomes, which are unstable and can generate genome instability. A well-studied example of shelterin importance in telomere maintenance is TRF2 depletion (**Figure 18**).
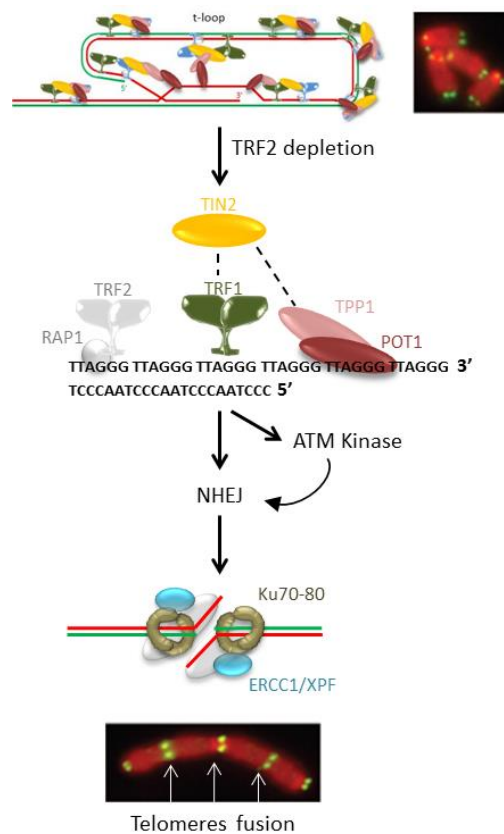
Figure 18| Repression of NHEJ by shelterin. Schematic representation of the NHEJ pathway, responsible for telomeres fusions upon TRF2 inhibition. TRF2 is required for the inhibition of NHEJ through prevention of Ku70-80 loading to chromosome end. ERCC1/XPF nuclease has been implicated in the removal of the G-strand overhangs upon inhibition of TRF2 in human cells. This function of TRF2 is proposed to depend on its ability to remodel telomeres into t-loops. How the telomere termini are processed during NHEJ is not yet fully understood. *Adapted from : Palm_2008_Annu.Rev.Genet*

e/ End replication problem

Biochemical characteristics of DNA polymerase prevent it from fully replicating the linear ends of eukaryotic cells. Hence, at each cell division in the absence of telomere length maintenance mechanisms a somatic cell will undergo telomere shortening (Whatson 1972; Levy et al. 1992). Indeed, lagging strand synthesis is initiated by RNA primers that are replaced by DNA. However, DNA polymerase is unable to fill in the gap left by the most distal primer. In consequence, the 5' end will shorten by 50-200nt with each cell division (Wai 2004). This was called the "end replication problem" (**Figure 19**).
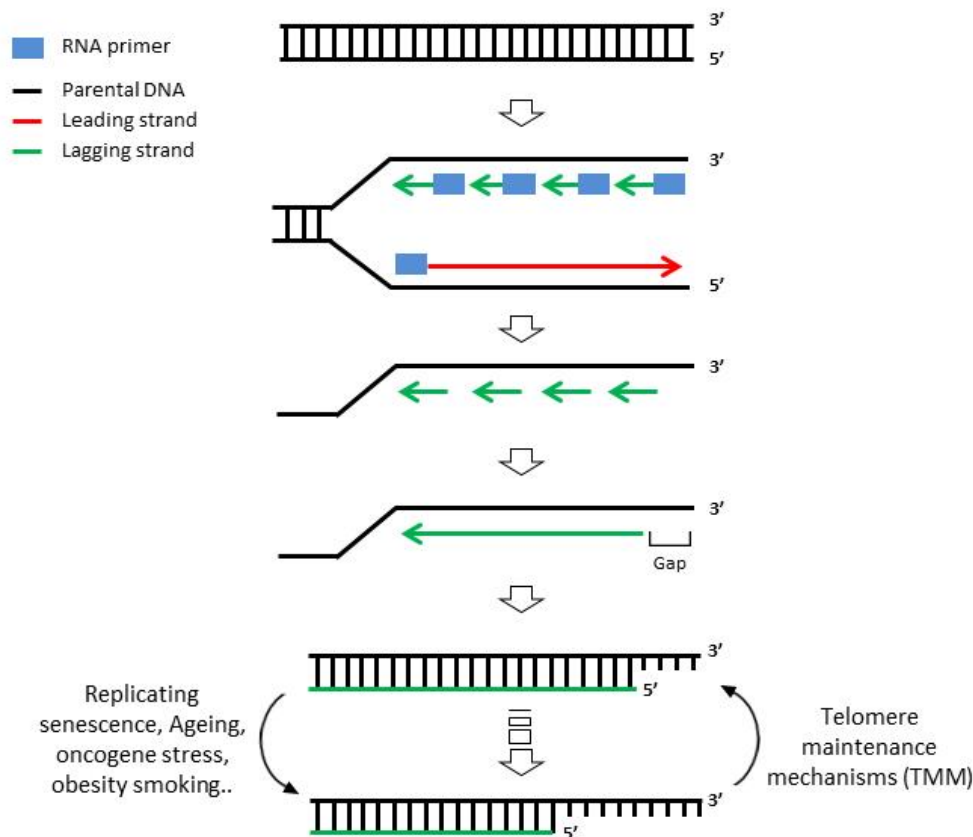
Figure 19| Cell division related telomere shortening. The chromosome end replication problem results from the most proximal RNA primer degradation on the lagging strand. The remaining "gap" 3' overhang cannot be completed by the DNA Pol, causing telomere shortening at each cell division and risking finally replicative senescence and ageing. To prevent telomere shortening and senescence, majority of cancer cells reactivate telomere via telomere maintenance mechanisms (TMM).

During metazoan evolution there may have been a strong selective advantage for programmed senescence of essentially all non-germline cells. Cellular mortality confers a strict level of growth control and reduces the probability of deleterious hyperplasia or cancer (Harley et al. 1990). In animals, cells of many somatic tissues have a finite replicative lifespan which contributes to senescence and ageing of the organism (Stanulis 1987). Olovnikov and colleagues (Olovnikov 1973; Yu et al. 1990; Levy et al. 1992) proposed that somatic cells may not overcome the "end-replication problem" and thus telomeric deletions would accumulate at each generation until a critical deletion is made that causes cell death (**Figure 19**). This hypothesis was supported by data showing that telomeres become shorter during aging of human cells *in vitro* during cell culture and *in vivo* upon mitogenic signals or with age (Harley et al. 1990; Allshire et al. 1986; Levy et al. 1992).

More recent studies directly measured telomeres length in patient's white blood cell population showing striking correlation between aging and cells telomere shortening (Vaziri et al. 1994; Hochstrasser et al. 2012). Interestingly, it was observed in humans, that the average rate of telomere shortening is higher after birth in 0 to 3 years old children (~170-270 bp/year), before eventually reaching to rates observed in adults (~30-50 bp/yr) (Slagboom et al. 1994; Zeichner et al. 1999). In some particular cases, cells are able to bypass replicative senescence upon telomere shortening thanks to inhibition of the tumor suppressor P53 and pRb proteins. Indeed, normally when telomeres reach critical short length, telomeres are uncapped and are detected as dsDNA breaks hence triggering P53 activation and cell cycle arrest. When cells bypass cell cycle arrest and continue to proliferate and to undergo telomere shortening, they enter a "crisis" state. This particular phenotype was described to undergo multiple chromosome fusions and bridge-breakage-fusion cycles associated with high level of apoptosis. Surviving to crisis will only be possible for the cell thanks to the activation of telomere maintenance mechanisms (TMM). Once TMM is activated, cells acquire the unlimited capacity to divide and become immortal; this is a strict condition for development of metastasis in cancer. The different TMM are discussed below.

I. 4. Telomere maintenance mechanisms

a/ Telomerase

Eukaryotic telomeres end replication can be efficiently mediated by original molecular machinery called telomerase. This unique enzyme contains a catalytic subunit, telomerase reverse transcriptase (TERT) and an RNA template (TERC) ("CCCAAUCCC" in vertebrates) which is used when it elongates telomeres (**Figure 20**).
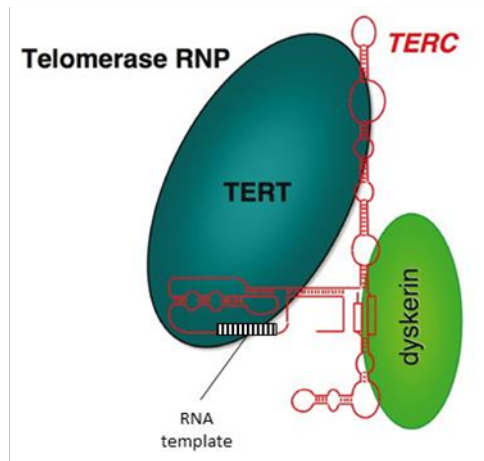
Figure 20| Structure of human telomerase. Telomerase active ribonucleoprotein is a holoenzyme composed of a catalytically active TERT subunit, the telomerase RNA (TERC), and dyskerin. Mutations in one of the three components of active telomerase lead to the clinical disease of dyskeratosis congenita.

Direct evidence that telomerase maintains telomere length *in vivo* comes from studies of mutations in the template region of the RNA component of Tetrahymena telomerase, which caused both an altered telomere sequence and altered telomere length (Yu et al. 1990). By synthesizing multiple tandem repeats of telomeric DNA encoded by its RNA template, telomerase compensates for the erosion of DNA ends during replication and provides the docking sites for telomeric proteins that bind specifically to the ends of chromosomes to distinguish them from broken DNA ends (**Figure 21**).

Human telomere synthesis occurs early in development (Collins and Mitchell, 2002; Cong et al., 2002). The majority of adult somatic cells lack telomerase activity entirely or have very low levels (Masutomi et al. 2003) hence, telomeres gradually shorten, limiting cell division capacity (Levy et al. 1992). However, in the majority of human cancers, telomerase is reactivated and provides the sustained proliferative capacity of these cells (Artandi & DePinho 2009).
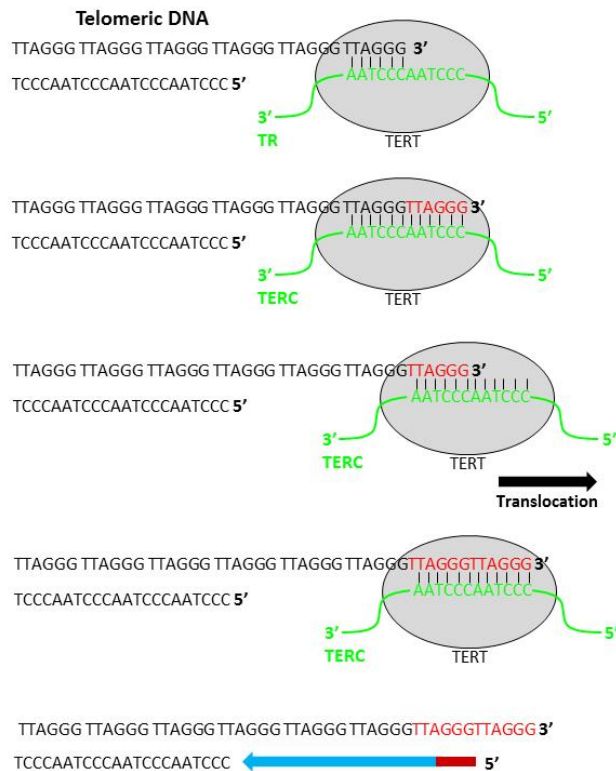
Figure 21| Telomere elongation by telomerase. TERT (grey) binds to telomeric DNA repeats (red) through its own (TERC) RNA template (green), adds one TTAGGG repeat to the 3' end of the chromosome end. Next, a translocation step is necessary to realign the TR template and allow synthesis of additional telomeric repeats. Finally after telomere repeats have been added to the 3' end, C-rich strand (blue arrow) synthesis is initiated by the addition of an RNA primer and carried by classical replication machinery.

This belief led to the hypothesis agents capable of inhibiting telomerase activity might cause the telomeres of cancer cells to erode and limit their proliferation. Successful anti-telomerase drugs would act by a mechanism different from all existing drugs, where cells will not be killed immediately. Instead, they would cause steady telomere shortening until a critical point was reached, potentially providing a valuable new tool for treating cancer (Corey 2010). An understanding of telomerase biology thus has important implications for both cancer and aging. Indeed, telomerase has also been linked to ageing, as telomere loss may result in tissue atrophy, stem cell depletion and deficient tissue regeneration. In humans, loss-of-function mutations in either TERT or TR have been associated with dyskeratosis congenita and cases of aplastic anaemia and pulmonary fibrosis (Armanios & Blackburn 2012).

Evidence have been presented that telomerase may exert several "non-canonical" functions that do not imply or depend on telomere elongation. Subsequently to the data published by Stewart et al. (Masutomi et al. 2003) showing that the ectopic expression of

catalytically dead telomerase was able to facilitate tumorigenicity, several non-canonical roles have been proposed for telomerase. Namely, telomerase was shown to play a role in cell proliferation, genome stability and protection against apoptosis (Saretzki 2009).

b/ ALT

Although in most organisms telomeres are being maintained by telomerase (mammals, fish, plants, yeast, birds) (Sýkorová & Fajkus 2009), an exception have been observed in D. melanogaster which maintains its telomeres thanks to a retrotransposon-based system. Thus, in contrast with other species drosophila telomeric sequences are made of arrays of retrotransposons instead of the unusual G-rich sequences (Abad et al. 2004). Drosophila and some other insects a side, another mechanism of telomere length maintenance is the ALT (Alternative lengthening of telomeres) pathway which drives telomere elongation via homologous recombination (HR). Although it is generally agreed that telomere elongation in ALT cells requires a DNA recombination step, the mechanism of the lengthening step is uncertain. Two suggested mechanisms for telomere elongation, which are not mutually exclusive, are described in **Figure 22**.
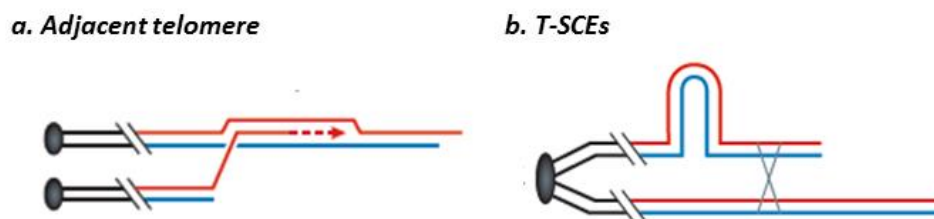
Figure 22| Models for alternative lengthening of telomeres. Suggested mechanisms for telomere elongation: **a.** Recombination-mediated synthesis of new telomeric DNA, using an existing telomeric template sequence from an adjacent chromosomal. **b.** Unequal telomere sister chromatid exchanges (T-SCEs) can occur and result in one daughter cell that has a lengthened telomere and therefore a prolonged proliferative capacity, and another daughter cell with a shortened telomere and decreased proliferative capacity. This could result in the unlimited proliferation of the cell population. *Adapted from: Cesare et al_2010_Nat Rev.*

Employed in 10-15% of "telomerase negative cancers" and in sarcomas of complicated karyotypes (Nowak et al. n.d.), ALT could provide cancer cells with an escape exist to elongate their telomeres even under telomerase-targeted therapies. Indeed, Hu et al. demonstrate that in conditional TERT knockdown mice that developed tumors, telomerase extinction led to the apparition of ALT phenotype in the tumor cells (Hu et al. 2012). Investigating ALT molecular mechanisms implicated in telomere elongation are highly investigated in the field of telomere-targeting cancer therapies.

ALT cells display several hallmarks that allow to distinguish them from telomerase positive cells. The ALT positive cancer cells are characterized by very long heterogeneous telomere DNA, the presence of extra-chromosomal repeats (ECTR), extensive genomic instability and DNA damage signaling, and deficient G2/M checkpoint of the cell cycle (Bryan et al. 1995; Cesare & Reddel 2010). Second, ALT positive cancer cells are also known to associate with promyelocytic leukemia (PML) bodies which are dynamic nuclear structures involved in many cellular processes, especially in DNA repair and proteins post translational modifications (Yeager et al. 1999). Yet, the observed tight correlation between ALT activation and PML bodies formation was suggested not be critic for ALT (Pickett et al. 2009). One of the hallmarks of ALT cancer cells is also their strong chromatin decondensation including hypomethylated DNA at subtelomeric regions correlated with telomere increased transcription and recombination (Episkopou et al. 2014). Indeed, various nuclear receptors binding to variant repeats and expressing their genes are a unique property of the ALT cells. Such activation may change the heterochromatic condition of the ALT telomeres and further more help in de-repression of telomeric recombination.

II.   Telomere transcripts – TERRA

Despite their compact heterochromatin state telomeres were surprisingly found, in 2007, to generate long non-coding RNAs named TERRA (TElomeric Repeat containing RNA). Telomeric transcripts have been identified as the third entity of the telomere nucleoprotein complex, providing newer insights into the regulation of telomeres (Azzalin et al. 2007).

II. 1.   Biogenesis

The first discovery of telomere-originated transcripts, in mammals, showed telomeres were transcribed into heterogeneous long non-coding RNA called TERRA (Azzalin et al. 2007; Blasco & Schoeftner 2008). However further studies in other eukaryotes proved the existence of other lncRNA species originate from chromosome ends and form, together with TERRA, the telomeric transcriptome (**Figure 23**) (Luke & Lingner 2009; Azzalin & Lingner 2015; Martínez-Guitarte et al. 2008).
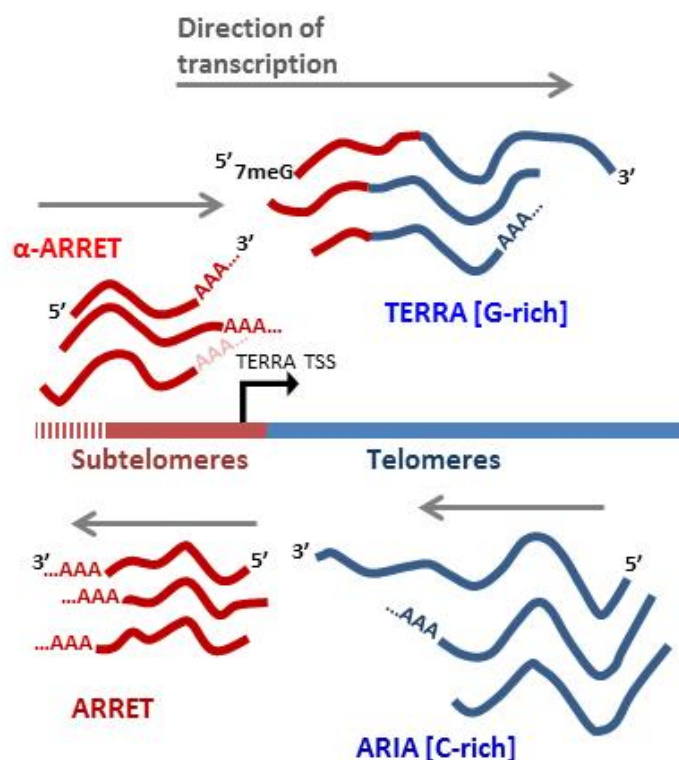
Figure 23| The telomeric transcriptome. Schematic representation of RNA species generated by transcription of eukaryotic chromosome ends. RNA and DNA sequences of telomeric (blue) or subtelomeric (red) origin are indicated. AAA. . . indicates poly(A) tails present at the 3' end of a fraction of TERRA and ARIA, and of all ARRET and α-ARRET species. 7meG indicates 7- methyl-guanosine caps present at TERRA 5' ends, while their presence on other RNA species has not been tested yet. Grey arrows indicate the direction of transcription. TERRA transcription start-sites (TSS) are located in the subtelomeric regions. While in mammals only TERRA RNAs were detected, the totality of depicted telomeric transcripts has so far only been identified in *S. pombe*. *Adapted from: Azzalin & Ligner_2015_Cell Press.*

TERRA transcription was found to be initiated within a subset of subtelomeric regions containing CpG islands and to end at the telomeric tract (Nergadze et al. 2009; Negishi et al. 2015). Importantly, the lengths of the telomeric repeat tracts of TERRA are heterogeneous. Consequently, TERRA molecules all share a G-rich telomeric repeats part and can be differentiated thanks to their subtelomeric-originated chromosome specific sequences (**Figure 23**). TERRA levels were found to be regulated through the cell cycle, with a visible accumulation pick in early G1 and a lowest expression levels at the transition between late S and G2. Subcellular localization of TERRA molecules by northern blot after cell fractionation or by *in situ* RNA-FISH experiments showed TERRA molecules were nearly exclusively nuclear and form discrete foci that partially localizes to telomeres (Azzalin et al. 2007). Less is known on the dynamics of TERRA localization in human cells. Mammalian telomeric transcripts were shown to associate with only a subset of chromosome ends at a given time (Azzalin et al. 2007; Lai et al. 2013), while a fraction of telomeric RNAs also resides within the nucleoplasm (Porro et al. 2010) suggesting, that TERRA molecules are not constitutively bound to telomeres. Telomeric RNA localization at telomeres is modulated by the nonsense-

mediated decay machinery in vertebrates (Azzalin et al. 2007; Luke & Lingner 2009). TERRA foci size and number was shown to be variable according to cell type, species and to environmental conditions (Azzalin et al. 2007; Blasco & Schoeftner 2008).

Human TERRA size ranges from~100 bases up to at least 9 kb and the vast majority of human TERRA 5' ends harbor a canonical m7G cap (guanosine is methylated on the 7 position) structure, suggested to protect TERRA from the action of 5'-to-3' exonucleases. The majority (90%) of TERRA molecules are not polyadenylated at their 3'end. Differences in biochemical behavior between the poly(A) negative and positive TERRA fractions were observed. Polyadenylated TERRA is more stable and was found to be mostly in a non-chromatin-associated pool of RNAs. Thus, the majority of TERRA (poly (A)-) are bound to telomeric chromatin. How the canonical poly(A) polymerase may polyadenylate TERRA remains to be explored as the classical cleavage and polyadenylation signals appear to be missing in the TERRA sequence. It has been recently suggested that telomeric transcription represents a challenge for polymerases and that only a fraction of telomeres are fully transcribed (**Figure 24**) (Azzalin & Lingner 2015). Indeed, measurement of the UUAGGG tract length by reverse transcription in the absence of dGTP indicated that a large fraction of human TERRA molecules does not contain cytosine-lacking stretches that exceed 400 bases, even though telomeres in the same cells extend for several kilo-bases (Porro et al. 2010). This suggests that the pure UUAGGG-tract length is considerably shorter than its C-rich telomeric DNA template. It is important to note, one cannot rule out that TERRA transcription may also start within telomeric repeats or within immediate proximity thereof, leading to underestimation of the real number of transcribed chromosome ends; similarly, cell line-specific effects that could impact the distribution of TERRA-expressing telomeres (Porro et al. 2014).

RNAPII (RNA Polymerase II) was shown to binds to TERRA promoters *in vivo* and to be the principal polymerase responsible for telomere transcription in mammals (Azzalin et al. 2007; Blasco & Schoeftner 2008). Very little is known about transcription factors involved in TERRA regulation. The transcription regulator CTCF (CCCTC-binding factor) and the cohesin Rad21 (radiation-sensitive 21) were found to be involved in TERRA regulation since, depletion of CTCF diminished TERRA levels as well as RNAPII and cohesin binding to

subtelomeres (Deng, Wang, Stong, et al. 2012). However, recent bioinformatics analysis of putative TERRA promoters were recently found to harbor numerous potential transcription factors binding sites (Porro et al. 2014) (**Figure 24**).

II. 2.   TERRA transcriptional regulation

a/ By promoter methylation

Human TERRA promoters are characterized by high density of methylated-CpG dinucleotides under normal conditions, suggesting TERRA expression could be regulated by DNA methylation of subtelomeric promoters (Nergadze et al. 2009) (**Figure 24**). Interestingly and consisting with this, subtelomeric DNA is hypomethylated in human sperm and ova, regions are subjected to de novo methylation during development (Brock et al. 1999). These results are nicely coincident with recent discovery of increased TERRA expression in human germ cells (Reig-Viader et al. 2013; Reig-Viader et al. 2014). Moreover coincident with these findings, research on ICF (Immunodeficiency, Centromere instability, Facial anomalies) syndrome showed patients' cells harbor drastically hypomethylated subtelomeres due to a DNMT3B mutation accompanied by~4 fold increase in TERRA global transcription (Yehezkel et al. 2008). Similarly human cancer cells (HCT116, HeLa) depleted for DNMT1 and 3B also display subtelomeric hypomethylation and RNAPII-PS2 enrichment resulting in increased TERRA levels (Nergadze et al. 2009; Farnung et al. 2012). Finally as mentioned before, one of the hallmarks in ALT cancer cells is subtelomeric loss of DNA methylation accompanied by high level of telomeres expression (Episkopou et al. 2014). Taken together these data underline DNA methylation in human cells as a major regulator of telomere transcription. It is noteworthy, that early studies in mice embryonic cell lines (ES) DNA methyl-transferases deficiency did not drastically impact TERRA expression proposing a species-specific pathway for TERRA regulation (Blasco & Schoeftner 2008) (**Figure 24**).

b/ By associated epigenetic histones modifications

Histone modification marks were also shown to be implicated in TERRA regulation. Trichostatin A-treated (global histone deacetylase) human HeLa cancer cells display increased TERRA levels (Azzalin & Lingner 2008). Moreover, disturbance of histone methyl-transferases (SUV39H1, SUV4-20H) or HP1 both in human and murine cell resulted in general

decrease (including at telomeres) of histone methylation at corresponding histones and an elevated TERRA levels (Blasco & Schoeftner 2008; Benetti, Gonzalo, et al. 2007; Arnoult et al. 2012). Also, in the case of ES and induced pluripotent cells telomeric chromatin were found to have lower levels of the heterochromatin marker (H3K9me3, H4K20me3) and increased transcription of telomeric repeat-containing RNA in comparison to differentiated cells (Marion et al. 2009). In ES cells, the histone chaperone ATRX was also attributed a role in TERRA regulation and in H3.3 histone variant insertion to telomere since ATRX depletion led to a reproducible 1.7 fold upregulation of TERRA (Goldberg et al. 2010). Together these data, suggest a role for a more open chromatin in TERRA upregulation. It was hypothesized that telomere heterochromatic status may affect telomere expression, possibly by inhibiting transcriptional elongation.

However the precise different epigenetic signatures leading to TERRA modulations remain unclear and set controversy in the field. Since, in telomerase-deficient mice with short telomeres lacking DNMTase or Dicer activities, TelRNA levels were slightly reduced concomitant with an increased density of histone trimethylation marks (Blasco & Schoeftner 2008). Consistently, Caslini et al. shown that the MLL (Mixed Lineage Leukemia) dependent-H3/K4 methylation at telomeres is a mark associated with active TERRA transcription. Indeed, MLL depletion in human diploid fibroblasts affected heterochromatin marks at telomeres and decreased overall TERRA levels by 28% (Caslini et al. 2009). Thus, open telomeric chromatin does not necessarily correlate with an increased level of telomere transcription and may instead rely on other factors such as the association of MLL at telomeres. Thus, heterochromatin regulation mediated by DNA methylation and histone modifications as well as telomere length directly impacts on TERRA expression.
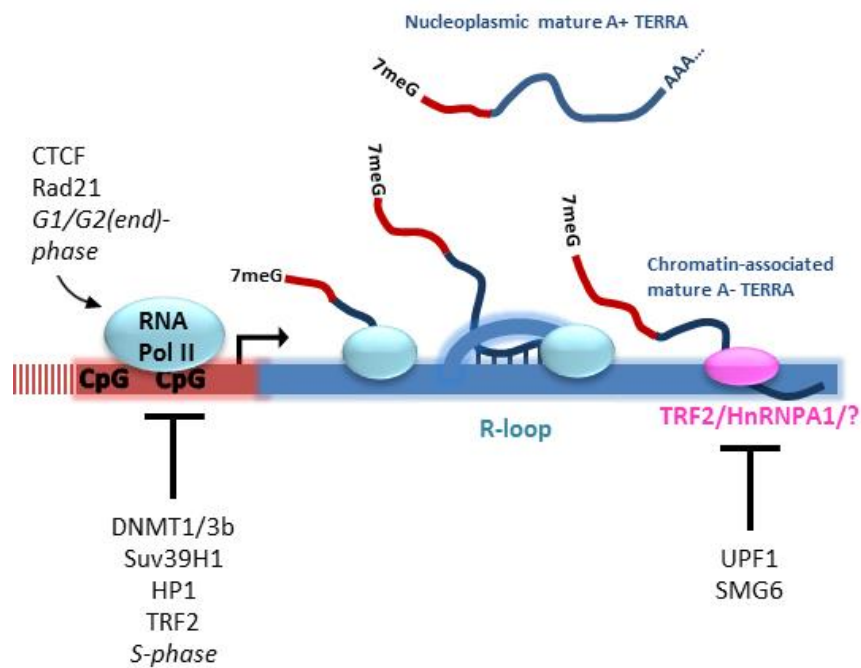
Figure 24| Regulation and biogenesis of TERRAs (telomeric repeat-containing RNA). Human TERRA is expressed during G1 and G2(end) phases of the cell cycle but is repressed during S-phase. CTCF and Rad21 promote human TERRA transcription whereas DNMT1 and 3b, SUV39H1 and HP1α, and TRF2 repress TERRA. TERRA was found to be able to form RNA:DNA hybrids with telomeric repeats. HnRNPA1 (A1), TRF2, or other unknown factors may promote TERRA association with human telomeres. Human TERRA association with telomeres is negatively regulated by the NMD components UPF1 and SMG6. In human cells, polyadenylated TERRA is not chromatin-associated whereas poly(A) TERRA is largely bound to chromatin. ***Adapted from: Azzalin & Ligner_2015_Cell Press.***

c/ By telomere length and shelterin

Telomere length and associated shelterin complex integrity was shown to impact TERRA levels. In addition to the demonstration that telomere elongation regulates TERRA transcription via increase in heterochromatin marks at telomeres (Arnoult et al. 2012) in mammals, both TRF1 and TRF2 were shown to be directly implicated in TERRA transcriptional regulation. While TRF1 binding to RNAPII was shown to be important to TERRA transcription, in human cell lines TRF2 was shown to directly bind and repress telomeric transcripts at telomeres. Indeed, TRF1 siRNA-mediated inhibition resulted in a twofold decrease of TERRA level in MEFs cells (Blasco & Schoeftner 2008). On the other hand, TRF2 was shown to physically interact with TelRNA *in vitro* and *in vivo* (Deng et al. 2009; Poulet et al. 2012). Moreover, experiences reproducing telomere uncapping processes in different human cell lines, through partial or complete TRF2 depletions were correlated with telomere shortening and drastic increase in TERRA levels (Blasco & Schoeftner 2008; Caslini et al. 2009; Porro et al. 2014). In line with these data, ICF cells that present

abnormally elevated TERRA levels also show fourfold decrease of TRF2 at CpG demethylated-subtelomeric regions (Deng et al. 2009). Thus TRF2 was proposed to negatively regulate TERRA transcription *in vivo* (Porro et al. 2014) (**Figure 25**).
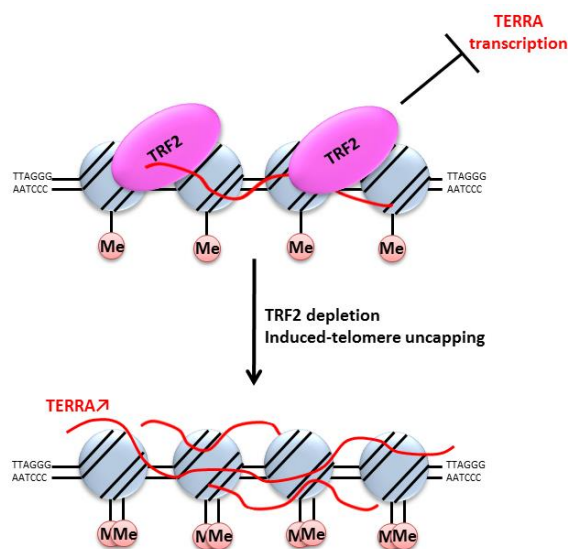
Figure 25| Model for TRF2 regulation of TERRA transcription. *In vivo*, the telomeric shelterin member TRF2 directly binds TERRA transcripts and negatively regulates its constitutive expression level. When TRF2 is removed, the higher order telomere structures are disrupted and the telomeric chromatin becomes accessible to RNAPII. Thus, increased TERRA levels can be detected and correlate with increased repressive epigenetic marks (Me) at telomeres. Arnoult et al. (2012) suggested, TERRA may represent a negative-feedback-loop mechanism that would prevent telomeres from hyper formation of heterochromatin. ***Adapted from: Porro et al_2014_Nat.Com.***

II. 3.   TERRA attributed functions

Initially, lncRNA TERRA has been implicated in telomere maintenance in a telomerase-dependent and a telomerase-independent manner during replicative senescence and cancer. However accumulating studies now show, TERRA's proposed activities are diverse (Azzalin & Lingner 2015).

The majority of mammalian TERRA molecules remain associated to telomeres (Azzalin et al. 2007); hence, it was suggested, a key for understanding TERRA functions may be the analysis of the numerous recently discovered TERRA-associated proteins (Azzalin et al. 2007; Porro et al. 2010). Indeed, the importance of telomeric proteome variations has been highlighted not only during telomere length changes, but also during cell cycle progression, normal development, aging, and during the development of pathologies such as cancer.

Several different facts about TERRA led scientists to the hypothesis it may play a pivotal role in telomere protein composition changes via an RNA-mediated interaction: I.

TERRA levels and telomere-binding capacity were shown to be tightly regulated through the cell cycle (Azzalin et al. 2007; Porro et al. 2010), II. Several TERRA molecular partners were all identified to have essential role in telomere biology (Deng et al. 2009; Lopez de Silanes et al. 2010; Redon et al. 2013) and III. TERRA expression was interestingly shown to be modulated in response to environmental stimuli such as stress (Blasco & Schoeftner 2008; Martínez-Guitarte et al. 2008; Eymery et al. 2009). The following paragraph will try to go through the main data supporting TERRA functions.

a/ Telomere length regulation

Among the different functions that have been assigned to TERRA transcripts, the first was a role in telomerase regulation (Cusanelli et al. 2013; Redon et al. 2010; Blasco & Schoeftner 2008) and in telomerase-mediated telomere elongation (Moravec et al. 2016).

Interestingly, some clues led to those hypotheses even before TERRA discovery. Aiming to dissect telomere properties and inspired by the fact centromere activity was disturbed by induced transcription. Sandell et al. reported in yeast *S. cerevisae* that artificial activation of telomere transcription induced a subtle and reversible shortening of telomeric length (Sandell et al. 1994). Later on with the discovery of TERRA, several observations such as cells from ICF patients harboring very short telomeres and several fold higher TERRA expression supported that same idea (Yehezkel et al. 2008; Deng et al. 2009). Moreover, the complementarity between TERRA-UUAGGG repeats and the template sequence within TERC (telomerase RNA template) opened the possibility that these TelRNAs could inhibit telomerase activity by blocking the TERC template region. Reinforcing the hypothesis TERRA exerts its function on telomere length via an impact on telomerase was supported by different *in vitro* studies where telomerase activity was completely abolished in mouse ES cells and human HeLa cells on addition of 2 picomol [UUAGGG]3 RNA oligonucleotide (Blasco & Schoeftner 2008). In line with these studies, Redon et al. demonstrated TERRA was able to bind hTERT *in vivo* and both telomerase subunits *in vitro* suggesting TERRA as a ligand and natural direct inhibitor of human telomerase (Redon et al. 2010) (**Figure 26**).

Although the promising potential of TERRA functions as a negative telomerase regulator, contrasting *in vivo* studies were published and challenged this hypothesis. Indeed,

human cancer cells harboring important TERRA upregulation, due to DNMT depletion, did not present any difficulty to continue and elongate telomeres in telomerase positive cells (Farnung et al. 2012). Moreover, inducible promoter inserted upstream to a chromosome specific telomere did not present any telomere shortening throughout cell culture and restoration of telomere length after telomerase chemical-inhibition (BIBR1532) was still possible (Arora et al. 2012). Possible explanations for these discrepancies could be that *in vitro* studies do not account for TERRA regulation through cell cycle neither for TERRA-binding proteins. Indeed, TERRA is repressed under S phase whereas telomerase is expressed and active at that same moment (Masutomi et al. 2003; Tomlinson et al. 2006) suggesting *in vivo*, TERRA and telomerase subunits may not bind and localized to telomeres as proposed. A second possibility could consider the implication of hnRNPA1 ribonucleoproteins suggested to co-act with TERRA to regulate telomerase and telomeres length (Redon et al. 2013) (**Figure 26**).

Figure 26| Telomerase-dependent telomere length regulation by TERRA. In human, whether if TERRA is implicated in telomerase-dependent telomere length regulation is still discussed. TERRA-sequence complementarity with the telomerase TERC RNA was showed to inhibit telomerase *in vitro*. However, *in vivo*, the presence of TERRA-binding proteins such as the hnRNPA1 was suggested to limit or prevent telomerase interaction and thus inhibition by TERRA. *Adapted from: Azzalin & Ligner_2015_Cell Press*

While TERRA functions as a negative telomerase regulator remains unclear in mammals it is noteworthy that very recent publication in yeast *S. pombe*, give direct evidence that TERRA stimulates telomerase recruitment and activity at chromosome ends.

In their model Moravec et al. propose TERRA produced upon telomere shortening is polyadenylated, largely devoid of telomeric repeats and furthermore, telomerase physically interacts with this polyadenylated TERRA *in vivo*. Their model, speculate TERRA plays a role

in mediating telomerase-dependent elongation of short telomeres (Moravec et al. 2016) suggesting TERRA may have species-specific roles.

b/ Telomeric heterochromatin formation

A second mechanism was proposed for telomere length-regulation by TERRA in a telomerase independent manner. Arnoult et al. used diverse human cell lines harboring short or long telomeres and demonstrated TERRA transcription is down regulated upon telomere elongation. Their results are the first to highlight a TPE (Telomere Position Effect) in human cells that they suggest to occur through telomere elongation-associated heterochromatinization negative feed-back loop (Arnoult et al. 2012). Their data also highlight a correlation between the cell cycle variations of TERRA expression and telomeric HP1 as well as H3K9me recruitment, supporting TERRA implication in telomeric heterochromatin formation (**Figure 27**).
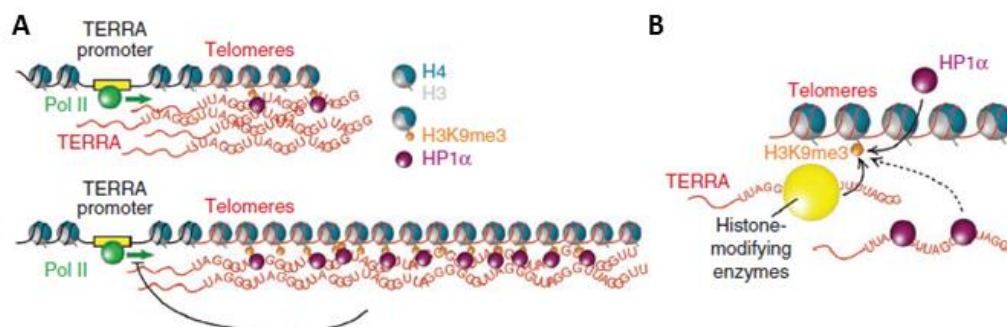
Figure 27| A. Proposed model for HP1α-dependent TPE on TERRA. In their model Arnoult et al. suggest telomere lengthening results in TERRA upregulation that, in turn, induce its own transcriptional repression in a negative-feedback loop that would prevent telomeres from hyper formation of heterochromatin. **B. Hypothetical role of TERRAs in TPE**. Increasing TERRA UUAGGG repeats number favors the recruitment of more HP1α and increases H3K9me3 at telomeres. Consequently TERRA upregulation contributes to telomeric heterochromatin formation and TPE. *Adapted from: Arnoult et al_2012_Nat Struct & Mol Biol.*

Deng et al. (Deng et al. 2009) identified two ORC (chromatin silencing complex) subunits, ORC1 and ORC5, and showed that the ORC complex is recruited to TERRA by TRF2, in Raji cells (human lymphoma). In addition they showed that TERRA directly interacts with HP1 subunits, TRF2 and H3K9me3. These results suggest a model where TERRA may participate in heterochromatin formation at telomeres via ORC recruitment (**Figure 28**).
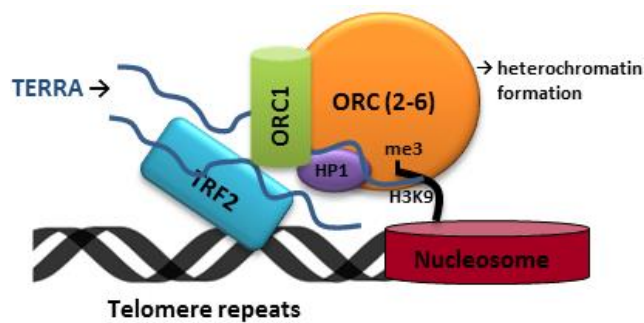
Figure 28| Model for TERRA involvement in telomeric heterochromatin formation. Deng et al. (2009) proposed TERRA is recruited to telomeres via its interaction with telomeric TRF2 and H3K9me3. TERRA presence at telomeres favors the recruitment of HP1 and ORC complex hence, leading to telomere heterochromatinisation. *Adapted from: Deng et al_ 2009_Mol Cell.*

A more recent example that may support this particular TERRA function was published this year by Lieberman's lab. They provide evidence that the Tumor Suppressor protein TP53 (P53) can be found associated with non-canonical binding sites located at subtelomeric regions in mouse and human cells under stress-induced nutrients deprivation. In their publication Tutton et al. (Tutton et al. 2015) propose a model where stress-induced telomere damage is prevented via a direct-chromatin binding of P53 to subtelomeric regions leading to histone acetylation and concomitant TERRA transcription.

c/ Telomere Replication

In mammals, the single-stranded telomeric DNA is bound by POT1/TPP1 during most stages of the cell cycle. In S-phase, however, ssDNA may also be bound by RPA (Replication Protein A), which promotes semiconservative DNA replication but can also induce ATR-dependent DNA damage repair (DDR). TERRA has been proposed to prevent RPA displacement during early stages of the S-phase ensuring proper telomere replication, while in late-S, TERRA is repressed and the POT1/RPA switch can occur and contribute to the essential telomeric cap (Flynn et al. 2011). Hence, TERRA can contribute to telomere replication but also protection (**Figure 29**).
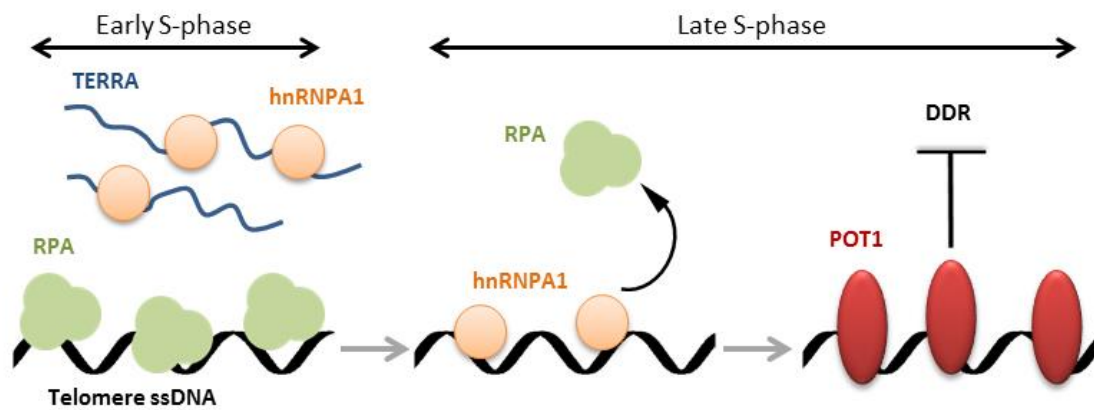
Figure 29| RPA-to-PO1 switch at telomeric ssDNA during replication. In early S-phase, RPA binds to telomeric ssDNA in order to ensure DNA replication. TERRA high levels during early S-phase prevent hnRNPA1 from displacing RPA from telomeres. During S-phase progression TERRA levels are at the minimum, liberating hnRNPA1 that displaces RPA from the telomeric repeats, subsequently, POT1 is allowed to bind ssDNA and prevent DDR machinery from recognizing telomeres. *Adapted from: Flynn et al _ 2011 _ Nature*.

d/ Processing of uncapped telomeres

Results from Lingner's group suggested a direct role for TERRA in the processing of uncapped telomeres. As mention before, telomeric DNA is associated with shelterin complex that protects telomeres. TRF2 depleted telomeres as well as critically short telomeres elicit a robust ATM DNA damage repair which involves formation of TIFs (Telomere dysfunction-Induced Foci) that can undergo telomere fusions events by NHEJ (Non homologous End Joining) repair and require prior removal of the telomeric 3'G overhang by the nuclease activity of Mre11/Rad50/NBS1 complex. Lingner's lab with others, with other, they demonstrate that in human cells telomeres deprotection, through TRF2 depletion, is correlated with TERRA upregulation (Caslini et al. 2009; Porro et al. 2014) and coinciding with lysine demethylase LSD1 (lysine demethylase 1) recruitment. TERRA is able to bind to LSD1 allowing its association with Mre11 that stimulates Mre11 catalytic activity and nucleolytic processing of uncapped telomeres. Whether LSD1 directly activates MRE11 through demethylation of lysine remains unknown. Together, these data suggest that TERRA may also assist telomere-remodeling events (Porro et al. 2014) (**Figure 30**)
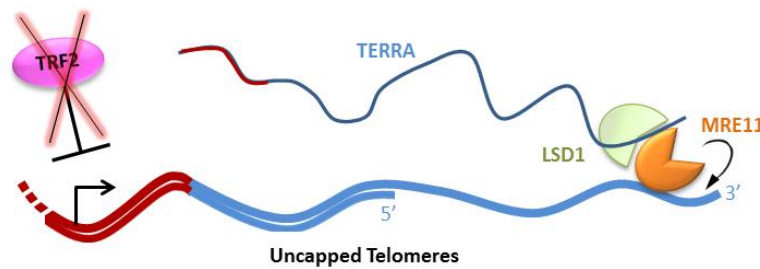
Figure 30| TERRA role in uncapped-telomere processing. B. Depletion of TRF2 in human cells leads to upregulation of TERRA coinciding with recruitment of the lysine demethylase LSD1. LSD1 binding to telomeres in TRF2-depleted cells depends on the DNA repair protein MRE11 and its complex partners RAD50 and NBS1. The LSD1–MRE11 interaction is strongly stimulated by TERRA *in vitro*. LSD1 is required for efficient removal of 3' overhangs at uncapped telomeres possibly through its ability to activate MRE11 nuclease activities. ***Adapted from: Azzalin & Ligner_2015_Cell Press***

Although there have been substantial advances in understanding the biogenesis and regulation of TERRA in cells from eukaryotes including yeast and humans (recently reviewed by (Maicher et al. 2014; Azzalin & Lingner 2015; Cusanelli & Chartrand 2015), the functional relevance of telomere transcription remains to be determined.

e/ Implications in immunity cancer and disease

As discussed previously and even though all aspects of TERRA biogenesis are not yet resolved, TERRA has already been connected to crucial telomeric roles including telomere length regulation, telomere replication and telomere protection. Therefore, if the tight regulation of TERRA biogenesis is disturbed it may sustain key events implicated in different diseases such as ICF or cancer.

The ICF syndrome may represent an example in which TERRA deregulation may cause disease. ICF patients' derived cells show increased TERRA levels likely to derive from augmented transcription of hypomethylated TERRA promoters. Telomeres are also much shorter in ICF cells. Although it has not yet been tested, it was suggested that increased TERRA transcription is responsible for telomere shortening possibly through inhibition of telomerase, increased accessibility to exonuclease 1, or accruing excess of telomeric R-loops that compromise telomere replication (Yehezkel et al. 2008; Deng et al. 2010).

Recent evidence has established that endogenous TERRA transcripts can base-pair with their template DNA strand forming RNA:DNA hybrid structures known as R-loop (Balk et

al. 2013; Arora et al. 2014; Yu et al. 2014). In the case of telomerase-deficient so-called ALT cancer cells, it was hypothesized that TERRA can form R-loop structures within the genome that may be able to trigger recombination and replication fork stalling (Aguilera & García-Muse 2012; Cesare & Reddel 2010), for telomere maintenance and eventually cell proliferation. The high level of TERRA detected in ALT cells raised the question, to what extent TERRA contributes to the indispensable R-loop induced homologous recombination (HR) events in those cells, in order to prevent telomere loss. Development of anti-ALT cancer therapeutics may rely in the future, on the identification and our understanding of factors regulating telomeric RNA/DNA hybrids in ALT cells (Arora et al. 2014; Azzalin & Lingner 2015).

In addition, recent publication has suggested that, in human malignant cells, TERRA can harbor a G4 quadruplex structure to downregulate innate immune genes like STAT1, ISG15 and OAS3 supposed to counteract malignancy (Kyotaro Hirashima & Seimiya 2015). Hirishima et al. propose a physiological role for TERRA in regulating gene expression in a genome-wide manner. In line with this, TERRA was recently identified as a component of extracellular inflammatory exosomes in mouse tumor and embryonic brain tissue, as well as in human tissue culture cell lines (Wang et al. 2015). These findings imply a previously unidentified extrinsic function for TERRA and a mechanism of communication between telomeres and innate immune signals in tissue and tumor microenvironments.

Finally, the expression and function of TERRA in the context of high proliferative cells remains poorly understood. However, data start to accumulate, showing TERRA expression levels are upregulated both in mammalian iPS (induced-Pluripotent Stem cells) cells (Marion et al. 2009; Yehezkel et al. 2008) and in cancer cells (Z Deng, Wang, Xiang, et al. 2012). These data highlight the fact TERRA expression may correlate with proliferative capacity and contributes to nuclear reprogramming (Z Deng, Wang, Xiang, et al. 2012)

THE PhD PROJECT'S ORIGINS

AND OBJECTIVES

I. The project's origins

- **Living organisms facing environmental stress**

Organisms, tissues and cells are constantly being challenged by their exposure to stressful environmental cues. In response to stress, cells from yeast to man have developed a series of events termed the cellular heat shock response (HSR). The cellular HSR involves, transcriptional changes associated with genome-wide chromatin remodeling, activation of protein chaperones and DNA damage response pathway, that are all induced to assist the cell in its recovery and survival.

The mammalian transcription factor Heat Shock Factor 1 (HSF1) was discovered 20 years ago and highlighted as the master regulator of the well-conserved cellular response to stress (Damberger et al. 1994; Jurivich et al. 1995; Shi et al. 1998; Akerfelt et al. 2010). HSF1 was first showed to exert its function by binding *HSP* (Heat Stress Proteins) gene promoters, encoding chaperone proteins that participate to cell survival and protein homeostasis (Wu 1984; Perisic et al. 1989; Clos et al. 1993; Wu 1995).

Interestingly, more recent data pointed out HSF1 as an activator and remodeler of repetitive genome sequences such as pericentromeres (9q12) (Metz et al. 2004; Eymery et al. 2010). Function and regulation of these pericentromeric ncRNA SatIII are still enigmatic and the understanding of mechanisms associated to their activation will surely shed light in the raising field of ncRNAs functions in the context of cellular stress response.

Even more interestingly, actual publications revealed HSF1 functions can extend to facilitating malignant transformation in mice models and human cancer cell lines by driving a transcriptional program, distinct from heat shock, implicating a new set of genomic targets (Dai et al. 2007; Tang et al. 2015; Su et al. 2016; Mendillo et al. 2012). These findings opened an all-new set of possibilities for HSF1 functions and more particularly for the identification of other heterochromatin targets with a potential role in cancer development.

- **The challenge of guarding telomere integrity:**

Like pericentromeres, telomeres belong to constitutive heterochromatin and play a vitally important part in protecting the end of linear chromosomes from degradation and recognition as double-strand breaks by the DDR (DNA Damage Repair) machinery. In human pathologies, telomeres are of crucial importance because of their role in cellular senescence, genome stability and their implication in cancer (Blackburn et al. 2006; Sfeir 2012). Telomeres have evolved into fascinating and complex molecular structures combining specific proteins complexes, secondary structures and maintenance mechanisms that all contribute to the challenge of preserving telomere integrity.

Importantly, telomeric chromatin was shown to be sensitive to HS-induced stress (Romano et al. 2013; Velichko et al. 2015; Blasco & Schoeftner 2008). In their recent publication, Romano et al. demonstrate that the yeast *S. cerevisiae* telomere length can be modulated in response to various environmental stimuli (caffeine, ethanol, temperature, hydrogen peroxide). Particularly, chronic exposure to high temperature (37°C) was responsible for significant telomere length decrease throughout generations (Romano et al. 2013). In mammalian cell lines, whereas a causal relationship between HS and telomere length was not yet determined, it has been shown that TRF2, an important telomere binding protein that protects telomeres, can be dissociated from telomeres upon heat shock (Petrova et al. 2014). Altogether, these results strongly suggest that the integrity and the stability of telomeric heterochromatin are impacted by heat shock.

Similarly to other heterochromatin loci, telomeric chromatin was thought to be transcriptionally silent until the discovery of telomeric ncRNA in 2007 (Azzalin et al. 2007). Telomeres are transcribed by RNA Polymerase II (RNAPII), into heterogeneous long non-coding RNAs called TERRA (TElomeric Repeats containing RNA) (Azzalin et al. 2007; Blasco & Schoeftner 2008). TERRA RNA has been assigned multiple functions, most of which are supporting the idea it is relevant for telomere maintenance and regulation (See introduction Chapter II). In the context of cancer, TERRA was shown to down regulate innate immune genes (K. Hirashima & Seimiya 2015) and to contribute to telomere maintenance in ALT cancer cells (Alternative Lengthening of Telomeres) (Arora et al. 2014). Interestingly, we and others have observed an accumulation of TERRA after HS in different model organisms

including human cell lines (Eymery et al. 2009; Martínez-Guitarte et al. 2008; Blasco & Schoeftner 2008).

Altogether, these results support the idea that HS can significantly impact telomere integrity and telomere transcription. Thus, telomeres appear as promising targets for HSF1 transcription factor and chromatin remodeler in the context of stress. Several exciting questions can therefore be raised: what are the mechanisms activated at telomeres to protect them from stress? Is HSF1 directly implicated in TERRA upregulation under stress? Does TERRA transcription have a role in telomere protection under stress? And finally, could these investigations lead us to the understanding of telomere biology in the context of other stress-inducing conditions like cancer for instance?

II.   Objectives

My PhD project focused on investigating the impact of HSF1 on telomeres during the telomeric stress response.

The preliminary work of my PhD consisted in confirming and further characterizing prior observations obtained in our lab, pinpointing an accumulation of TERRA transcripts upon heat shock (HS) in human cell lines (Eymery et al. 2009). Thus, we investigated TERRA nuclear pattern, foci number and volume prior and post HS.

Our first aim was to assess whether HSF1 plays a role in TERRA regulation during the cell response to stress. To dig into this issue, we took advantage of an inducible cell model, generously donated by Sistonen's lab and extensively explored by our team, providing a stable knock-down of HSF1 human protein in HeLa cells. Using this tool, the impact of HSF1 was first tested on TERRA accumulation during HS kinetics. Next, we aimed to characterize HSF1 progressive enrichment on subtelomeric regions (putative TERRA promoters) *in vivo* and to correlate this data with chromosome specific-TERRA accumulation during HS kinetics. To complete our data, we next assayed for TERRA stability and transcription upon HS in order to solve the question of transcription or stability-induced accumulation of TERRA by

HSF1. Finally, experiments of TERRA promoter methylation allowed us to open perspectives concerning a potential mechanism of TERRA upregulation.

A second aim of this PhD work was to get a view, at the molecular level, on the way HS impacts telomeres integrity and to distinguish a putative role of HSF1 in telomere maintenance. Various studies show telomeres integrity can be estimated by different molecular markers such as TRF2, H2A.X-P or 53BP1 for example. The presence of these three markers to telomeres was estimated before and after cells exposure to HS in WT and KD HSF1 cells. To go further, we analyzed the impact of HSF1 in the elimination of DNA damage at telomeres after HS. In addition, we proceeded with the characterization of HSF1 impact on telomeres distinctive epigenetic mark H3K9me3, on telomerase activity and on telomere length.

Our third aim was to address the question of a possible correlation between HSF1 function in telomeres protection and TERRA upregulation upon HS. We propose and discuss a molecular model and different experiments that should be finalized in order to validate our hypothesis.

Results will be presented in two distinct chapters and the submitted article presenting a part of this work (actually being revised) will be joined to this manuscript.

RESULTS

Chapter I| HSF1 impact on TERRA upon HS

I.   Control of HSF1 knock down model

To examine the effect of environmental stress stimuli on TERRA regulation and telomere integrity we used the Heat Shock (HS) model. Extensively used and studied, cell exposure to HS was shown to induce major yet potentially reversible changes in the cell like drastic changes in transcription and global chromatin remodeling (Richter et al. 2010; Boulon et al. 2010; Miozzo et al. 2015). One of the most spectacular effects of stress, in human, is the massive concentration of HSF1, a key transcription factor of the HSR, at subnuclear structures termed nuclear Stress Bodies (nSBs) clearly visible with immunofluorescence (**Figure 31 A**) (Jolly et al. 1997; Morley & Morimoto 2004; Biamonti & Vourc'h 2010).

Indeed under physiological conditions (37°C) HSF1 nuclear signal is diffused (**Figure 31 A**) while after HS, a massive reorganization of HSF1 nuclear distribution in a variable number of bright nuclear foci harboring different sizes can be distinguished. Our group has contributed to the deeper understanding of the Heat Shock Response mediated by HSF1 and consistent studies show that HSF1 upon various stress stimuli (heat, infection, heavy metals) is mostly located on the 9q12 genomic region (Jolly et al. 1997; Dengeri et al. 2002).

In HeLa aneuploid cells the mean number of large foci also called "primary stress granules" (► **Figure 31 A**) is ranging from 2 to 4 corresponding to the various copy number of chromosome 9 percentromeric region (9q12). The smaller foci also called "secondary stress granules" (* **Figure 31 A**) are more numerous and were showed not to localize with 9q12 loci (Eymery et al. 2010). This comes in agreement with data showing HSF1 genomic targets are not limited to the pericentromeric region, 9q12.

Highlighting the role of HSF1 at telomeres in our study was made possible using KD HSF1 and WT human HeLa (immortal, derived from cervical cancer) cells. Stable HSF1 knock down cell lines, kindly donated by Dr. Lea Sistonen's lab (Östling et al. 2007; Sandqvist et al. 2009) were validated by western blot and immunofluorescence as shown in (**Figure 31 A** and

B). HSF1 molecular weight increase (band shift) upon HS corresponds to multiple post translational modifications accompanying HSF1 activation. Among those post translational modifications a significant hyper-phosphorylation of HSF1 is known to occur upon HS and is frequently used as a molecular marker for HSF1 activation. (**Figure 31 B**) (Anckar & Sistonen 2011).
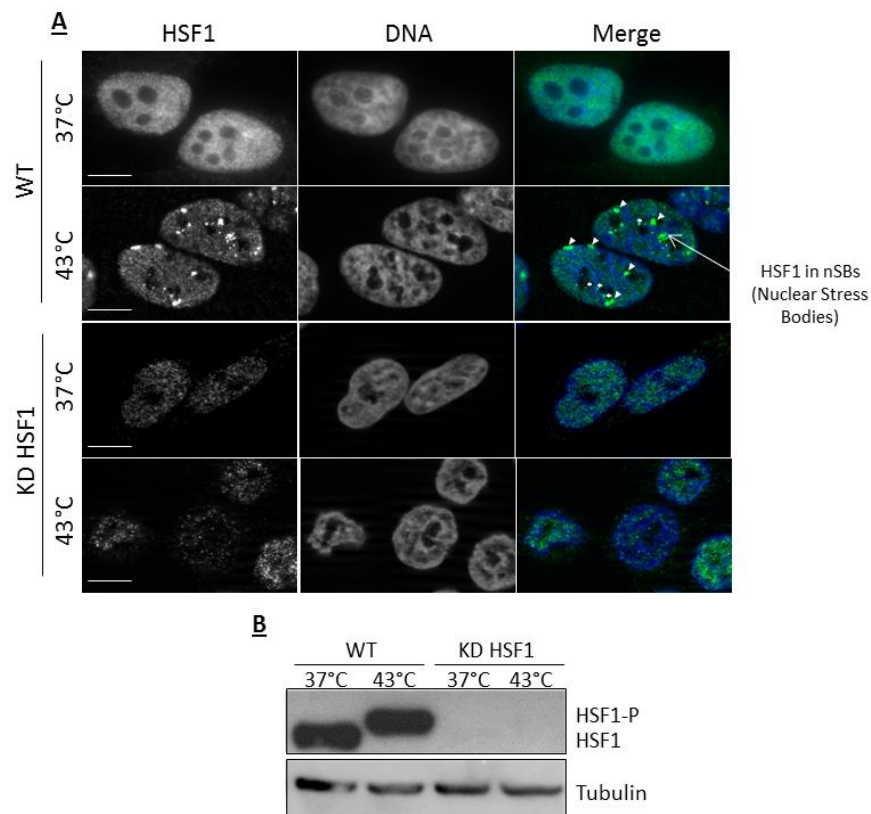
Figure 31| HS impact on HSF1 nuclear distribution and validation of HSF1 Knock Down (KD HSF1) in HeLa cells. A. Representative immunofluorescent labeling of HSF1 protein in HeLa cells heat treated (43°C, 1h) or not (37°C). DNA was stained with DAPI. HSF1 accumulation to nuclear Stress Bodies (nSBs) upon HS is indicated (white arrow) as well as "primary stress granules" (triangles) and "secondary stress granules" (stars). Scale bar =5 μm. **B.** HSF1 total protein expression level in WT and KD HSF1 cells was analyzed before and after HS using western blot. Tubulin is shown as a loading control.

Constitutive telomere transcription was shown to be sensitive to various environmental or patho-physiological conditions like tumorigenesis, ICF syndrome or HS (Eymery et al. 2009; Yehezkel et al. 2013; Blasco & Schoeftner 2008; Tutton et al. 2015). Interestingly a reproducible 2 to 4 fold increase of telomeric transcripts in response to HS was observed in independent studies using various models (Blasco & Schoeftner 2008; Martínez-Guitarte et al. 2008; Eymery et al. 2009) but no regulatory pathway was suggested.

To determine whether HSF1 could play a role in telomere transcription, we analyzed TERRA (TElomeric Repeat containing RNA) global and specific transcription level respectively by RNA dot-blot and RT-qPCR in WT and HSF1 KD cells as well as TERRA nuclear pattern by RNA FISH.

II.   HSF1 is required for TERRA accumulation upon HS

II. 1.   HSF1-dependenet accumulation of global TERRA upon HS

Global effect of HS and HSF1 on total TERRA RNA level was first estimated using RNA dot-blot technique (**Figure 32 A**). RNA dot-blot was performed using TERRA C-Rich (left panel) probe or U2 control (right panel) probe. Signals obtained upon HS were quantified and normalized to WT TERRA expression at 37°C. WT HeLa cells showed a robust 1.8 fold increase in TERRA level upon HS (**Figure 32 B**) and interestingly this upregulation was abolished in KD HSF1 cells. U2 transcripts used as negative control showed, as expected, no variations upon HS (**Figure 32 B**). TERRA signals were shown to be sensitive to RNase A treatment (bottom line left and right panel **Figure 32 A** and **C**) confirming probes specificity to RNA. A quality control and total RNA quantification were validated on BET-stained agarose gel (**Figure 32 C**).
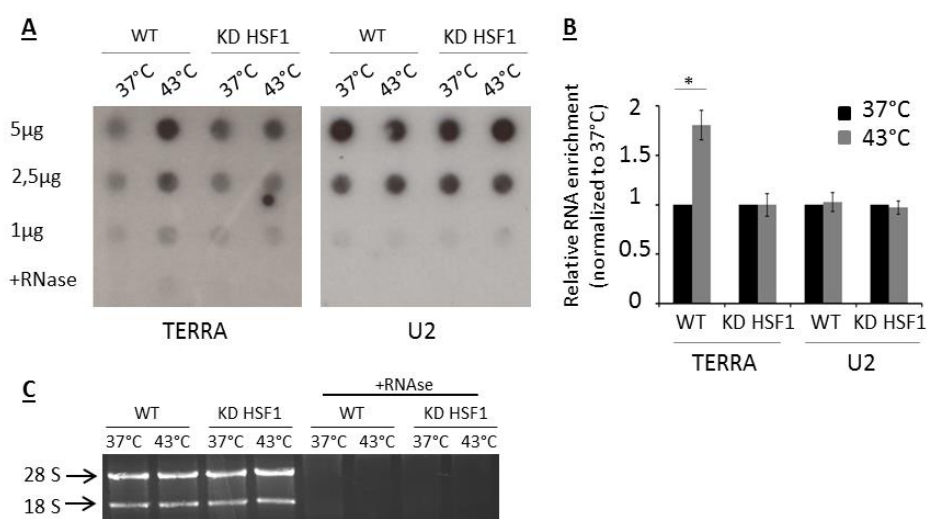
Figure 32| TERRA accumulation requires HSF1 upon heat shock. RNA was extracted from WT or KD HSF1 HeLa cells before and after HS (1h at 43°C) and treated with DNAse before **A.** 1µg, 2.5µg or 5µg of total RNA fractions were subjected to RNA dot blot analysis using a C-Rich radioactive TERRA oligonucleotide probe (left panel). The same membranes were stripped and hybridized with a control probe specific for U2 transcripts (right panel). **B.** TERRA and U2 RNA-blot signals were quantified and normalized to 37°C condition. S.d. were calculated from 3 independent experiments. P-value was calculated by unpaired Student's *t*-test with Welch's corrections (*) p<0.05. **C.** Quality of RNA extracts, treated or not with RNAse A (1mg/mL), was estimated after migration on ethidium bromide stained-agarose gel.

Hence our data confirm TERRA global level is upregulated by HS and suggest an HSF1 dependent regulation of TERRA.

In order to validate HS and HSF1 impact on global TERRA level is not cell line specific in our hands two other cell lines were controlled for TERRA expression. RNA extraction was carried out on HT1080 human fibrosarcoma and HFF2-TERT telomerase-immortalized human fibroblasts, used as such (WT) or after siRNA-induced HSF1 transient depletion (siHSF1). TERRA RNA dot-blot was performed (**Figure 33 A**) and signal quantification reported on graph (**Figure 33 B**) was normalized by 37°C. Wild-type (WT) HT1080 and HFF2-TERT cells respectively show a 2 and 2.5 fold increase of TERRA global level upon HS, thus confirming HS impact on telomeric RNA level (**Figure 33 B**). Similarly to HeLa cells, HSF1 downregulation in HT1080 and HFF2-TERT completely abolished TERRA upregulation upon HS (**Figure 33 B**). Taken together, our results support an HSF1-dependent mechanism of TERRA upregulation upon HS in different human cell lines.

Control of HSF1 protein downregulation after transient siRNA transfection in both cell lines was validated by western blot (**Figure 33 C**). Tubulin was used as loading control. Validation HSF1 activation upon used HS conditions was validated in both lines by western blot (**Figure 33 C**) and by HSF1- immunolabeling (**Figure 33 D**) with the formation of nSBs.

In human, TERRA transcription is initiated at the subtelomeric adjacent region (Nergadze et al. 2009; Negishi et al. 2015). Contrasting with the telomeric tract, subtelomeric regions contain both telomeric repeats (TTAGGG) and unique sequences that vary among chromosomes. Thus, a heterogeneous pool of TERRA RNAs is continuously produced and chromosome specific transcripts can be distinguished when using subtelomeric primers. However, specific TERRA primers design and validation for RT-Q-PCR purposes represents a real challenge in such heavily repeated regions. Therefore, among the available human subtelomeric sequenced regions, primers were designed and tested for their specificity. Only specific primers showing unique target region were further used for single TERRA molecules analysis. An exception is to be noticed concerning the known subtelomeric duplicon known to exist on chromosomes 10p and 18p, which share a high

degree of sequence homology and thus a common set of primers (Stong et al. 2014) (**Figure 34**).
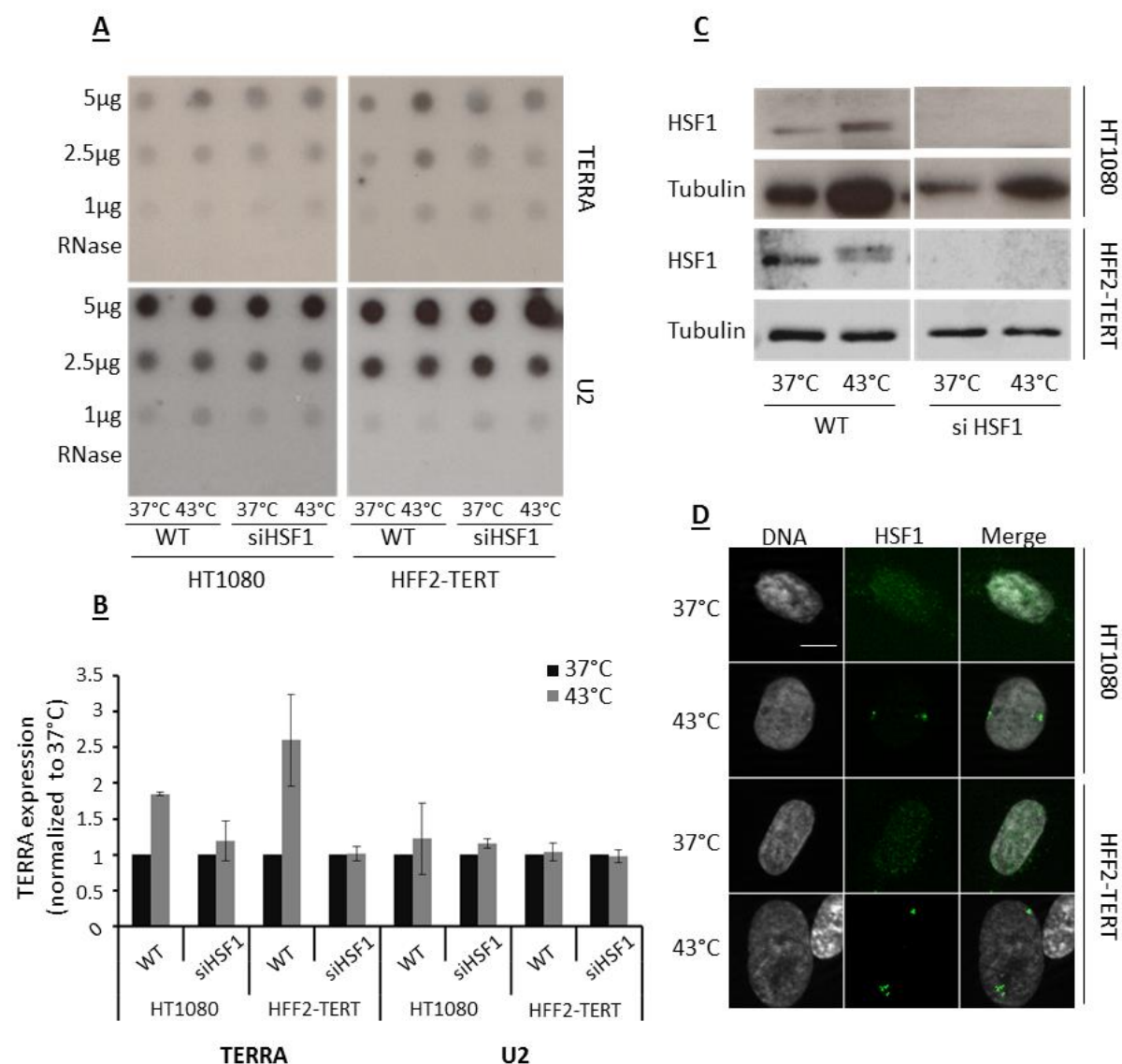
Figure 33| HS-dependent TERRA accumulation requires HSF1 in different cell lines. RNA was extracted from WT or siHSF1 HT1080 and HFF2-TERT cells before and after HS (1h at 43°C) and treated with DNAse before **A.** 1µg, 2.5µg or 5µg of total RNA fractions were subjected to RNA dot blot analysis using a C-Rich radioactive TERRA or U2 oligonucleotide probe (respectively upper and lower panels). RNase treatment validates the specificity of TERRA signal (bottom lines of all panels). **B.** Quantification of TERRA levels with non-heat-shocked (37°C) conditions was done using ImageJ software. S.d correspond to experimental replicates with different RNA concentrations **C.** Western blot analysis of HSF1 expression in HFF2-TERT and HT1080 cell lines transiently transfected or not with an siRNA against HSF1 in normal conditions and after 1 hour of HS at 43°C. Tubulin was used as a loading control. **D.** Representative confocal images of HSF1 immunostaining (in green) in HT1080 and HFF2-TERT cell lines before and after HS. DNA was stained with DAPI. Scale bar = 10µm.

II. 2. HSF1-dependent accumulation of chromosome-specific TERRA upon HS

Chromosome specific analysis of TERRA was estimated using RT-Q-PCR technique and subtelomeric designed primers (**Table 1**). Relative quantification of all Q-PCR signals was normalized to the corresponding "WT 37°C" condition. Consistently with our previous data, HS showed no impact on TERRA level for all tested chromosomes in KD HSF1 cells whereas WT heat shocked cells presented a 1.5 to 3 fold upregulation of specific TERRA molecules (Chromosomes: 3p, 17q and 18p-10p). However several tested chromosomes (1q, 2p, 11q, 14q) showed no significant variation in TERRA level upon HS in WT cells (**Figure 34**). These results confirm that TERRA level upregulation upon HS is an HSF1 dependent process and furthermore that TERRA upregulation upon HS is chromosome dependent.
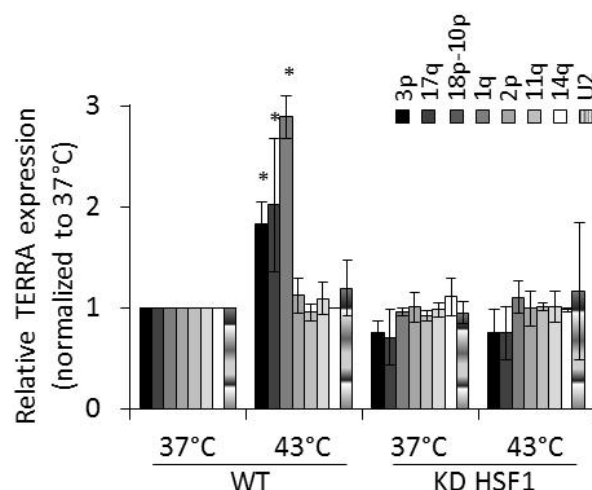
Primer name	Primer sequence
TERRA 3p-F	CCTGGAGGCAAGGGAAGAC
TERRA 3p-R	TGCCCATGAAGCTTTTGGTC
TERRA 17q-F	GAAAATAAGGTCGGGATTGCTGTGCTCAC
TERRA 17q-R	CTATCCCCTCAAATGCCTGTGTTCCTTG
TERRA 18p-10p-F	TCGTACACTCTCTGGTAGGT
TERRA 18p-10p-R	TCATCTCCACCTGCAATCCA
TERRA 1q-F	TGTTCTGTATCCCACCAGCA
TERRA 1q-R	GGTCAAAGAAAAGGCTGACG
TERRA 2p-F	GTGCTGCAAACAGGAAGTATTTTATT
TERRA 2p-R	GGGCACTGCCTCGCTTT
TERRA 11q-F	CCCTGATTATTCAGGGCTGCAAAG
TERRA 11q-R	ACAGACCTTGGAGGCACGGCCTTCG
TERRA 14q-F	ATGGGATCTTGGGTCAGAGT
TERRA 14q-R	ATTTCCCATGTAGCCGCAAC
U2-F	GGCTAAGATCAAGTGTAGTATCTGTTC
U2-R	GCTCCTATTCCATCTCCCTGCTC

Table 1 : Primers used for RT-Q-PCR

Figure 34| HSF1-dependent accumulation of chromosome specific TERRA upon HS. Chromosome specific TERRA quantification arising from 3p, 17q, 18p-10p, 1q, 2p, 11q and 14q chromosome arms in WT and KD HSF1 cells before and after HS (1h at 43°C) was performed by RT-Q-PCR. U2 transcripts were used as a negative control. S.d. was calculated from 3 independent experiments. RNA levels were normalized to 37°C conditions. P-values were calculated by unpaired Student's *t*-test with Welch's corrections ((*), $p < 0.05$).

III. HSF1-dependent dynamics of TERRA foci upon HS

HS was showed not only to impact TERRA RNA level but also TERRA subnuclear occupancy (Blasco & Schoeftner 2008). Indeed it was published that exposure of MEF cells to HS (42°C, 1h) resulted in a significant change in the number of TERRA foci after 3 hours of recovery compared to cells grown at 37 °C. Moreover a visible increase in TERRA foci size

was noticed but not quantified (**Figure 35 A** and **B**). Based on these data on mouse fibroblasts, we decided to analyze the nuclear pattern of TERRA with our conditions in human HeLa cells.
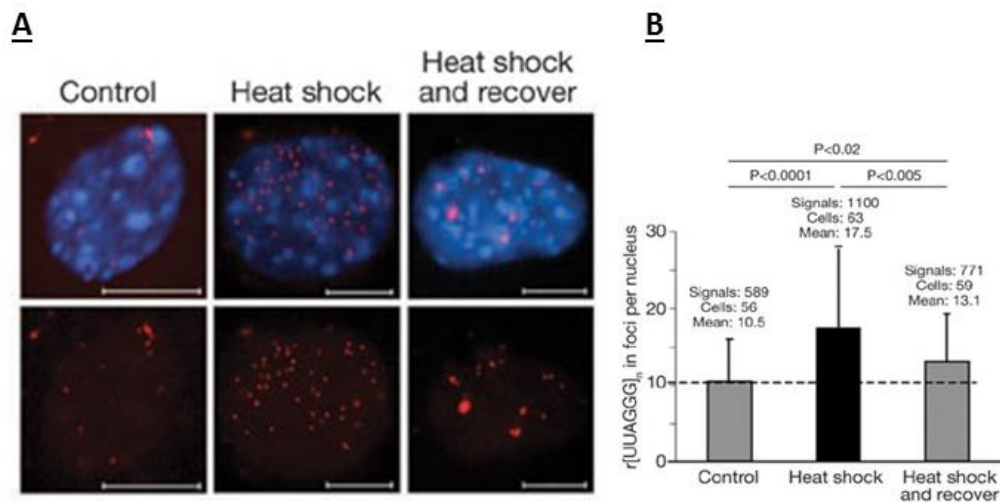
Figure 35| TelRNAs are upregulated on Heat Shock. A. Tel RNA foci localization by FISH analysis in normal conditions (control) after heat shock treatment (1 hour 42°C) and after 3 hours of recovery in mouse fibroblast cells. **B.** Number of analyzed cells, total number of TelRNA signals, mean number of TelRNA foci per nucleus (mean s.d., *n* = 3) and *P* values are indicated. ***Extracted from: Schoeftner & Blasco_2008_Nature Cell Biology.***

In situ techniques showed TERRA is able to form more or less discrete nuclear foci in various normal and cancer cell lines and tissues. To extensively characterize HS and HSF1 impact on telomeric transcripts' nuclear pattern, we employed RNA-FISH using optimized method for detection of rare and unstable RNA such as TERRA (Arnoult et al. 2012). A Cy5-conjugated PNA C-rich probe was used under non-denaturing conditions to selectively distinguish telomere RNA from telomere DNA. We proceeded to TERRA nuclear pattern analysis, monitoring for TERRA foci volume and number in individual nuclei using 3D reconstituted image stacks. RNA-FISH experiments were performed on WT and KD HSF1 HeLa cells before and after HS. (**Figure 36 A** and **B**) RNase A treated cells were used as a control for TERRA RNA probe specificity and showed as expected no TERRA FISH signal (**Figure 36 C**). 3D assessment of TERRA foci number and volumes per nucleus were collected and mean values were calculated for each condition.
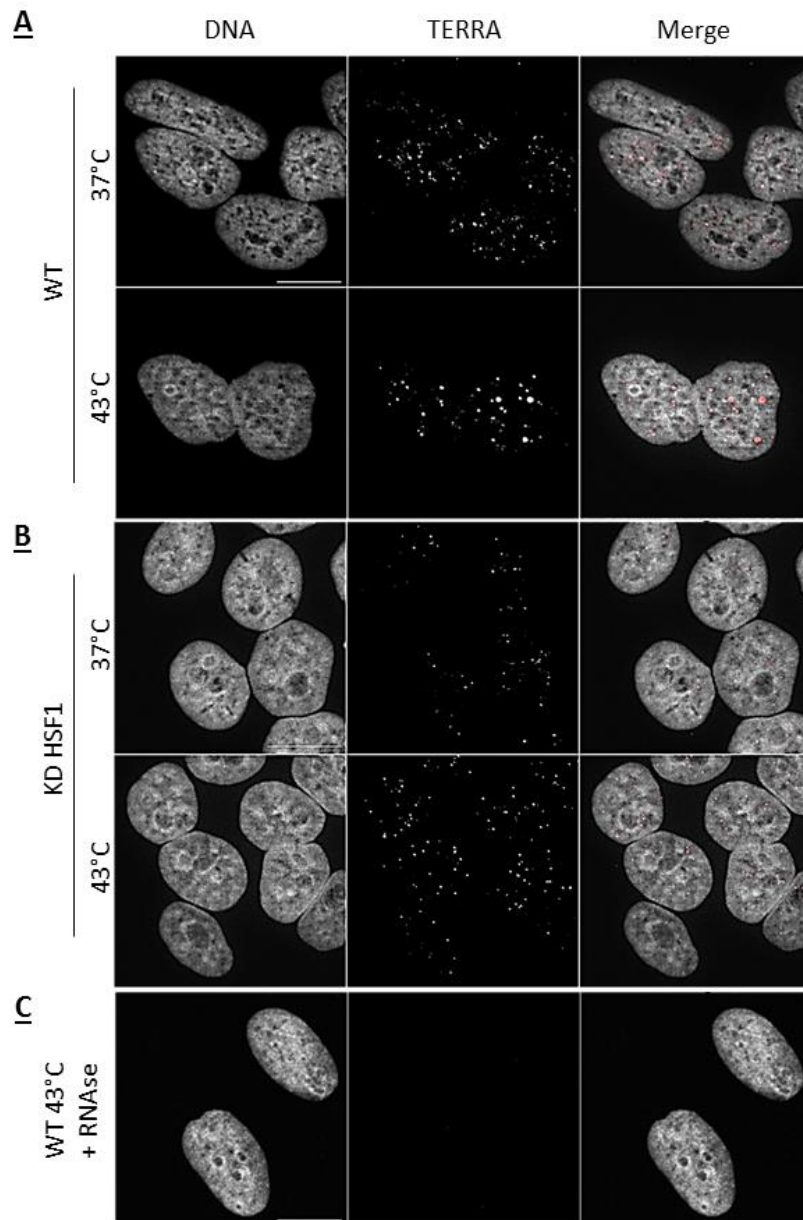
Figure 36| HS induces variations of TERRAs foci nuclear pattern. Representative images of TERRA RNA-FISH analysis. Strand-specific telomeric DNA probes were used. Experiments were performed in native conditions. DAPI-stained nuclei are in gray **A.** on HeLa WT. **B.** or KD HSF1 cells before or after HS. Scale bar = 10 μm. **C.** RNA-FISH experiments performed with RNAse A treated cells were used as a control.

We next analyzed TERRA foci volume and number using *in situ* FISH technique. Exposure of WT HeLa cells to HS revealed a significant, on average 2-Fold, increase in foci 3D volume (μm^3) per nucleus compared to cells grown at 37°C (**Figure 37 A**) confirming exogenous stimuli such as HS is capable of modulating TERRA's subnuclear pattern in human cancer cell lines. Interestingly KD HSF1 cells do not present a significant increase in TERRA foci volumes following HS suggesting an HSF1 dependent phenomenon.

Exposure of WT HeLa cells to HS revealed a visible (~ 50%) decrease in the mean foci number per nucleus compared to cells grown at 37°C. However, KD HSF1 cells present a stable TERRA foci number per nucleus before and after HS (**Figure 37 B**). Taken together we

were able to observe that upon HS in WT HeLa cells the mean volume of TERRA foci is increased accompanied with a decrease of TERRA foci number which may suggest several hypotheses including an accumulation of TERRA transcripts at certain subnuclear location or confirming previous observations showing exogenous stimuli such as HS are able to modulate TERRA subnuclear occupancy. Indeed, no variation in the number of telomeres per nucleus was observed in unstressed and stressed WT and HSF1 KD cells suggesting that HS does not induce any clustering of telomeres (**Figure 37 C**). On the other hand HSF1-depleted HeLa cell line showed no variations of TERRA foci volume or number. These results strongly suggest TERRA foci modulations upon HS is, at least partially, HSF1 dependent.
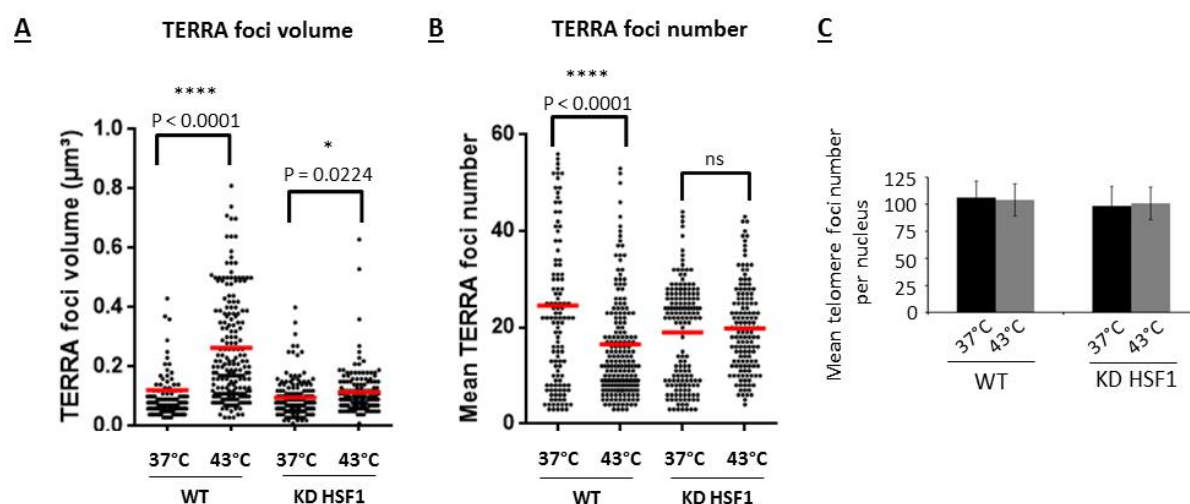
Figure 37| HSF1-dependent dynamics of TERRA foci upon HS. A. HS induces a significant increase of TERRA foci volume in a HSF1 dependent manner. Mean values of TERRA foci volumes (μm^3) per nucleus, are represented as dots and mean values were calculated before and after HS (red lines) in WT and KD HSF1 cell lines, between [130-200] nuclei were analyzed per condition. Indicated *P* values were calculated using a two-tailed T-test. **B.** HS induces a significant decrease of TERRA foci number in a HSF1 dependent manner. Total number of TERRA foci per nucleus and represented on graph as dots. Mean values are designated (red lines). Between 130 and 200 nuclei were analyzed per condition. Indicated *P* values were calculated using a two-tailed T-test. **C.** Quantification of telomeric foci per nucleus by DNA FISH in WT and HSF1 KD cells before and after HS. n ≥ 150 cells.

In order to approach the mechanisms associating HSF1 to TERRA upregulation under stress, we first hypothesized that HSF1 could play a direct role in telomere transcription upon HS, therefore, suggesting subtelomeric promoters regions may constitute new HSF1 genomic targets.

Active HSF1 is known to homotrimerize and to bind DNA sequence consisting of inverted repeats of the pentameric sequence nGAAn, known as heat shock elements (HSE) (Akerfelt et al. 2010). HSF1 binding to HSE present within promoter regions of target genes will result in their transcription. The number of HSE elements and their sequence homology with the canonical HSE is thought to account for differences in HSF1 affinity for its targets (Perisic et al. 1989; Sorger 1991). The majority of TERRA subtelomeric promoters harbor CpG islands which presence allowed localizing TERRA's transcription start sites. Therefore, we started by an in Silico screening subtelomeric human sequences analysis for the presence of HSE. The proximity between subtelomeric CpG islands and potential HSEs could suggest HSF1 exerts a transactivating function at those regions.

IV.   Subtelomeric promoters regions constitute new HSF1 targets

IV. 1.   Potential HSF1-binding sites at human subtelomeres

We screened for the presence of HSEs as well as CpG dinucleotides at the majority of human, recently sequenced, subtelomeric regions (Stong et al. 2014). The distribution of HSE and CpG islands is shown in (**Figure 38**).
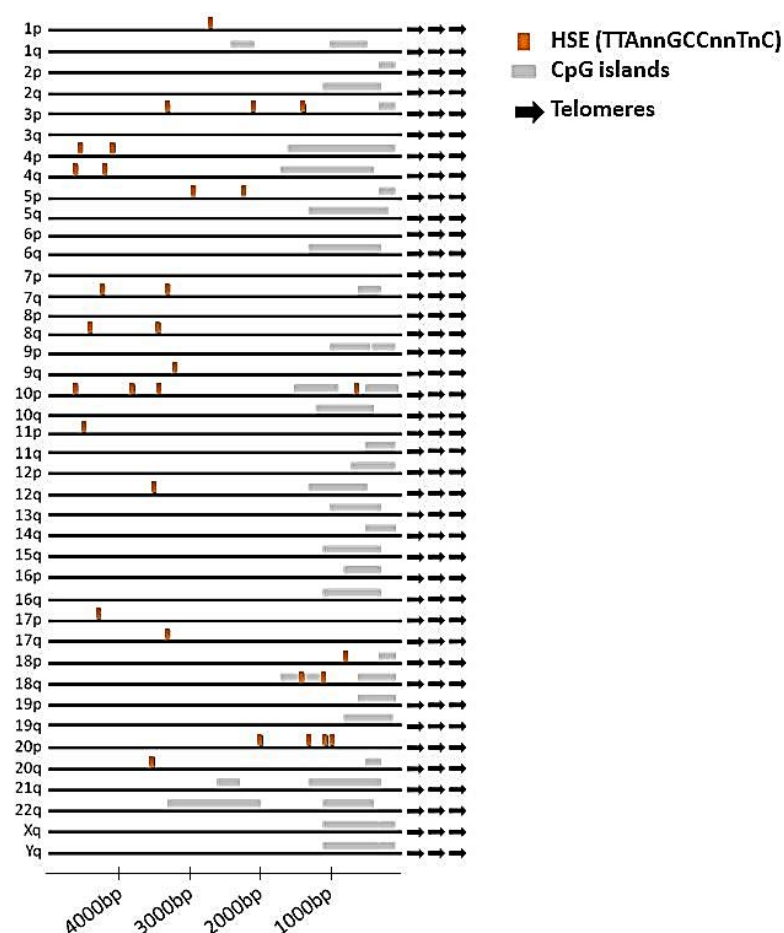
Figure 38| Potential HSF1-binding sites at human subtelomeres. Scheme showing potential Heat Shock Elements (HSE), HSF1 binding sites, CpG islands positions and their distance from telomeres on various human chromosomes.

Legend:
- HSE (TTAnnGCCnnTnC)
- CpG islands
- Telomeres

Our analysis revealed the existence of putative HSE in more than 40 % of human subtelomeres, within a region encompassing 5000bp upstream of the TTAGGG repeats (telomeric tract). Moreover, about 25% of chromosome extremities displayed both HSE and CpG islands. This analysis was therefore complementary to the analysis performed by Lingner's laboratory (Porro et al. 2014), that also identified HSE within TERRA proximal promoters onto seven chromosome ends, in a 1kb window around TERRA proximal 5' end, and the presence of HSE motifs within 80% of TERRA proximal transcription start sites identified by RNA-Seq analysis. Results obtained with in silico approach encouraged us to proceed to an *in vivo* validation of HSF1 binding to subtelomeric chromatin by ChIP. These findings suggest that HSF1 might only bind to a subset of subtelomeric regions.

IV. 2.   *In vivo* HSF1 enrichment at subtelomeres upon HS

To validate HSF1 *in vivo* enrichment and binding kinetics on TERRA promoters during the HSR, we next performed ChIP experiment against HSF1. Representative image of DNA

sonication control are presented (**Figure 39 A**) as well as HSF1 immunoprecipitation western blotting controls before and after HS kinetics (**Figure 39 B**).

Five subtelomeric regions were selected that either displayed HSE and CpG island (3p and 10p-18p) or were devoid of HSE elements (2p, 14q), and thus served as negative controls (**Figure 39 C**). Specific primers were designed for ChIP analysis performed against HSF1 that revealed a specific enrichment only on subtelomeric regions containing at least one HSE (**Figure 39 C** and **D**), supporting a chromosome-specific binding capacity of HSF1 at subtelomeric regions, upon HS. In addition, HSF1 binding to subtelomeric regions was slightly delayed (30 min of continuous HS) when compared to the well-characterized HSF1 target gene, *HSP70* (5 min of a continuous heat shock) (**Figure 39 D**).
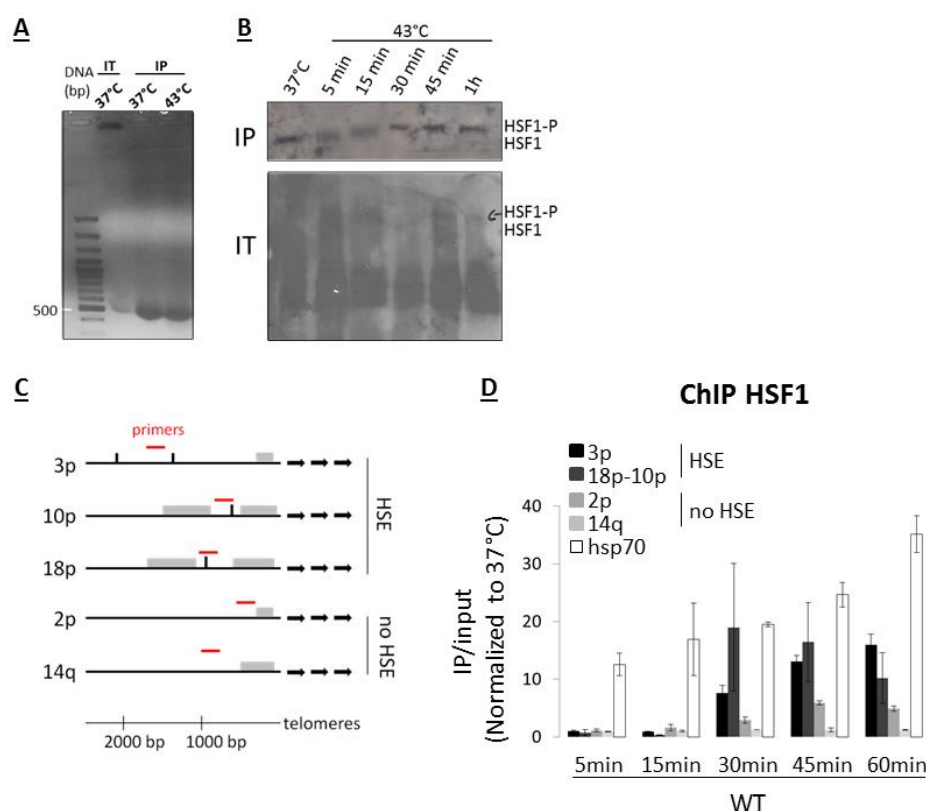
Figure 39| *In vivo kinetics of* HSF1 enrichment at subtelomeres upon HS. **A.** Representative image of DNA sonication control. Input (IT) or sonicated DNA (destined to immunoprecipitation (IP)) from WT HeLa cells at 37°C or after HS were migrated on agarose gel and revealed with Ethidium Bromide. **B**. Western blot validation of the HSF1 protein presence in IT and IP form WT HeLa cells before and during HS kinetics. **C.** Relative positions of primers selected for Q-PCR analysis. Primers were designed on subtelomeric regions of chromosomes 18p, 14q, 10p, 3p and 2p, containing or not HSEs, up to 3000bp from telomeres. **D.** Chromatin isolated from WT cells heat treated at 43°C from 5 to 60min was subjected to ChIP-HSF1 experiments. HSF1 enrichment during HS kinetics to subtelomeres was analyzed by Q-PCR. S.d. was calculated from 2 independent experiments. HSF1 enrichment to *HSP70* promoter was used as a positive control.

These results were also supported through co-detection of telomeres and HSF1 by DNA FISH and immunofluorescence (**Figure 40**). As expected, in physiological conditions, HSF1 displays a heterogeneous and diffuse nuclear signal, with no obvious enrichment at telomeric regions.
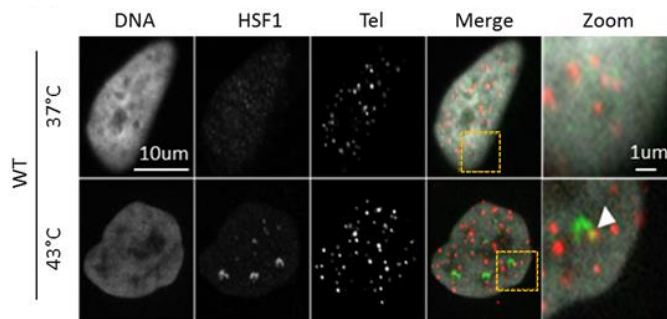
Figure 40| Partial Telomere-HSF1 localization *in situ*. Representative images of co-localization between telomeres (red) and HSF1 nuclear foci (green) detected by combined Immuno-DNA FISH in normal and heat shocked WT HeLa cells.

In heat-shocked cells, HSF1 harbors a discrete nuclear foci distribution also described as nuclear stress bodies (nSBs). We found that ~46% of the cells displayed at least 3 large HSF1 foci partially colocalizing with telomeres.

IV. 3. Kinetics of chromosome-specific TERRA transcription and subtelomeric HSF1 binding

Agreeing with a role of HSF1 in HS-dependent up-regulation of telomere transcripts and a chromosome specific HSF1 binding at subtelomeric regions upon HS, a chromosome specific analysis of TERRA level under identical HS kinetics showed only specific TERRA transcripts coming from HSE-containing subtelomeres accumulated after 30 to 45 minutes of HS (**Figure 41 A**). SatIII and U2 transcripts were used respectively as positive and negative controls. The level of SatIII non-coding RNA (9q12 locus) was rapidly upregulated starting from 5 to 15 minutes of HS and going up until ~100 fold at 1h of HS. U2 transcripts used as negative control showed no variations (**Figure 41 B**). Significant induction of TERRA transcripts was found to occur between 30min to 45min of HS, demonstrating a striking parallel between the kinetic of HSF1 binding to subtelomeres and that of TERRA upregulation.
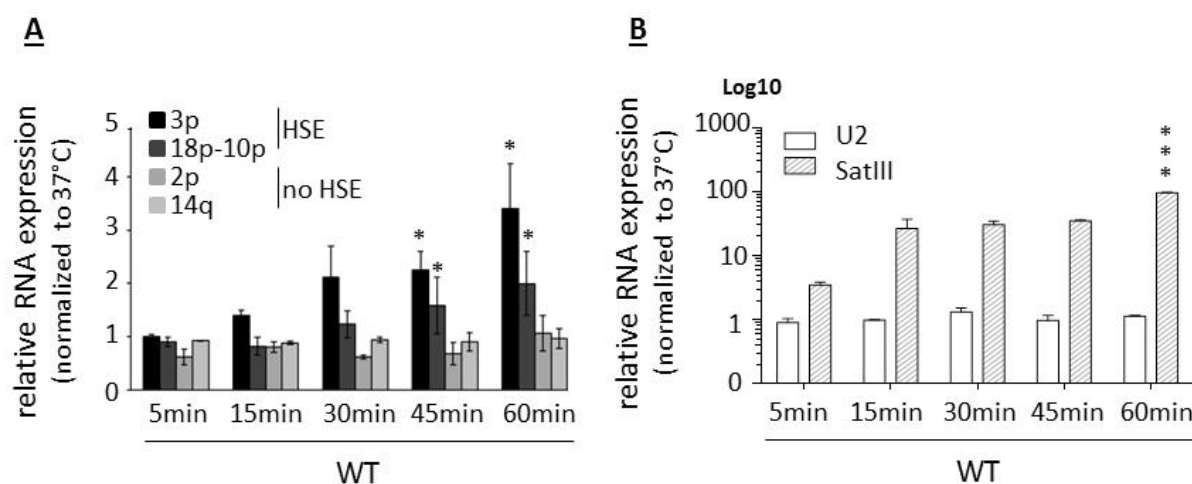
Figure 41| kinetics of TERRA upregulation upon HS. A. TERRA transcripts from selected chromosomes were quantified by RT-Q-PCR during HS kinetics. **B.** Quantification of U2 and SatIII transcripts was used respectively as negative and positive controls, in the same experimental conditions as described above. S.d. was calculated from 2 independent experiments. P-values were calculated by unpaired Student's *t*-test with Welch's corrections T test: ((*) p<0.05, for TERRA and (***) p<0.0001 for SatIII).

Altogether, our results reveal the existence of a clear correlation between HSF1 binding to subtelomeric HSE and chromosome-specific TERRA up-regulation under stress.

V. TERRA RNA stability is not impacted upon HS

Despite strong evidence suggesting that HSF1 directly controls the level of TERRA expression in heat-shocked cells, the possibility that HSF1 affects TERRA stability could not be excluded. To clarify this point, we monitored the impact of HSF1 on both TERRA stability and active RNA Polymerase II (RNAPII) binding at subtelomeres. RNAPII was showed to associate with telomeric DNA and largely transcribe TERRA in human, mouse and yeast cells (Azzalin et al. 2007; Luke et al. 2008; Blasco & Schoeftner 2008).

We first assayed TERRA stability in the presence of triptolide, an RNAPII inhibitor, during recovery from HS (**Figure 42 A**). Relative TERRA expression was estimated using RT-Q-PCR and TERRA specific primers (**Table 1**) immediately after HS and then from 2 to 8h of recovery (**Figure 42 A**). TERRA level quantification, using a specific primers pairs, was normalized to the relative 37°C condition. No significant difference in TERRA stability from chromosomes 14q, 18p-10p, 3p and 2p was observed in WT neither in KD HSF1 after HS (**Figure 42 B**). HSF1 global protein level and "shifting" upon HS and triptolide treatment were

monitored using western blot (**Figure 42 C**). As expected, HSF1 protein level remained unchanged upon triptolide application before or after HS and did not disturb HSF1 activation characteristic "shifting" upon HS in WT cells. To conclude, these experiments encouraged our hypothesis of an HSF1-dependent TERRA transcription, resulting in the observed TERRA upregulation upon HS.
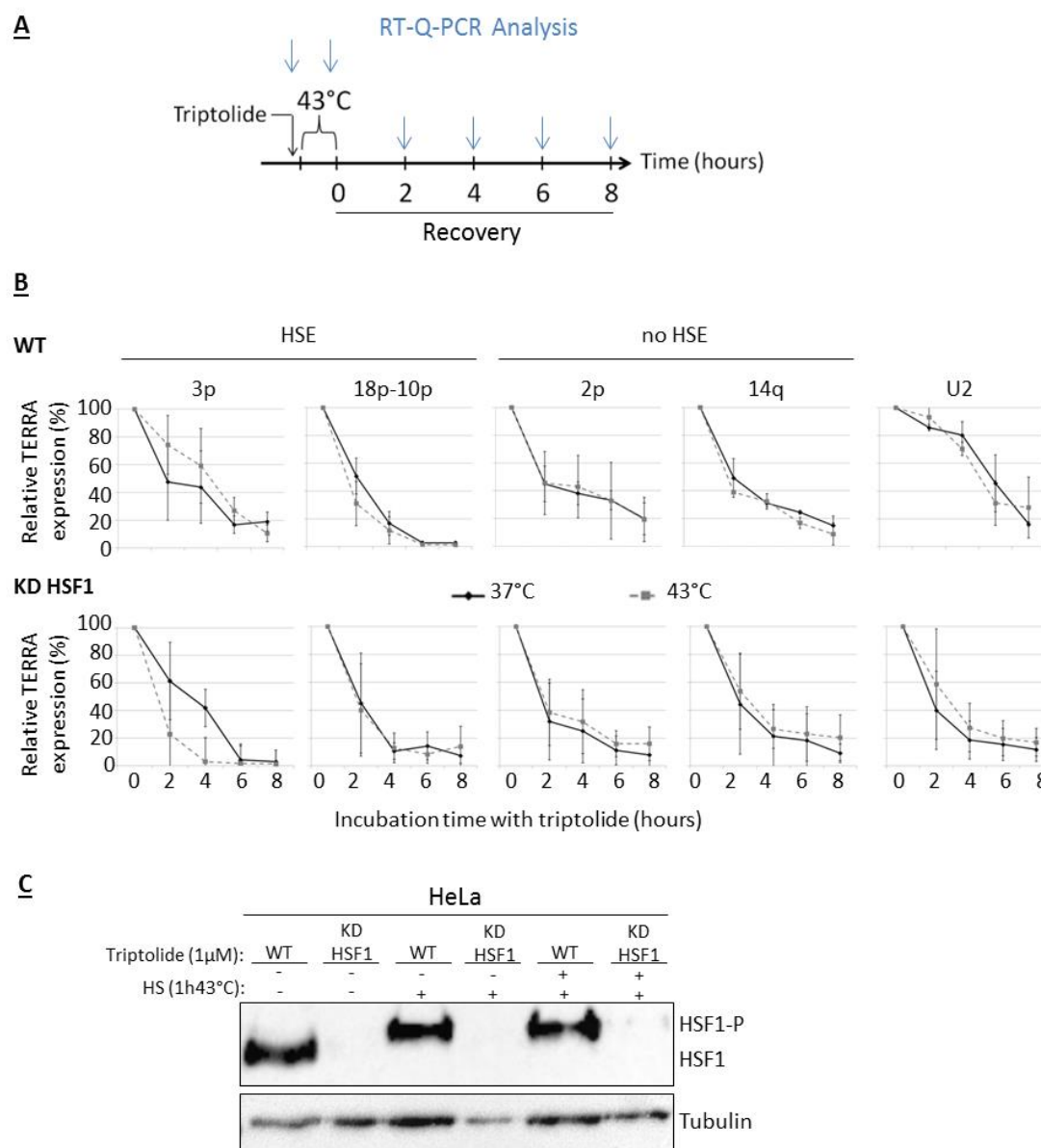
Figure 42| HSF1 upregulates telomere transcription upon heat shock. A. Scheme of experimental procedure testing for TERRA stability. **B.** To estimate TERRA stability, RNA extracted from triptolide treated cells was purified and subjected to RT-Q-PCR analysis. TERRA encoded by chromosomes 14q, 18p, 10p, 3p and 2q and U2 transcripts were quantified, in cells treated or not with HS. Results were normalized with an act1 cDNA coming from an exogenously added yeast RNA, and were depicted as percentages of the time point 0 (*n* = 3). **C.** Control of Triptolide treatment impact on HSF1 total protein expression level and shifting (post translational modifications associated to HSF1 activation) in WT and KD HSF1 cells was analyzed before and after HS using western blot. Tubulin is shown as a loading control.

VI. HSF1 activates chromosome specific, RNAPII-dependent TERRA transcription, upon HS

Telomeres are known to be transcribed by RNA Polymerase II (RNAPII). To validate our proposition that TERRA accumulation is due to an HSF1-dependent transcription, we next performed ChIP experiment against elongating RNA Polymerase II (RNAPII P-S2). The phosphorylation status of RNAPII has been shown to correlate with its activities in promoter assembly (S5 phosphorylation) and transcriptional elongation (S2 phosphorylation) (Nechaev & Adelman 2011; Selth et al. 2010). DNA sonication (**Figure 43 A**) and RNAPII-P-S2 immunoprecipitation were validated (**Figure 43 B**), before and after HS.
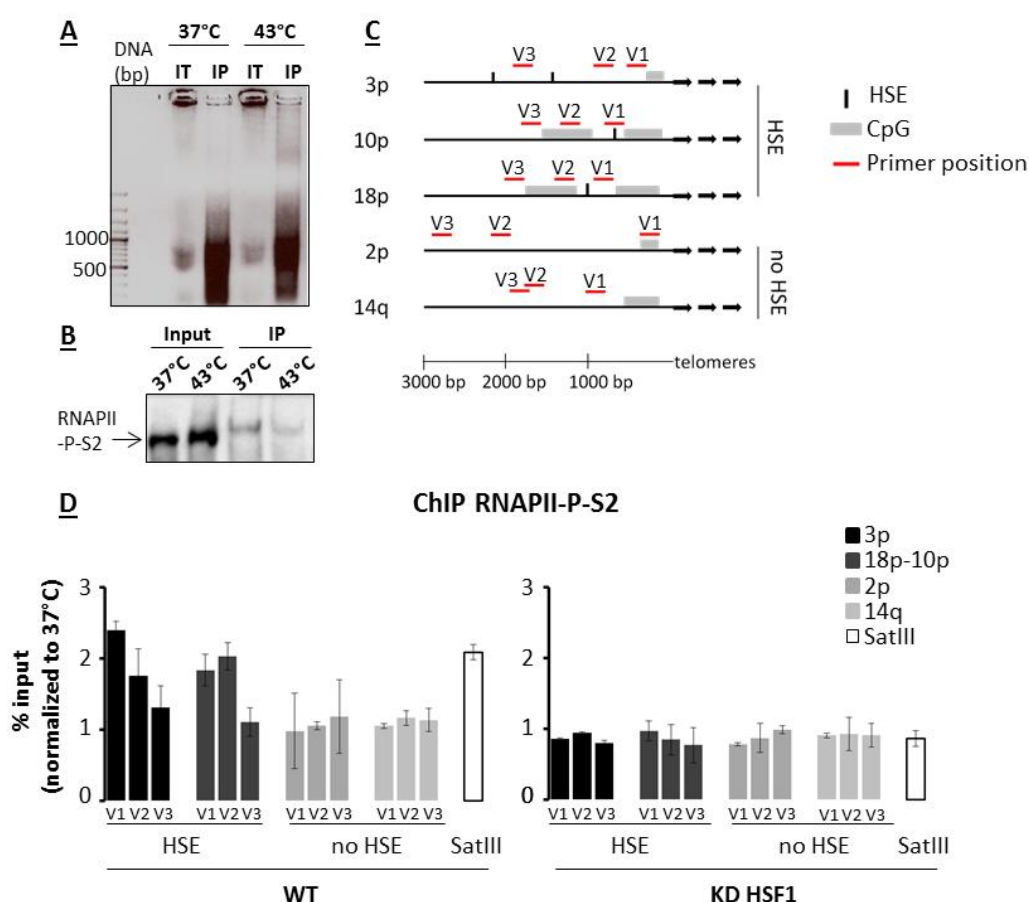
Figure 43| Chromosome specific RNAPII-PS2 enrichment at subtelomeres upon HS A. Representative image of DNA sonication control. Input (IT) or sonicated DNA (destined to immunoprecipitation (IP)) from WT HeLa cells at 37°C or after HS were migrated on agarose gel and revealed with Ethidium Bromide. **B**. Western blot validation of the RNAPII-PS2 protein presence in input and immunoprecipitated extracts performed with chromatin form HeLa cells before and after HS. **C.** Q-PCR primers position, selected for ChIP-RNAPII-PS2 experiments, designed on subtelomeric regions of chromosomes 18p-10p, 14q, 3p and 2p up to 3000 bp from telomeres. **D.** Relative enrichment of RNAPII-PS2 at subtelomeric and control region (chromosome 9q12 (SatIII)) was quantified in stressed WT and KD HSF1 HeLa cells by Q-PCR and normalized with input and 37°C conditions .

The position of the different primers used in this ChIP experiment across subtelomeric regions is shown in (**Figure 43 C**). For each chromosome arm, three primers couples were specifically designed and tested. Indeed, it has been shown that elongating RNAPII binds as a diffuse peak with higher intensity at positions close to telomeric TTAGGG repeats and overlapping with CpG islands (Deng, Wang, Stong, et al. 2012). RNAPII P-S2 enrichment upon HS was only observed at subtelomeric regions displaying HSE (**Figure 43 D**). No RNAPII P-S2 telomeric enrichment was observed in stressed cells deficient for HSF1. Thus, elongating RNAPII seems to be specifically enriched on HSE-containing subtelomeres upon HS and demonstrating the role of HSF1 in heat-induced initiation and/or elongation of TERRA transcription. Heat-induced enrichment of RNAPII P-S2 at pericentromeric region of chromosome 9 (SatIII) was used as positive control (**Figure 43 D**). The specificity of antibodies used for ChIP, including α-RNAPII P-S2, was assessed with all primers using a control antibody α-IgG (**Figure 44**).
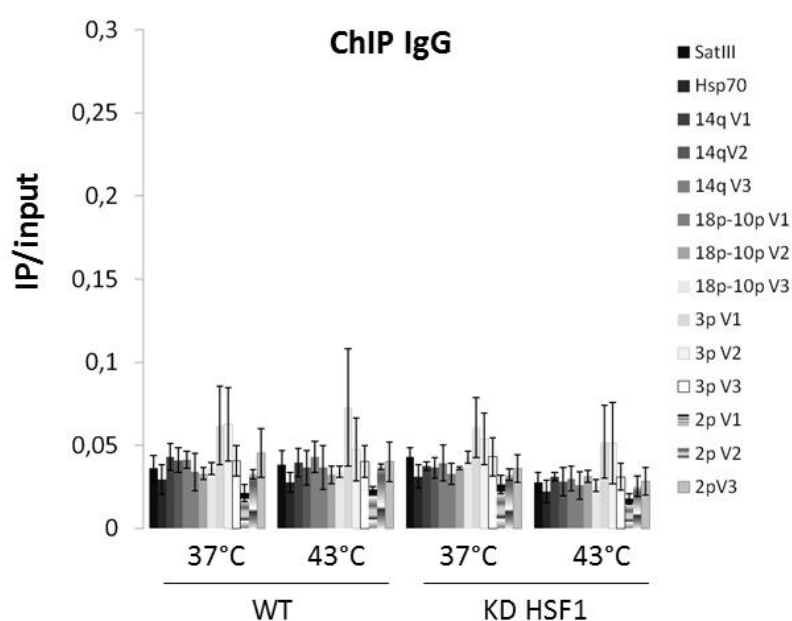
Figure 44| Validation of antibodies' specificity used for ChIP analysis. In parallel to all antibodies used for ChIP experiments an IgG control antibody (see table 2 in Material and Methods p.) was used and all primers were tested by Q-PCR, in the four experimental conditions.

HSF1 is a primary mediator of stress-responsive transcription that regulates the expression of many pro-survival genes and for the first time our data report a direct role of HSF1 in telomere transcription under stress.

VII.   HS-induced subtelomeric TERRA promoter DNA demethylation

One of the few mechanisms previously described to be involved in regulation of telomere transcription is DNA methylation. Subtelomeric regions are characterized by a high density of methylated CpG dinucleotides under normal physiological conditions (Maeda et al. 2012). A decrease in CpG island methylation of TERRA subtelomeric promoters induced (or endogenously present in ICF patients) by DNMT3B mutation, was shown to be tightly correlated to high TERRA level in human cancer cells and ICF patients (Yehezkel et al. 2008). To investigate whether the methylation status of TERRA promoter regions changes under HS we performed a preliminary analysis of CpG methylation at the HS-induced TERRA promoter located on subtelomeric region 10q, using bisulfite sequencing. Previously validated primers furnished by our collaborators, were used to analyze DNA methylation on chromosome 10q subtelomeric region. Analysis was performed on HeLa WT (**Figure 45 A**) and HT1080-ST cell lines under normal or HS conditions, in collaboration with Anabelle Decottignie's lab (**Figure 45 B**). Our HeLa cell model harbors relatively short telomeres (approximatively 2000bp see **figure 58 A**). It has been shown that elongated telomeres are positively associated to DNA CpG methylation level (Deng1 et al. 2010; Buxton et al. 2014).

In order to be able to distinguish all the variations in CpG methylation at TERRA promoters after HS, we used in parallel another cell line: HT1080-ST with longer telomeres. Super-Telomerase HT1080 presents elongated telomeres thanks to a stable expression of hTERT and hTR inducing a "Super-Telomerase" activity (Mattiussi et al. 2012). 10q promoter CpG methylation percentage was reported on graph (**Figure 45 C**). Although HeLa cells show greater heterogeneity, our analysis confirm HeLa cells degree of subtelomeric methylation (69%) is lower than detected in HT1080 cells (84%) under normal 37°C condition. Interestingly, upon heat shock both cell lines presented a relative decrease (~15%) of CpG residues methylation.
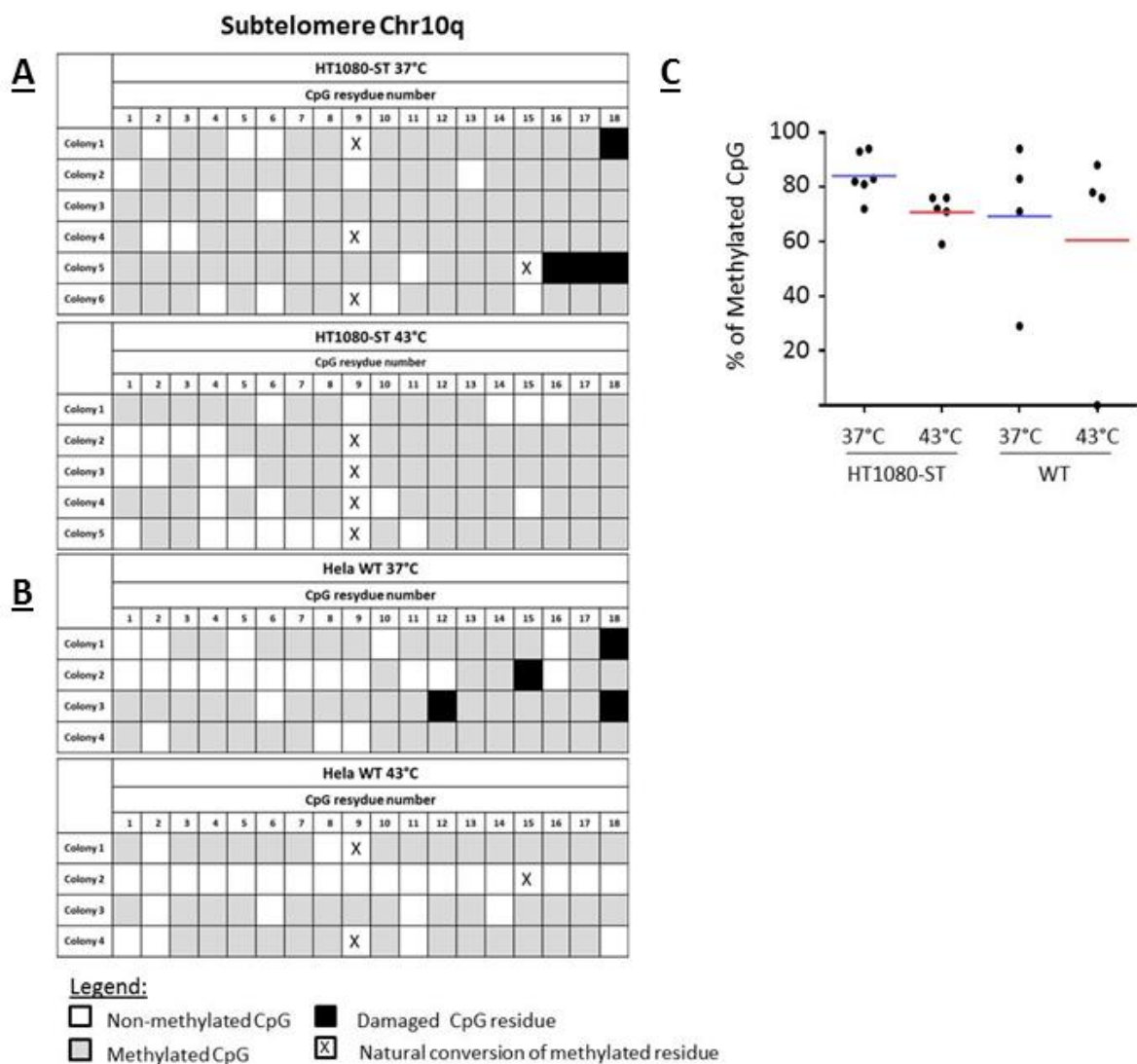
Figure 45| Preliminary data showing CpG-methylation loss of on subtelomeric 10q locus, upon HS. Subtelomeric (Chr 10q) CpG methylation analysis was performed using bisulfite sequencing. DNA was extracted from HT1080-ST and HeLa WT cells before or after HS. The PCR-selected 10q subtelomeric region was isolated then cloned and different colonies were analyzed. **A.** Subtelomeric CpG-residues methylation was estimated in HT1080-ST (harboring elongated telomeres) and in HeLa WT cells **B.** before or after HS. **C.** % of CpG methylation at the 10q locus was calculated per colony (black dots) and mean value was estimated under 37°C (blue line) and HS (red line).

Thus these preliminary data seem to confirm Hs can impact DNA methylation status in human cells (Tilman et al. 2012). Yet, a correlation between DNA hypomethylation and the upregulation of TERRA transcription should be further investigated. In order to dig into such perspectives, previous results should be reproduced and supplementary experiments and controls are imperatively required. To start with, a similar analysis of CpG methylation should be performed in KD HSF1 cell line to correlate HSF1-dependent TERRA transcription to a loss of CpG methylation. In addition, other subtelomeric promoter regions need to be

analyzed by bisulfite sequenced in the three cell lines in order to confirm a general or a chromosome specific CpG methylation loss upon HS at TERRA promoters. Finally other CpG islands-containing promoters regions should be carefully selected and sequenced as positive and/or negative controls.

Chapter II| HSF1 impact on telomeres upon HS

I. HS and HSF1 impact on telomere integrity

Telomeres protect chromosomes ends and thus ensure genome stability. This vital function is tightly associated to telomere integrity. Maintenance of telomere integrity was shown to be mediated through a subtle combination of factors: telomere length, epigenetic status, telomeric binding proteins, T-loop structure and TERRA expression.

In this second part of my PhD work we attempted to first understand to which extent HS could affect telomeres integrity and in a second time, to determine if active HSF1 could play a role in telomere protection under stress.

I. 1. Telomeric Repeat-binding Factor 2 (TRF2)

One of the major complexes related to telomeres integrity is the so-called "shelterin". Telomeres are described as a nucleoproteic complex due to the presence of telomeres-associated proteins that were shown to play essential functions associated to telomere integrity. Among the shelterin complex members, the most extensively studied is TRF2 (Telomere Repeats binding Factor 2). TRF2 binds double-stranded telomeric DNA in a sequence- and structure-dependent manner and was highlighted as an essential element of telomere maintenance through its involvement in T-loop formation, telomere length regulation and ATM repair pathway inhibition (Takai et al. 2003; Palm & de Lange 2008; Sarek et al. 2015).

To evaluate the impact of HS and HSF1 on telomere integrity we analyzed TRF2 protein level, telomeric binding and nuclear localization in WT and KD HSF1 cells. We first controlled that TRF2 total protein level was not impacted by HS and/or HSF1 using western blot analysis (**Figure 46 A**). Tubulin detection was used as loading control (**Figure 46 A**). Quantification of WB signals showed no impact of HS and HSF1 on TRF2 total protein level in WT and KD HSF1 (**Figure 46 B**).

A

B
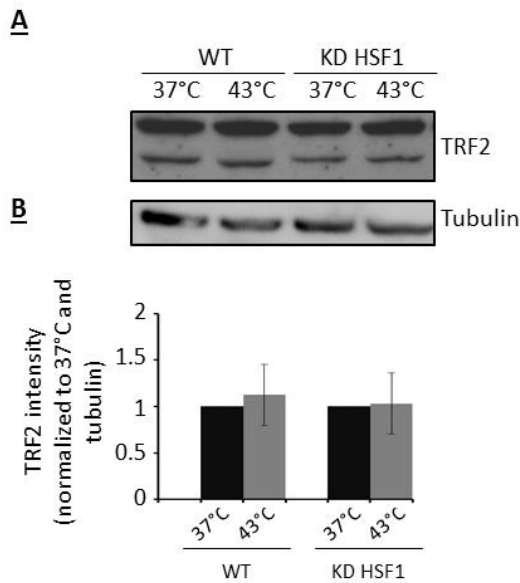
Figure 46| Heat shock and HSF1 depletion do not impact TRF2 global protein level. A. TRF2 protein level was visualized by western blot, using whole-cell extracts derived from HeLa WT and KD HSF1 cells, submitted or not to HS. Tubulin was used as loading control. **B.** Graph bars represent western blot signals quantified and normalized to Tubulin. S.d are based on 3 independent experiments.

HS and HSF1 impact on TRF2 were investigated in terms of nuclear localization, using *in situ* approaches combining detection of telomeric DNA by FISH and of TRF2 by immunostaining (**Figure 47 A**). As expected under physiological conditions, TRF2 foci were detected as small foci localized to telomeres and under HS, the TRF2 foci nuclear distribution were maintained. Minimal distances between TRF2 and telomeres foci centers, in both stressed and unstressed cells, were estimated from 3D reconstituted images. Mean values of TRF2-telomere distances were calculated per nucleus and reported on graph (**Figure 47 B**). The mean distance separating TRF2 from the closest telomere seemed to be increased upon HS in WT cells or in other words, the tight subnuclear colocalization normally observed for TRF2 and telomere DNA at 37°C, is being altered following HS. To a lesser extent, KD HSF1 cells show a similar effect of HS on TRF2 subnuclear distribution. These results suggest that partial TRF2 delocalization from telomeres upon HS is an HSF1 independent phenomenon. Antibody used for TRF2 signals detection by immunofluorescence and western blot were controlled for its specificity. WT cells or cells transiently transfected with a siRNA against TRF2 were subjected to TRF2 immunofluorescence (**Figure 48 C**) or western blot. siTRF2-transfected cells showed a significantly decreased TRF2 signals therefore validating the specificity of TRF2 labeling (**Figure 48 A**).
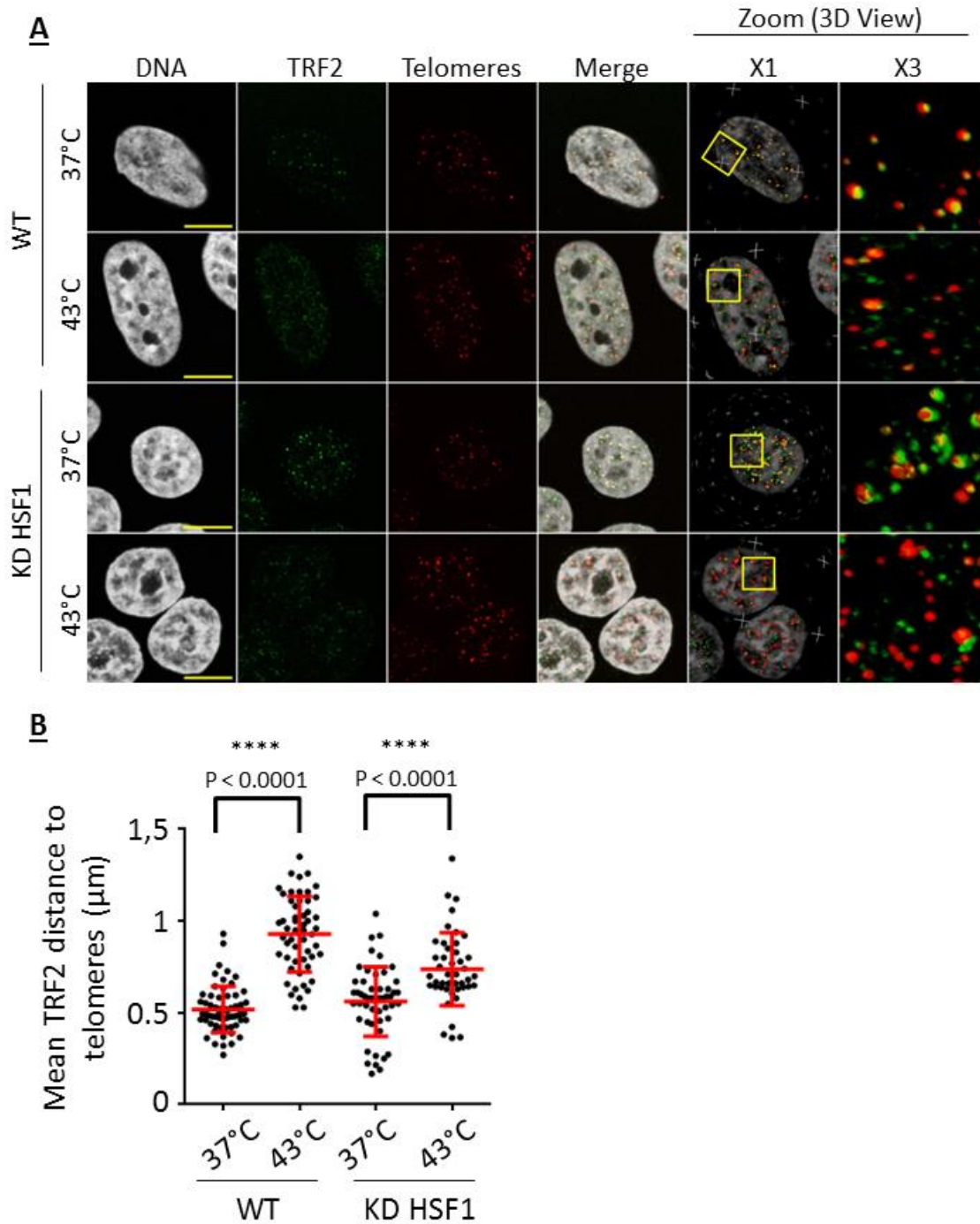
Figure 47| Heat shock induces a partial delocalization of TRF2 from telomeres in a HSF1 independent manner. A. Fixed HeLa WT and KD HSF1 cells were subjected to combined DNA-FISH IF experiments where telomeric repeats were labeled using a Cy3-(TTACCC)$_3$ probe (red) and TRF2 protein was immuno-labeled (green). DAPI-stained nuclei are shown in gray. Representative images of subnuclear TRF2 protein and telomeres localization are shown **B.** Reconstructed 3D images were submitted to analysis of minimal distance between TRF2 and telomeres foci in individual nuclei. Mean values of minimal distances were calculated and are reported on graph for 37°C and 1h at 43°C conditions. Between 45 and 65 nuclei were analyzed per condition. Indicated *P* values were calculated using a two-tailed T-test.
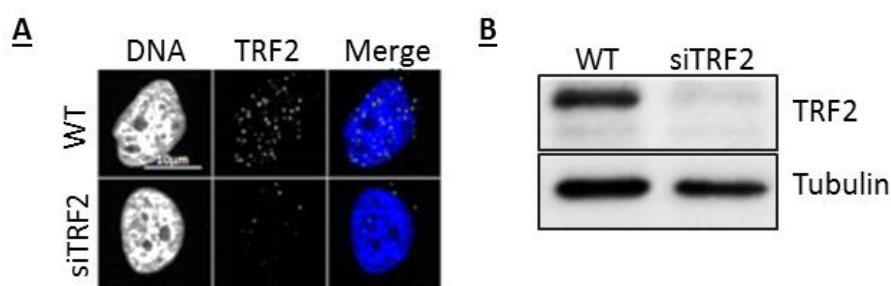
Figure 48| Validation of TRF2 depletion. In parallel WT HeLa cells were transiently transfected with a siRNA against TRF2 in order to validate TRF2 antibody specificity. Scale bar = 10μm. **A.** Immunofluorescence and **B.** Western blot analysis were performed in WT or siTRF2 transiently transfected HeLa cells Tubulin was used as loading control.

Next, the impact of HS and HSF1 on specific TRF2 abundance at telomeres was monitored by ChIP on chromatin extracts from WT HeLa cells heat shocked or not (**Figure 49 A**).
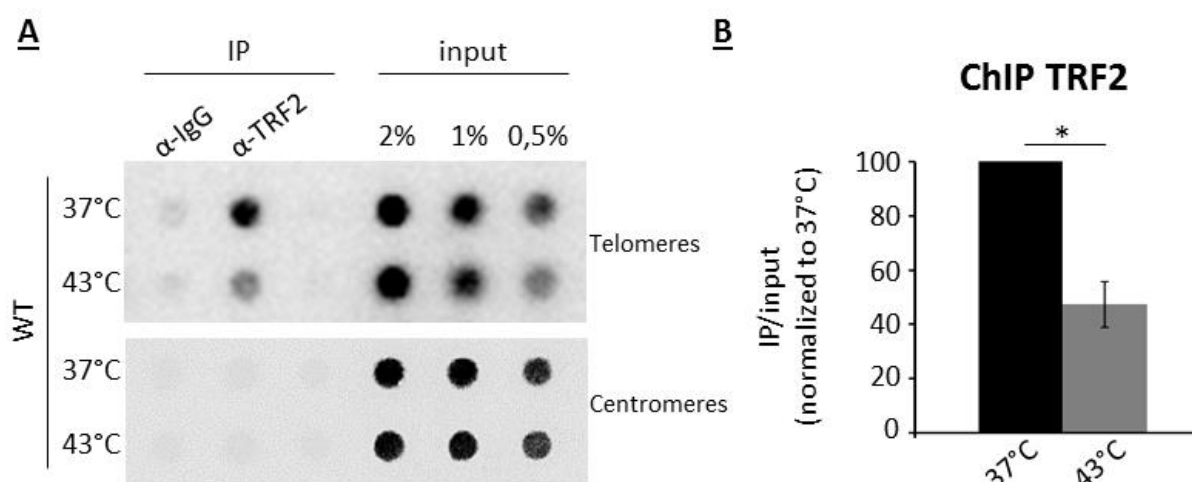
Figure 49| A. Representative image of ChIP TRF2, DNA Dot-blot analysis. ChiP experiments were performed on cell extracts from unstressed (37°C) and heat-shocked cells (43°C). Membranes were hybridized with radioactive telomeric probe. IgG-immunoprecipitated DNA and centromeric probe were used respectively as a control for antibody and probe specificity. **B.** Quantifications of ChIP-TRF2 telomeric enrichment are reported on graph. Data were successively normalized with input and values obtained at 37°C conditions. S.d are based on 3 independent experiments. P-values were calculated by unpaired Student's *t*-test with Welch's corrections ((*) $p<0.05$).

Centromeric probe was used as control. DNA obtained after ChIP-TRF2 was subjected to DNA dot-blot and signals were quantified, normalized (to input and 37°C conditions) and reported on graph (**Figure 49 B**). As expected, TRF2 was efficiently immunoprecipitated from telomeres in WT physiological conditions while TRF2 enrichment was not detected at centromeric regions thus demonstrating the specificity of this observation. Interestingly, WT

cells present a partial decrease of TRF2 binding to telomeric sequences, involving 50% of the total TRF2 fraction, upon HS (**Figure 49 A** and **B**) supporting the idea HS may destabilize elements of the telomeric architecture, such as TRF2, and induce a partial telomeric deprotection. In the case of KD HSF1 cells, we encountered difficulties reproducing results concerning the telomeric TRF2 binding. To conclude, we were able to demonstrate a reproducible and significant impact of HS on telomeric TRF2 binding, whether or not HSF1 plays a role in that process is still to be investigated.

Taken together our results show HS does not impact TRF2 level but induces a partial TRF2 dissociation from telomeres that seems to be HSF1 independent. Increase in temperature may impact protein conformation and consequently protein function. It has been shown that partial depletion (Cesare et al. 2013) or complete deletion of TRF2 (Takai et al. 2003) in human cell lines, leads to telomere deprotection and induction of telomeric damages. The next step was to analyze the impact of HS and HSF1 on telomeric integrity.

I. 2.   H2A.X histone variant phosphorylation (H2A.X-P)

▪   Upon HS

To evaluate the impact of HS and HSF1 on telomere integrity, we next controlled for stress induced-telomeric DNA damages. Dysfunctional, uncapped telomeres, created through inhibition or partial dissociation of TRF2 were showed to be associated with DNA damage response factors, such as 53BP1 and H2A.X-P (Takai et al. 2003; Cesare et al. 2013). In addition, it has been shown that HS induces DNA damages within the genome. (Velichko, N. V Petrova, et al. 2012; Velichko et al. 2013; Velichko et al. 2015) We started by investigating H2A.X histone variant phosphorylation (H2A.X-P) at serine 139 which is a well-known molecular marker of DNA damage (Fernandez-capetillo et al. 2002). Domains of telomere-associated DNA damage factors were termed "Telomere Dysfunction-Induced Foci (TIF) (Takai et al. 2003).

A preliminary control of H2A.X-P total protein level was made in both WT and HSF1 KD cell lines before and after HS. Tubulin was used as loading control (**Figure 50 A** and **B**). Western blot analysis of H2A.X-P signals show HS application in both WT and KD HSF1 HeLa

cell lines induced a significant increase of total protein level suggesting increased global genomic damages independently of HSF1.

A

B
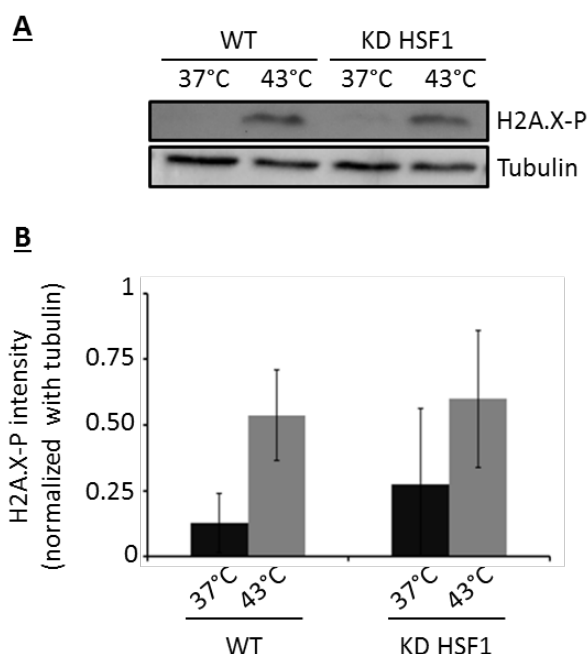
Figure 50| Heat shock induces a global H2A.X-P increase, independently of HSF1. A. H2A.X-P protein level was visualized by western blot, using whole-cell extracts derived from HeLa WT and KD HSF1 cells, submitted or not to HS. Tubulin was used as loading control. **B.** Graph bars represent western blot signals quantified and normalized to Tubulin. S.d are based on 3 independent experiments.

To evaluate the impact of HS and HSF1 on telomere integrity, we next monitored for H2A.X-P accumulation to telomeres by ChIP dot-blot in both WT and HSF1 KD cells (**Figure 51 A**).
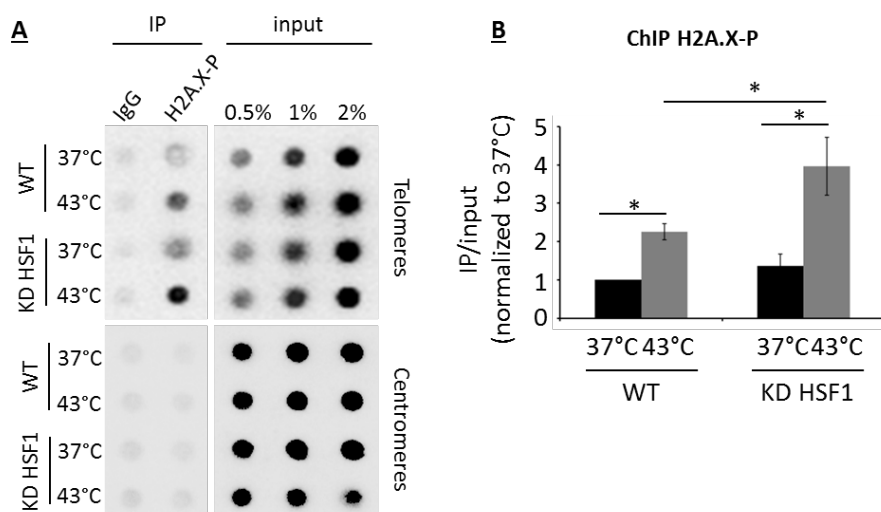
Figure 51| HSF1 depletion results in extended telomeric damage upon HS. A. Representative DNA Dot-blot of ChIP H2A.X-P analysis. ChiP experiments were performed on cell extracts from unstressed (37°C) and heat-shocked (43°C) cells. Membranes were hybridized with radioactive telomeric probe. IgG-immunoprecipitated DNA and centromeric probe were used respectively as a control for antibody and probe specificity. **B.** Quantification of H2A.X-P telomeric enrichment is reported on graph. Data were successively normalized with input and values obtained at 37°C conditions in WT cells. S.d are based on 3 independent experiments. P-values were calculated by unpaired Student's *t*-test with Welch's corrections ((*) p<0.05).

Dot-blot quantification revealed remarkably that previous results showing a 2-fold decrease of TRF2 binding correlated with a 2.25-fold enrichment of H2A.X-P at telomeres in heat-shocked WT cells. In parallel, we noticed a 2-fold increase of TIF in heat-shocked HSF1 KD cells compared to heat-shocked WT suggesting a role of HSF1 in telomeric protection upon HS (**Figure 51 B**). Centromeric probe was used as negative control and showed no H2A.X-P enrichment (**Figure 51 A**). To conclude our results show HS exposure induces a significant increase of telomere specific DNA damage in our cell model. Most importantly, our data support the idea that HSF1 depletion results in extended telomeric damage upon HS, suggesting a role for HSF1 in telomere maintenance under stress conditions. Heat-induced DNA damages at telomeres were similarly evaluated by *in situ* approaches (**Figure 52 A**).
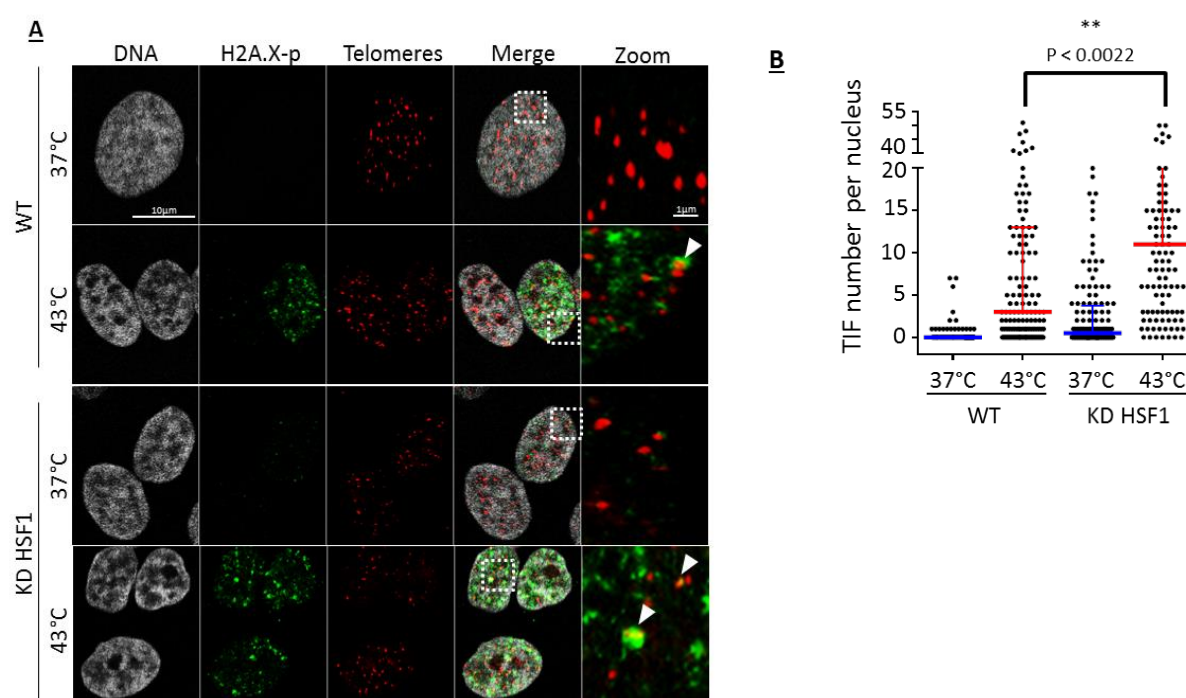
Figure 52| HSF1 depletion results in extended *in situ* Telomere damage Induced Foci (TIFs) upon HS A. Fixed HeLa WT and KD HSF1 cells were subjected to combined DNA-FISH IF experiments where telomeric repeats were labeled using a Cy3-(TTACCC)$_3$ probe (red) and H2A.X-P protein was immuno-labeled (green). DAPI-stained nuclei are shown in gray. Representative images of subnuclear H2A.X-P protein and telomeres localization are showed as well as estimated foci co-localization (arrow) known as Telomere damage Induced Foci (TIF). **B.** TIFs were quantified in 3D reconstituted nuclei images of WT and KD HSF1 cells. The number of counted TIFs per nucleus (black dot) was reported on graph, Median values of TIFs per nucleus are represented (thick bars) as well as the 3rd quartile (thin barres) designing respectively 50% and 75% of each cell population. Median and quartile are based on n=3 independent experiments, data were assembled (cell number per condition between [120; 180]. *P value is indicated and* was evaluated with Mann Whitney test.

Quantification of H2A.X-P foci associated with telomeres also called TIFs per nucleus revealed a 10-fold increase following HS (**Figure 52 B**) in HSF1 depleted cells while WT cells to a lesser extent also presented TIFs upon HS (3-fold). It is noteworthy that even though median values are similar under physiological conditions (37°C) in HSF1-depleted population shows a stronger heterogeneity in the number of TIFs per cell. As the number of cells accounted in both conditions is similar (WT, 37°C: 140 cells and KD HSF1, 37°C: 180 cells) this difference may reflects the populations heterogeneity in terms of HSF1 expression levels.

In order to control our observations are not limited to HeLa cell model, three other human cell lines HT1080, and HFF2-TERT were submitted to HS and analyzed, after fixation, to telomere DNA FISH combined to H2AX-P immune-labeling (**Figure 53 A**). 3D reconstructed IF-DNA FISH images were analyzed for TIF accumulation (**Figure 53 B**). Interestingly we observed three different intensity of response: the number of TIFs in WT HeLa and HT1080 increases around 10 times compared to 37°C, whereas HFF2-TERT cells show a lower increase of TIF level upon HS. The following results confirm HS negative impact on telomere integrity in two other human cancer cell lines and underline the fact that the number of HS induced damages depends on cellular strains.
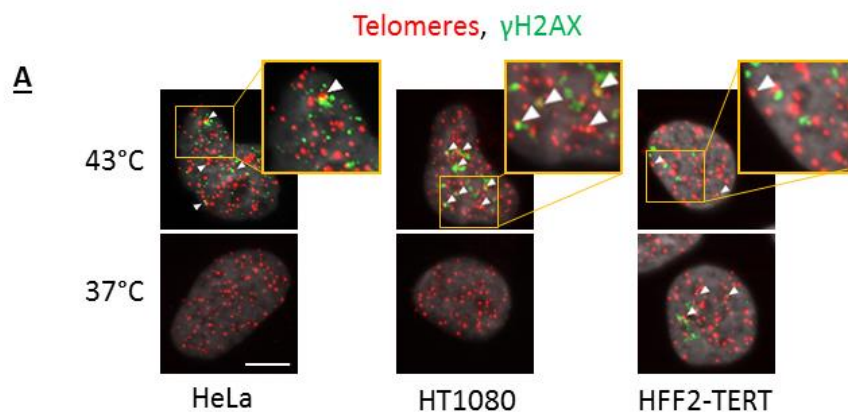
B
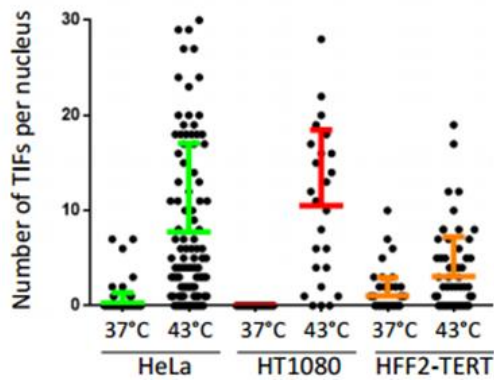
Figure 53| A. Heat shock impacts telomere integrity in various cell lines. HT1080 and HFF2-TERT cell lines were used to control HS effect on telomere integrity in different cell lines. Cells were subjected to DNA FISH-IF experiments in order to evaluate TIF (arrow) accumulation. **B.** H2A.X-P accumulation to telomere was estimated per nucleus (3D images) and reported on graph. Mean values of TIFs per nucleus are represented. Mean standard deviation bars represent the population's heterogeneity.

■   During recovery

Heat shock is a reversible process and HSF1 is involved not only in balancing core cellular processes during stress but also in their rapid re-establishment once conditions suitable for proliferation have been restored. After 1 hour of heat shock at 43°C, the active phosphorylated form of HSF1 begins to disappear in favor to its inactive form at 2h of recovery at 37°C (**Figure 54 A**) To assess the impact of HSF1 depletion on telomeric recovery after stress, we quantified the progressive disappearance of TIFs, in WT and HSF1 KD cells, during a kinetic of recovery (2 to 6 hours after HS) (**Figure 54 B** and **C**).
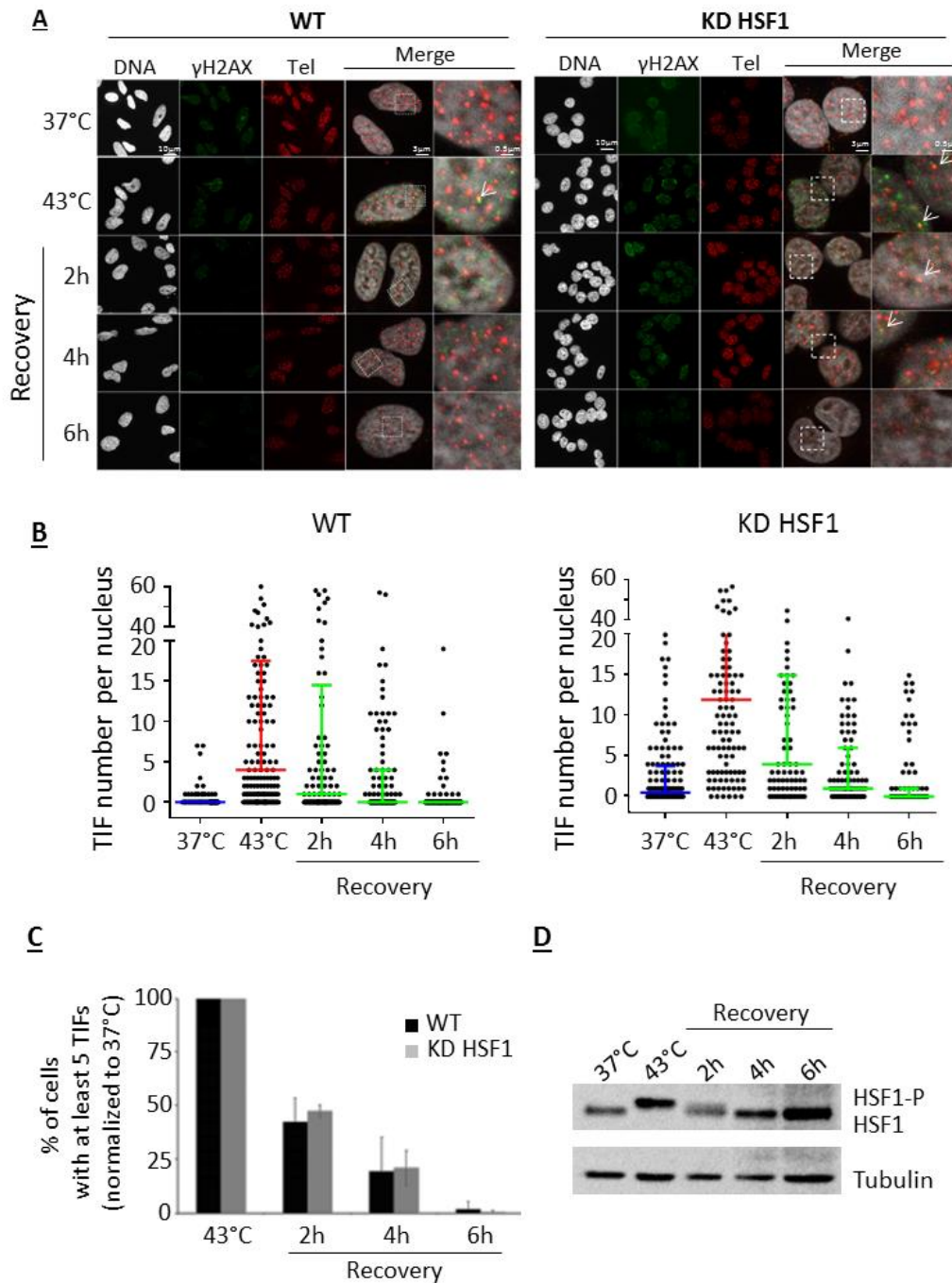
Figure 54| No impact of HSF1 on the resolution of TIFs after HS. A. Representative images of co-localization between H2A.X-P (green) detected by IF and telomeres (red) detected by DNA-FISH with Cy3 (TTACCC)$_3$ probe in WT cells and KD HSF1 cells before and after HS and during a recovery period (2h to 6h) at 37°C. **B.** Quantification of TIFs number per nucleus in WT and KD HSF1 cells before and after HS at 43°C and during the recovery period (n≥80 per condition). **C.** Quantification of the percentage of WT and KD HSF1 cells with at least 5 TIFs and normalized to the 1 hour HS and 37°C conditions, representing the rate of recovery. **D.** Western blot analysis of HSF1 during kinetics of recovery (from 2 to 6 hours) after HS in WT cells. Tubulin was used as loading control.

In order to evaluate the recovery rate for each one of our cell lines, TIFs quantification values were normalized both by (a subtraction of) 37°C values and 43°C

condition (**Figure 54 C**). Our results show that there is no significant difference between WT and HSF1 depleted cells, in the speed of telomeric damage resolution suggestion HSF1 is not implicated in that process. Indeed WT and KD HSF1 cell lines show 50%, 25% and almost a complete TIF loss, during 2, 4 and 6h of recovery respectively. HSF1 characteristic shifting under HS was controlled, in parallel to FISH-IF experiments, as well HSF1 inactivation under recovery kinetics (**Figure 54 D**).

I. 3. 53BP1

In a similar manner to H2A.X-P, the p53-binding protein 1 (53BP1) is an important regulator of the cellular response to DSBs and hence a reliable molecular marker of DNA damage (Fernandez-capetillo et al. 2002). Remarkably, upon HS, 53BP1 subnuclear localization was totally overturned in WT HeLa cells as shown (**Figure 55 A**) when using *in situ* immunofluorescence. The characteristic foci pattern of 53BP1 that can clearly be observed at 37°C was completely diffused after exposure to HS rendering TIFs colocalization analysis technically impossible. This phenomenon may be explained by 53BP1 protein structure sensitivity to HS. In contract to H2A.X-P, 53BP1 protein structure could have been denatured by heat and hence it's DNA binding properties are altered. In order to control 53BP1 protein level before and after HS, WB was employed (**Figure 55 B** and **C**). In our cells the basal 53BP1 protein level is relatively weak and remains unchanged after HS exposure, supporting the idea that the protein diffusion upon HS is not accompanied by protein degradation. Thus, we decided not to use 53BP1 as a marker for studying HS and HSF1 impact on telomeres integrity.
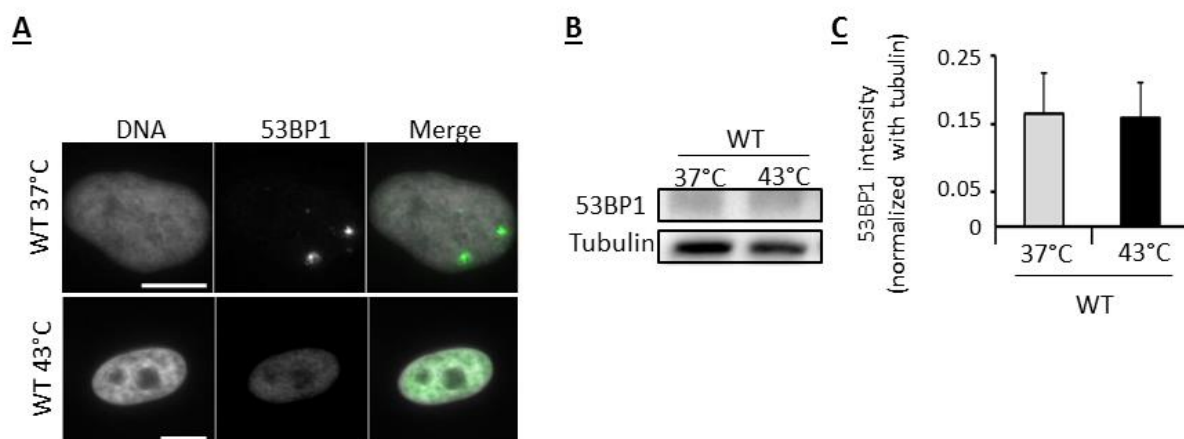
Figure 55| 53BP1 subnuclear compartments are disrupted upon HS exposure A. Representative images of 53BP1 immunofluorescent labeling in control (untreated) and heat-treated cells (43°C, 1h) HeLa WT. The DNA

was stained with DAPI. Scale bar = 5µm. **B.** 53BP1 protein level was visualized by western blot, using whole-cell extracts derived from HeLa WT cells, submitted or not to HS. Tubulin was used as loading control. **C.** Graph bars represent western blot signals quantified and normalized to Tubulin. S.d are based on 2 independent experiments.

II.   HSF1-dependent modulation of telomeric epigenetic status

II. 1.   Telomeric H3K9me3 and H3

Mammalian telomeric and subtelomeric heterochromatin is characterized by a specific epigenetic signature enriched in repressive histone marks such as H3K9me3, H4K20me3 and Heterochromatin Protein 1 (HP1) (Blasco & Schoeftner 2008; Yehezkel et al. 2008). In the case of the pericentromeric 9q12 locus, under HS, heterochromatin decompaction was shown to occur through the HSF1-dependent loss of epigenetic repressive marks (H3K9me3, HP1) and massive histone H3 and H4 acetylation followed by SatIII transcription (Biamonti & Vourc'h 2010). In addition, active TERRA transcription is accompanied by modifications in telomeric and subtelomeric heterochromatin marks (Blasco & Schoeftner 2009; Arnoult et al. 2012). The impact of HSF1 on the epigenetic status of telomeres and subtelomeric regions upon stress was thus assayed. The impact of HSF1 on the global protein amount of H3 and H3K9me3 was first examined in WT or HSF1 KD cells, submitted to heat-shock kinetics (experimental procedure described in **Figure 56 A**).

As expected, a delay in HSF1 mobility on gel was observed in heat-shocked cells due to its heat-induced post transcriptional modifications including hyper-phosphorylation but no significant impact of HS on the global protein amount of H3 and H3K9me3 was observed (**Figure 56 B**). We therefore proceeded to investigate the impact of HS and of a HSF1 knock-down on telomeric H3 and H3K9me3 levels by ChIP followed with DNA dot-blot analysis (**Figure 56 C**).

H3 and H3K9me3 dot-blot signals were quantified and normalized to input and 37°C respective conditions. H3K9me3 IP values were also subjected to H3 normalization to prevent a possible bias resulting from nucleosome occupancy changes. Although no significant change in H3 enrichment was observed during HS, a gradual increase in H3K9me3 occupancy at telomeres was detected in WT cells in response to HS exposure (**Figure 56 D**).
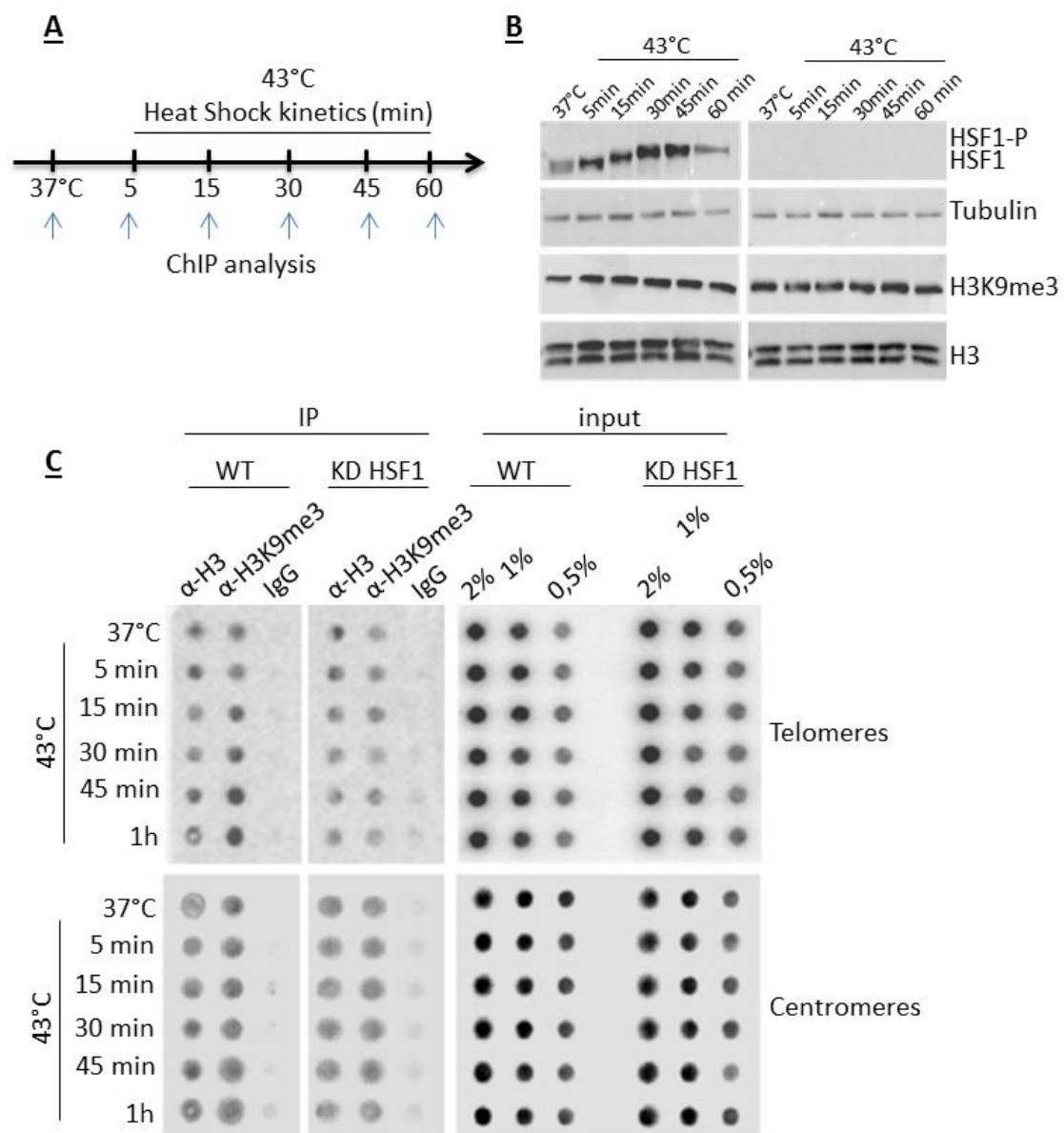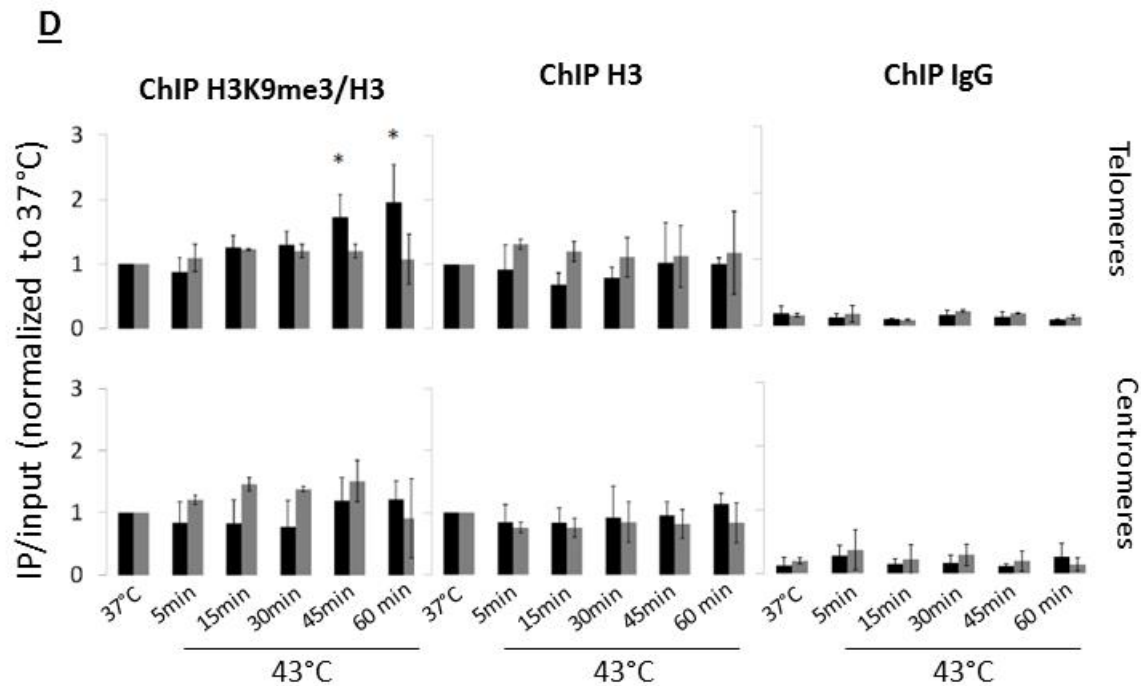
Figure 56| TERRA upregulation correlates with H3K9me3 enrichment at telomeres. A. Experimental procedure for ChIP analysis during heat shock kinetics. **B.** Western blot analysis of total HSF1, H3 and H3K9me3 protein level in WT and KD HSF1 cells upon HS kinetics. Tubulin was used as loading control. **C.** Representative image of analyzed DNA dot-blot of ChIP H3 and H3K9me3. ChiP experiments were performed on cell extracts from unstressed (37°C) and exposed to 43°C from 5 to 60min. Membranes were hybridized with radioactive telomeric probe. IgG-immunoprecipitated DNA and centromeric probe were used respectively as a control for antibody and probe specificity. **D.** Quantification of DNA dot-blot. H3 and H3K9me3 telomeric enrichment are reported on graph. Data were successively normalized with input and values obtained at 37°C conditions. S.d are based on n=3 independent experiments. P-values were calculated by unpaired Student's *t*-test with Welch's corrections ((*) $p<0.05$). IgG antibody was used as a negative control for ChIP.

Conversely, no variation in the level of either H3 or H3K9me3 was observed at telomeres of KD HSF1 cells upon HS. As no significant variation of H3K9 trimethylation levels was observed at centromeric regions in response to HS (Fig 53 D), our data suggest a specific and HSF1 dependent increase of telomeric H3K9me3 density upon HS that may be related to our prior observation of TERRA upregulation. Indeed, several publications underlined TERRA capacity to specifically bind H3K9 tri-methylated histone and HMTs like SUV39, suggesting TERRA upregulation favors heterochromatin formation and maintenance at telomeres (Deng et al. 2009; Arnoult et al. 2012). Next, we proceeded to a chromosome specific analysis of subtelomeric H3 and H3K9me3 epigenetic marks in order to determine if only transcribed telomeres undergo epigenetic modulation suggesting a cis-effect of TERRA or if a trans-effect of TERRA can by questioned.

II. 2. Subtelomeric H3K9me3 and H3

Subtelomeric H3 and H3K9me3 levels were evaluated using ChIP followed by chromosome specific Q-PCR analysis of selected regions using designed primers (**Figure 57 A**). ChIP values are represented as a percentage of total telomeric DNA in the input. H3K9me3 and H3 ChIP values were then normalized to those obtained at 37°C. No significant changes in H3 or H3K9me3 enrichment were observed during HS at selected subtelomeric regions containing or not HSE in both cell lines (**Figure 57 B**). Used respectively as a positive and negative control, a significant decrease in H3K9me3 enrichment at pericentromeric 9q12 region was detected (**Figure 57 C**) and an unchanged and very weak H3K9me3 signal was detected at *HSP70* promoter.
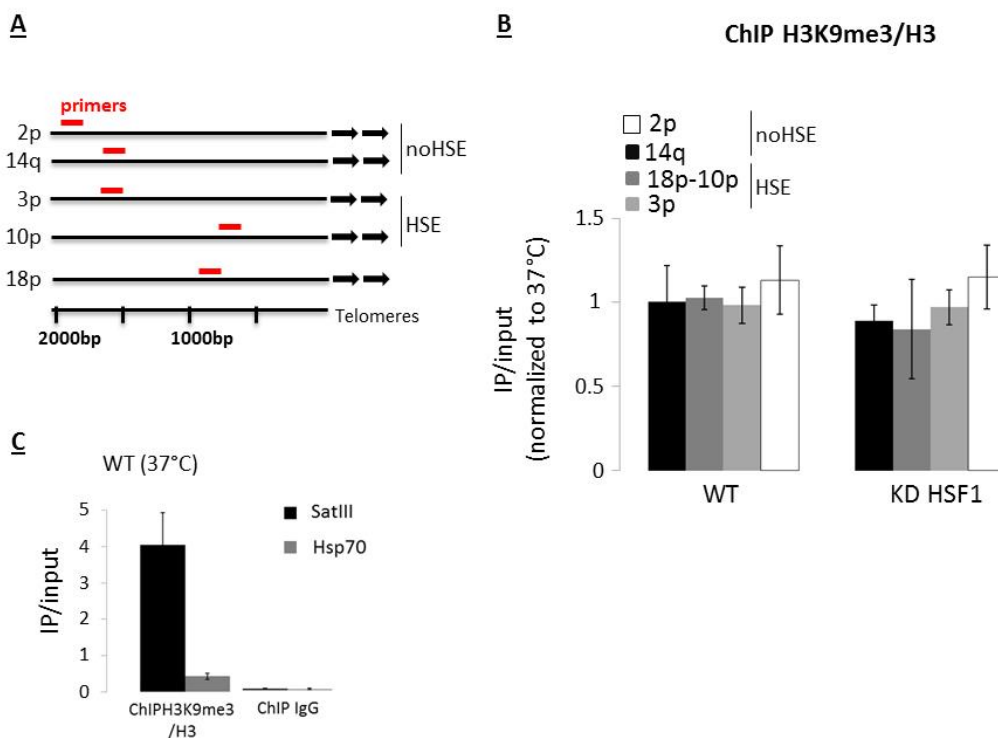
Figure 57| H3K9Me3 enrichment at subtelomeric regions is not impacted by heat shock or HSF1 deficiency. A. Set of subtelomeric primers used for ChIP Q-PCR Analysis. **B.** DNA extracted from WT or KD HSF1 cells submitted to 37°C or HS conditions was used for α-H3K9me3 and α-H3 ChIP experiments. H3K9me3 and H3 enrichment to subtelomeric regions (chromosomes 14q, 10-18p, 3p, 2p) was estimated by Q-PCR. S.d are based on n=3 independent experiments. **C.** WT cells immunoprecipitated DNA was also analyzed with primers recognizing SatIII 9q12 region and *HSP70* gene promoter, used respectively as positive and negative controls for H3K9me3 enrichment at physiological (37°C) conditions.

Our data showed no impact of HS or HSF1 on H3 and H3K9me3 epigenetic marks on subtelomeric regions flanked by chosen primers. However to insure our conclusions a larger panel of subtelomeric primers should be tested on selected chromosomes. Indeed, one

cannot predict the exact position of epigenetic marks on subtelomeric regions since, the precise H3K9me3 signature on TERRA promotors is not precisely defined and can vary among cells and experimental conditions. Moreover, human TERRA promoter regions can extend up to 5000bp and histone epigenetic marks can be found widely, yet not evenly, distributed along the subtelomere.

II. 3. Punctual HS exposure does not impact telomere length

Telomere shortening is directly associated with telomeres fragility, cell senescence, ageing and cancer. Evidence in the literature demonstrating an negative impact of heat induced–stress on telomere length was accomplished in yeast *S. cerevisiae*, exposed to chronic heat shock over 100 generations (Romano et al. 2013). In order to control that a short HS exposure (1h 43°C) does not directly impact telomere shortening, we performed terminal-restriction-fragment Southern blotting (**Figure 58 A**).
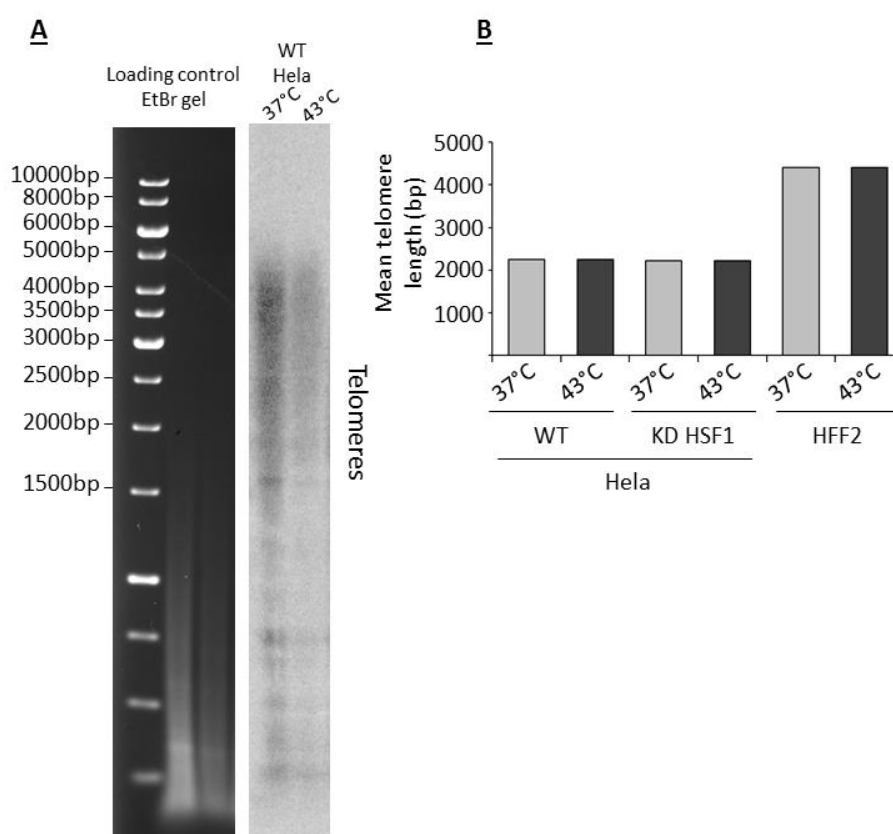
Figure 58| No impact on telomere length upon HS A. Telomere length was monitored using terminal-restriction-fragment Southern blotting. Telomeric repeats were detected with a radioactive specific probe; Ethidium Bromide-stained agarose gel was used to detect size markers and for DNA loading. **B.** Mean telomere length was estimated for WT and KD-HSF1 HeLa and HFF2 cells under 37°C or 43°C conditions.

Mean telomere length was estimated for the three tested cell lines HeLa WT or KD HSF1 and HFF2 cells and reported on graph (**Figure 58 B**). The three cell lines presented no visible difference in telomere length after 1 hour exposure to 43°C. As expected HFF2 cell line immortalized with TERT, presented a twofold longer telomeres compared to HeLa cells, known for their short telomeres.

II. 4.   HS induces telomerase activity decrease independently of HSF1

Telomerase activity has been shown to be specifically expressed in a large proportion of immortal cells cancer, where it compensates for telomere shortening during DNA replication and thus stabilizes telomere length. Thus telomerase activity was correlated with an efficient repair of unprotected telomeres. Telomerase activity was assessed using an *in vitro* Q-TRAP assay on WT and HSF1 depleted HeLa cell extracts submitted to 37°C or HS conditions. Cells exposure to HS resulted in a 50% decrease of telomerase activity independently of HSF1. Our data confirm telomerase is a heat-sensitive enzyme and excluding a correlation between HSF1 and telomerase activity upon HS. siRNA-dependent depletion of HSF1 was controlled by western blot (**Figure 59 A**) showing a significant decrease in HSF1 total protein level. Unchanged level of HSF1 was found in WT or siCTL-treated cells. Tubulin was used as loading control. TRAP assay quantification were reported on graph (**Figure 59 B**), telomerase activity is presented as a percentage of the 37°C WT condition. Based on our previous results on telomere length analysis, a relatively breve heat shock exposure is not sufficient to impact telomere homeostasis via the reversible partial telomerase inhibition.
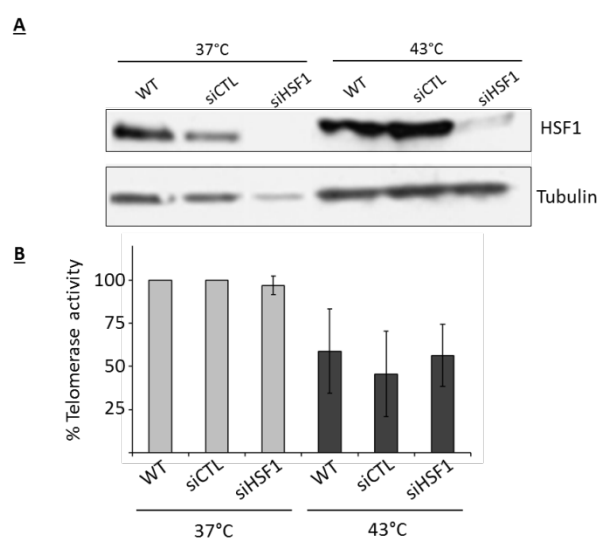
Figure 59| Telomerase activity loss upon HS is HSF1 independent A. HSF1 siRNA-dependent knock down was controlled by western blot at 37°C and 43°C conditions. **B.** Telomerase activity was evaluated using TRAP essay in WT, siCTL or siHSF1 HeLa cells before or upon HS. S.d are based on n=2 independent experiments

Result's synthesis:

Model: HSF1 dependent-TERRA function in telomere protection upon cellular stress response.
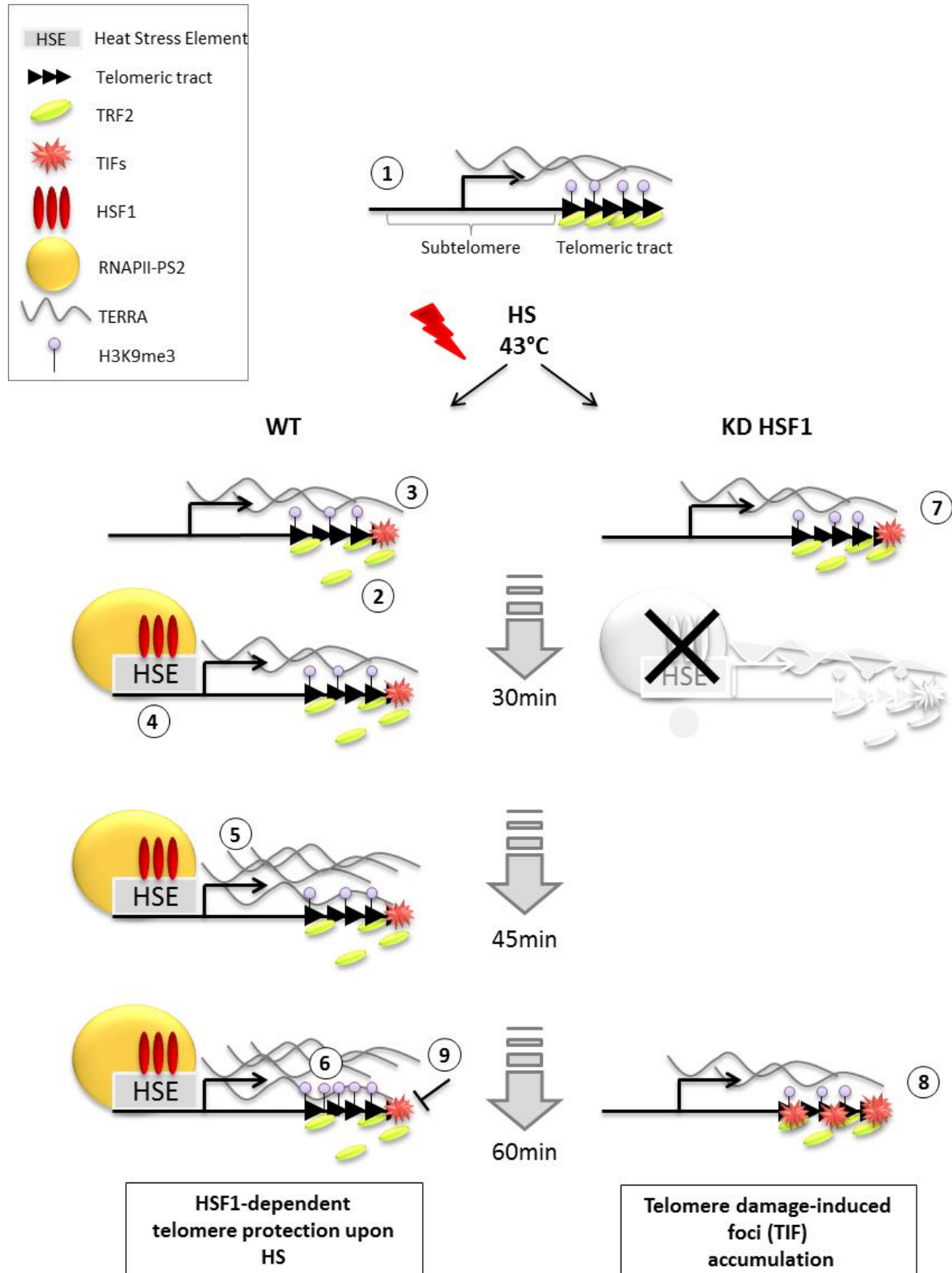
Figure 60| Model for HSF1 dependent telomere maintenance under stress conditions.

Telomeres and hence telomere maintenance mechanisms, play a central role in preserving the eukaryotic genome integrity. Telomeres are organized into a nucleo-proteic complex called the "shelterin". This complex is characterized by the presence of repeat-binding-factor 2 (TRF2), by a heterochromatin state enriched in repressive epigenetic marks such as H3K9me3 and by the presence of lncRNAs of telomeric origin known as TERRA (TElomere Repeat-containing RNA). Indeed, a portion of mammalian telomeres have been shown to be constitutively transcribed by RNA Polymerase II (RNAPII). TERRA has been found to play an essential role in telomere architecture, stability and protection. TERRA can be distinguished from other lncRNAs as they form a heterogeneous group of RNAs with regard to their nucleic acid sequences (their 5' end encompasses chromosome specific subtelomeric regions) and length (**1**). Our research aimed to question the impact of environmental stress on telomere integrity, at the molecular level, using the heat shock (HS) model as a stressing agent. Our results convey two main information, firstly, HS significantly alters telomere homeostasis and secondly, the molecular orchestrator of the cell response to stress, HSF1, directly contributes to telomere integrity maintenance upon HS. Indeed, exposure of HeLa human cancer cell lines to 1 hour HS (43°C) leads to a rapid and significant loss of TRF2 from telomeres (**2**) and to an accumulation of DNA damage induced-foci at telomeres (TIFs), characterized by the presence of γH2A.X (**3**). Additionally, our work brings clear evidence for a HSF1 and RNAPII-PS2 (elongating polymerase) enrichment after 30 min HS (**4**). Consequently to HSF1 binding, chromosome specific TERRA expression is enhanced starting from 30 to 45 minutes of HS (**5**), followed by an increase of telomeric H3K9me3 level after 45 to 60 minutes of HS (**6**). Use of HSF1 knock-down (KD HSF1) cells supports the existence of a partial HSF1-independent TRF2 dissociation from telomeres upon HS (**7**). In HSF1 KD cells, no accumulation of RNAPII-PS2 and of TERRA upon HS was observed at telomeres and loss of H3K9me3 was not detected either. However, a significantly higher number of TIFs was clearly detected after 1 hour of HS, compared to WT cells (**8**). Taken together, based on our observation and given the role of TERRA in telomeres heterochromatin formation and maintenance, we propose the following model: Upon environmental stress, insult telomere's integrity is at stake and HSF1-dependent TERRA accumulation plays a protective role, limiting telomeric DNA damage (**9**). We can imagine that TERRA may exert its protective role on telomeres upon HS by favoring heterochromatin (H3K9me3) formation. As no significant difference was detected between both cell lines in the kinetics of TIF resolution after HS, during recovery period, our data highlight HSF1's contribution to telomere integrity maintenance by limiting TIF accumulation during stress exposure. In contrast, our data suggest that one or several HSF1 independent pathways take over to promote TIF resolution during recovery from stress.

DISCUSSION AND PERSPECTIVES

In this last section of the manuscript, the main results obtained during my PhD will be resumed and discussed in light of other studies published in the related scientific fields. Conclusions and proposals for future investigations will be presented as perspectives of this work.

After years of investigating HSF1 molecular functions in modulating heat shock proteins expression, the discovery of new HSF1 genomic targets changed the field's perception and perspectives (Mendillo et al. 2012; Mahat et al. 2016).

In the first place, pericentromeric heterochromatin was found to be targeted by HSF1 under HS (Jolly et al. 1997; Denegri et al. 2001). Consequently, an HSF1-dependent chromatin remodeling of the pericentromeric 9q12 locus accompanied by a massive SatIII ncRNAs transcription was shown to occur under HS (Metz et al. 2004; Rizzi et al. 2004; Eymery et al. 2010). Specialized chromatin remodeling factors such as HATs or BETs proteins were found to be recruited to the 9q12 locus via HSF1 binding, to induce a transition from heterochromatin to a euchromatin-like state, and thus ensuring SatIII RNA efficient transcription (Fritah et al. 2009; Col & Hoghoughi 2016, data under revision). Based on this new networking between HSF1 and heterochromatin under HS, telomeric heterochromatin, have been investigated as a potential new HSF1 target.

Interestingly, telomere transcription can be regulated by developmental, environmental and stress-related signals (Blasco & Schoeftner 2008; Marion et al. 2009; Porro et al. 2010; Tutton et al. 2015). In pathological stress context such as cancer, TERRA expression was also found to be modulated depending on cancer types and grade (Ng et al. 2009; Sampl et al. 2012; Blasco & Schoeftner 2008; Zhong Deng, Wang, Xiang, et al. 2012).

In particular, our lab together with others, have observed a robust ~twofold upregulation of TERRA transcripts upon HS, in different model organisms (mouse cells, human cells, *C. Thummi*) (Eymery et al. 2009; Blasco & Schoeftner 2008; Martínez-Guitarte et al. 2008). The mechanism through which TERRA may be regulated under HS-induced stress has not been explored in molecular details, and was the starting point of my thesis. This work took advantage of the generously donated HSF1 knock down HeLa cell line

(Dr L. Sistonen, university of Turku, Finland) and of heat shock (HS) as a model to induce the Heat Shock Response (HSR), capable of transiently and reversibly inducing drastic changes in the cell transcriptome through various mechanisms. Both of these tools allowed us to address the impact of heat shock, and of HSF1, main actor in the cellular response to stress, on telomeric transcription and telomeres, in human cancer cell lines.

First, my work confirmed telomeric transcripts are upregulated upon HS and interestingly, subtelomeres-unique sequences analysis of TERRA molecules identified a chromosome specific pattern of TERRA expression, during stress application. Our results point out the essential role of Heat Shock Elements (HSE) at subtelomeric regions in redirecting the recruitment of HSF1, revealing that stress-induced HSF1 binding at telomeres is restricted to telomeres containing HSEs. Indeed, we found only TERRA from HSEs-containing subtelomeric regions were upregulated upon HS. Importantly, we found that HSF1 depletion has no impact on the constitutive level of TERRA expression, thus suggesting that the role of HSF1 on the transcriptional activation of heterochromatic regions is restricted to stressed cells. We also demonstrated that HSF1 impacts the telomeric heterochromatin environment by inducing H3K9me3 enrichment.

We bring evidence that HS affects telomere integrity by inducing telomeric damage and partial uncapping. HSF1 assures telomere protection by limiting the accumulation of telomere-associated H2AX-P foci in response to DNA damage induced by HS. We propose that HS-induced HSF1 activation secures telomeric DNA repair or telomere protection via TERRA upregulation, thus providing a previously unknown telomere maintenance function of HSF1.

HS disrupts telomere integrity

Several biological stressors are known to disrupt telomere length homeostasis (Romano et al. 2013). HS has also been shown to affect DNA replication (Velichko, N. V Petrova, et al. 2012) and to specifically inhibit homologous recombination (HR) involved in both DNA repair (Krawczyk et al. 2011) and in the formation of telomere-specific structures essential for telomeric functions (Verdun & Karlseder 2006). Here, we bring evidence that HS

impacts telomere integrity and that cell exposure to HS increases the number of telomere dysfunction induced foci (TIF) in the different cell lines analyzed. A possibility is that DNA damages we observe at telomeres upon HS may partially result from telomeres uncapping. We observe that a portion of TRF2 dissociates from telomeres upon HS and may be involved in telomeric deprotection. Indeed, several publications show that TRF2 depletion in mammalian cells is a critical event accompanied by TERRA upregulation, TIF accumulation and telomere shortening (Takai et al. 2003; Cesare et al. 2013; Porro et al. 2014).

Few hypotheses can be formulated in order to explain HS impact on telomeric integrity: HS-induced partial TRF2 dissociation may result from structural alterations affecting TRF2 or TRF2 partners. Likewise, we cannot exclude that stress-induced chromatin conformation changes at telomeres may also interfere with the efficacy of the DNA repair machinery. Finally, the presence of TIF may also reveal the existence of a small number of replication fork arrests since H2A.X-P is also thought to protect stress-induced arrested replication forks (Velichko, N. Petrova, et al. 2012). Whatever the exact mechanisms underlying the formation of TIF in HS cells, their presence correlating with TRF2 dissociation at telomeres suggests that HS specifically impacts the integrity of telomeres through a certain level of telomeric deprotection.

It is important to note that, Velichko et al. (Petrova et al. 2014) recently published data show that HS (45.5°C for 10 to 30min) induces TRF2 redistribution throughout the nucleoplasm, which does not initiate the DNA damage response at telomeres, in human primary and cancer cell cultures. The authors analyzed the DNA damage response by using exclusively *in situ* analysis that allows evaluating foci nuclear proximity between H2AX-P and TRF2, used as a telomeric marker. Based on these results, Velichko et al. conclude HS does not induce DDR at telomeres. As their data show TRF2 nuclear organization is drastically deranged upon HS it may be important to confirm their results by using telomeric DNA detection instead of TRF2 IF upon HS. On the other hand, our data bring *in situ* evidence that HS induces a significant delocalization of TRF2 from telomeres accompanied with significant accumulation of the DNA damage molecular marker H2A.X-P to telomeres, and this by two complementary methods (ChIP-DNA dot-blot and combined DNA-FISH/IF). Therefore, our results suggest HS does severely impair telomere integrity. Hence, we can hypothesise the

difference in experimental conditions used in both studies are at the origin of these discrepancy. To take this reasoning further, we can suppose the HS conditions used by Velichko et al. induced a drastic denaturation of TRF2 protein that could explain the massive nuclear redistribution we did not observe.

HSF1 impact on subtelomeric epigenetic status

In the framework of our study, we were interested in gaining insight into the mechanisms allowing HSF1 to promote higher TERRA transcription under HS. Among the described pathways regulating telomere expression, epigenetic remodeling of telomeric and subtelomeric chromatin was found to be strongly correlated with TERRA expression levels. Our goal, was to check if telomeres transcribed during HS show different epigenetic signature than non-transcribed telomeres and how this could be linked to HSF1, since it was demonstrated HSF1 can recruit chromatin remodelers and histone modifying enzymes to its other studied genomic targets, upon HS Col & (Hoghoughi 2016 unpublished data, Fujimoto et al. 2012; Sullivan et al. 2001; Jolly et al. 2004).

TERRA promoter DNA CpG-methylation

Particularly, TERRA-promoter's DNA methylation state was shown to be tightly associated with TERRA transcription (Nergadze et al. 2009; Deng1 et al. 2010). Thus, in collaboration with Anabelle Decottignie's lab, we initiated our study to evaluate the impact of HS on TERRA-promoter methylation status. One publication from Decottignie's lab showed hyperthermia was associated with DNA hypomethylation of SatII locous in human fibroblasts (Tilman et al. 2012). Consistently, our analysis of unique subtelomeric 10q region containing CpG islands showed HS induced approximatively 10% loss of CpG methylation at subtelomeric TERRA promoter in both tested cell lines. This data suggests subtelomeric DNA methylation could be implicated in the process of TERRA transcription upon HS. However, in order to consolidate our hypothesis it would be indispensable to extend our study. Further experiments should be realized, it can be very interesting to continue our study with the design of primers for similar analysis at CpG islands of chromosomes which we showed to be directly bound and upregulated by HSF1 like h3p or h18p, or in contrast chromosomes

where we did not detect any TERRA variations upon HS. Moreover, using cell lines where HSF1 is down regulated for these experiments would be an asset to investigate whether DNA methylation downregulation may be an HSF1-dependent mechanism. Finally, our collaborators showed a more important impact on DNA methylation (-27%) upon HS recovery period (Tilman et al. 2012). Thus, investigating subtelomeric DNA methylation upon HS and HS-recovery kinetics could generate precious information and shed light on the way this major epigenetic signature could modulate TERRA expression.

DNA hypomethylation on TERRA promoter we observed upon HS occurred on a subtelomeric region that does not contain any HSE. Thus, if HSF1 binding is able to contribute to gene promoter demethylation, as it was previously suggested (Tilman et al. 2012; Strenkert et al. 2013) it cannot be excluded that HS itself destabilizes to a certain extent heterochromatin region by promoting DNA demethylation. This may favor HSF1 access to previously-hidden HSEs. Thus, HSF1 binding will precede a first hypomethylation "wave" and in turn will play a role in a more extensive promoter demethylation, contributing to TERRA accelerated transcription.

Histone modifications associated with TERRA transcription

In this study we were also interested in investigating histone epigenetic marks shown to be implicated in TERRA regulation. Importantly, we found HS-induced upregulation of TERRA correlated with a significant increase of telomeric but not subtelomeric increase of H3K9me3. Thus, suggesting that the HSF1-dependent TERRA upregulation favors telomeric heterochromatinization under stress that may protect telomeres. Such situation was previously described by Arnoult et al. (Arnoult et al. 2012). They show that HP1α and H3K9me3 density to telomeres mediates TERRA transcriptional regulation, and that this occurs without spreading of these marks beyond the telomeric tract. They also suggest a negative feedback loop, where TERRA repressed its own expression by recruiting these specific marks to telomeres (Arnoult et al. 2012). Other telomeric marks were analyzed in the frame of the cited study such as H3K27me3 and H3ac, which did not show any variation correlating with TERRA regulation. We were able to detect a significant global increase in histone 3 lysine 9 tri-methylation at telomeres, starting from 45min of HS. Kinetics of

H3K9me3 enrichment at telomeres correlated nicely with TERRA upregulation kinetics, suggesting TERRA may be responsible for H3K9me3 accumulation upon HS (Deng et al. 2009; Arnoult et al. 2012). Moreover, we were interested in analyzing chromosome specific epigenetic modifications. Our analysis did not detect any changes of subtelomeric H3K9me3 after HS exposure, suggesting a distinct impact of HS and HSF1 on subtelomeric, versus telomeric regions. However, in our study only a small portion of the subtelomeric regions was taken into account using ChIP-Q-PCR technics. Therefore, a more extensive analysis of these subtelomeric regions is needed and will allow concluding on this part.

Telomeric heterochromatin region is characterized by other repressive marks, like heterochromatin protein 1 (HP1) subunits, H3K40me3 as well as low H3 and H4 acetylation marks (Blasco 2007). These markers were shown to account for TERRA transcriptional regulation and repression. Indeed heterochromatin loss at telomeres, in cells lacking DNMTs, SUV39 or SUV4-20 HMTases results in increased telomeric recombination and telomere elongation (Blasco & Schoeftner 2009). Hence, to complete our knowledge on TERRA regulation upon HS it should be necessary to consider other known telomeric and subtelomeric epigenetic marks as well as histone modifying enzymes showed to interact with TERRA (Porro et al. 2014). These experiments could contribute to precise the mechanism induced by HSF1-dependent TERRA transcription at telomeres.

Finally, it is important to keep in mind that HSF1 is a powerful gene activator with numerus genomic targets (Mendillo et al. 2012), hence it cannot be excluded that one or several factors activated by HSF1 may also contribute to TERRA expression and telomere protection upon HS. In order to clarify this issue, "rescue" experiments in HSF1-depleted cells can be imagined. Rescuing HSF1 knock down with hHSF1 protein to check for a complete or partial recovery of the observed telomeric "phenotype", but also recue with upstream major actors of the HSR for example (HSP70, HSP90).

TERRA function at human telomeres upon HS

Interestingly, we found that HSF1 plays a role in telomeric protection upon stress. Indeed, we show that HS triggers a more effective DNA damage response (DDR) in WT cells

than in KD HSF1 cells involving an early telomeric response. Published data support the idea that TERRA ncRNAs contribute to preserve telomere integrity (Deng et al. 2009; Arnoult et al. 2012). Thus, a rapid HSF1-dependent accumulation of TERRA may initiate an effective DDR pathways as demonstrated by Porro's work (Porro et al. 2014). Moreover, TERRA upregulation upon HS may be a favoring factor to facilitate TRF2 return to telomeres, reducing TIF formation in WT compared to KD HSF1 cells. In order to confirm this hypothesis, our analysis of telomeric TRF2 enrichment in KD HSF1 cells by ChIP technique should be consolidated and confirmed. Indeed, based on the new emerging concept of RNA playing a role as thermosensor during stress (Shamovsky et al. 2006), TERRA may elicit an appropriate response by transducing the stress signal to essential molecular actors with more directe roles in the stress response.

In addition, we can bring the hypothesis that WT cells accumulate less TIF upon HS compared to KD HSF1 cells due to a reinforced heterochromatin status, previously showed to participate to telomere integrity (Cusanelli & Chartrand 2015). However, it is noteworthy that TERRA upregulation associated with uncapped telomeres through TRF2 partial or complete depletion was shown to trigger telomeric DNA damage and telomere fusion. In this context, TERRA was proposed to participate to the DDR pathway and two mechanisms were previously described (Cusanelli & Chartrand 2015). The first one, propose that increased TERRA expression favors LSD1-MRE11 complex at telomeres promoting nucleolytic processing of uncapped telomeres thus, contribute to telomere fusion through NHEJ activation (Porro et al. 2014). The second model, suggests that TERRA can promote chromatin remodeling at dysfunctional telomeres by serving as a recruitment platform for SUV39H1 promoting H3K9 methylation and chromosome end-to-end fusions (Porro et al. 2014). Moreover H3K9me3 was proposed to serve as a docking site for histone remodeling complexes that may participate in ATM activation process at telomeres. Ataxia telangiectasia mutated (ATM) protein kinase is recruited by and activated following dsDNA breaks, it directly interacts with the NBS1 DNA damage recognition complex subunit and phosphorylates the histone variant H2AX on Ser139. Thus, our proposed model can also consider these proposed roles of TERRA and TERRA-induced H3K9me3 accumulation in telomere protection upon HS.

It would be interesting in our case to check if HS induces important increase in telomere fusion, and if this is exacerbated in KD HSF1 depleted cells, in order to reinforce our hypothesis of an HSF1-dependent telomere protection mechanism upon HS. We did started looking at mitotic aberrations in both of our cell lines using *in situ* technique, and found KD HSF1 cells harbor slightly higher level of mitotic aberrations. Although, mitotic aberrations can be caused by different factors, among which telomere fusion can be cited, this gives us a first encouraging clue that could be completed with in situ labeling of telomeric markers in mitosis to consolidate our hypothesis.

Since TERRA was discovered, only two different publications discuss the impact of a partial TERRA depletion in human and mouse cells. A complete repression of total or specific TERRA remains a great challenge in the field and will be of a great benefit to all labs seeking to gain deeper insights into TERRA functions. Both publications support the view that altered TERRA expression or localization is involved in the activation of DDR at telomeres (Deng et al. 2009; Lopez de Silanes et al. 2014). Our lab will start to develop an efficient approach to block TERRA expression based on the system Mmi1/exosome able to degrade ncRNA.

A possible contribution of the HSR activation to telomere protection

In addition, our results show that the resolution of TIF in the late recovery period from stress is not impacted by HSF1 KD suggesting that the role of HSF1 in telomeric protection is restricted to the early stage of the stress response. It is important to cite HSF1 function in telomere protection may also be due to heat stress-regulated chaperones proteins. HSPs like HSP70, HSP90 and the co-chaperone HSP40 were shown to regulate HSF1 activation cycle and play a major role in protein folding in the context of stress where proteotoxicity is generated (Bose et al. 1996; Bukau & Horwich 1998). Hence, DNA detection, repair, displacement and other cofactors conformation and thus functionality, can all depend on HSPs efficiency upon HS. *HSP*s are constitutively transcribed in almost all organisms but upon HS an HSF1-dependent activation of *HSP* genes enhances major upregulation of these proteins expression in the cells. This suggest that HSF1 depleted cells undergo HS and HS recovery periods with a basal level of chaperone proteins, meaning they are basically "unarmed" in the face of proteotoxic stress compared with WT cells. It would

be interesting to test if HSF1 activation uncoupling from *HSP* induction under HS by using translational inhibitors will generate more DNA damage accumulation to telomeres in WT cells. This may indicated *HSP*s and TERRA upregulation collaborate to ensure telomeric protection under the control of HSF1 activation.

Chromosome specific TERRA expression upon HS

An intriguing question is why HSE are only present at certain chromosomes and consequently why an up regulation of TERRA transcripts only occurs at a subset of chromosomes. A possible hypothesis is that HSF1-upregulated TERRA could diffuse from their sites of transcription and act in trans to protect telomeres and/or to promote chromatin remodeling at telomeres. Interestingly, in mouse and yeast, TERRAs are not expressed from all telomeres at a given time and TERRA molecules produced at a specific locus are able to relocate at different telomeres (Cusanelli et al. 2013; Lopez de Silanes et al. 2014).

Interestingly, partial depletion of TERRA expressed from the single telomere 18 in mouse cells leads to DDR activation at different chromosome ends and widespread telomere dysfunction (Lopez de Silanes et al. 2014). These data propose exciting possibilities where TERRA expressed from one chromosome is able to bind different chromosome extremities and to exert its protective function both in 'cis' and in 'trans' (**Discussion figure 1**).

It would be particularly interesting to analyze single TERRA molecules dynamics upon HS to better understand its mode of action on telomeres and TIF resolution. Indeed, our *in situ* data of total TERRA show total foci number decrease while single foci volume increase upon HS. However the hypothesis of telomere clustering during HS seems unlikely, since telomeric DNA-FISH data show no significant decrease in total telomere foci after HS. Interestingly, such nuclear pattern was previously observed in different murine cells. Large TERRAs nuclear foci were identified in MEFs cells under recovery, following HS and in proliferating mouse cerebellar neuronal progenitors or medulloblastoma and to occur as a consequence of a high level of TERRA expression (Zhong Deng, Wang, Xiang, et al. 2012). These foci have been proposed to represent new nuclear bodies with still unknown

Discussion figure 1| Chromosome specific TERRA upregulation upon HS. Upon HS, all tested chromosomes harboring subtelomeric HSEs are being targeted by HSF1 resulting in chromosome-specific TERRA upregulated transcription. HSE negative chromosomes did not show variations of TERRA transcription upon HS. In parallel, HS induces a significant increase of telomere dysfunction induced foci (TIF) and HSF1 depletion extends telomeric damage upon HS. Considering TERRA implications in heterochromatin formation upon telomere uncapping, we suggest a TERRA upregulated upon HS may contribute to protect telomere by preventing TIF formation. Damaged telomeres where TERRA is upregulated may imply a 'cis' function of TERRA another hypothesis could be that upregulated TERRAs are able to exert their protective function in 'trans' on other damaged telomeres.

functions. In human cells, a fraction of telomeric RNAs also resides within the nucleoplasm (Porro et al. 2010). Therefore, suggesting that TERRA molecules are not constitutively associated with telomeres.

These data raise multiple questions: First, does large TERRA foci result from chromosome specific TERRA clustering at precise nuclear loci? Second, could a TERRA molecule originated from one chromosome bind other telomeric regions like it was shown in mouse? Third, do large TERRA foci localize to damaged telomere, if not where do they localize? To resolve some of these questions we started to develop *in situ* TERRA specific probes in collaboration with Dr. Peter (Institute for Molecular Genetics in Montpellier). The technique is based on the detection of subtelomeric specific sequences of TERRA molecules by tandem specific fluorescent molecules. Usually used in the field of mRNA single molecule detection, applying this to human TERRA is actually in progress (**Discussion figure 2**).
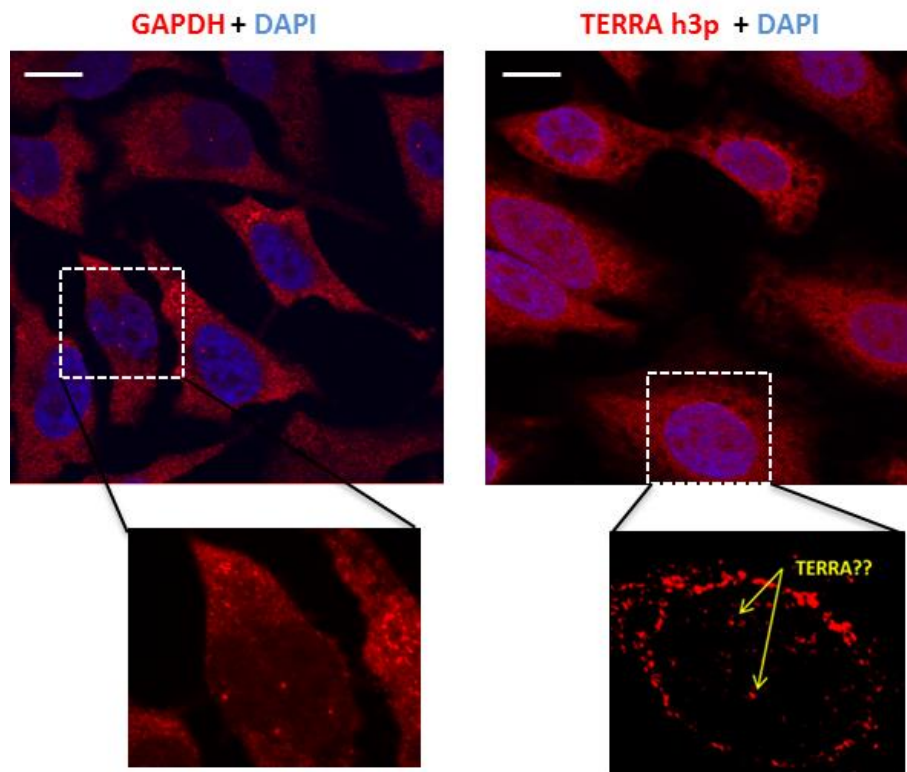
Discussion figure 2| Single TERRA molecule *in situ* labeling. RNA FISH experiments labeling GAPDH mRNA (red, left panel) and chromosome specific 3p TERRA molecules (red, right panel) were carried on WT HeLa cells under normal growth conditions. Combination of multiple specific nucleic probes was used to detect each RNA sequence. As expected mRNA coding for GAPDH (in red, left panel) was found abundantly in the cytosol and several foci were also found in the nucleus, representing multiple transcription sites indicating HeLa cells aneuploidy. A Subtelomeric h3p TERRA sequence (in red, right panel) was detected both in the nucleus and the cytoplasm although to a lesser extent compared to GAPDH as we should expect. Nuclear background reduction allowed identifying several discrete foci that may represent h3p TERRA RNA.

To conclude, HSF1 appears as a new transcription factor of TERRA, and as a new essential actor to protect telomere integrity upon stress. Based on our results and given the important role of HSF1 in tumor formation (Dai, Whitesell, Arlin B. Rogers, et al. 2007) and telomeres biology, defining the exact role of HSF1 with regard to telomere stability in tumor development already emerges as a promising challenge.

Discussing parallels between TERRA and SatIII non-coding transcripts accumulation upon HS and beyond

Pericentric SatIII transcripts are originated from juxtacentromeric regions known for their heterochromatic nature and for their strong HSF1 dependent-activation in response to different stress stimuli in human cell lines. If we take a step back looking at all the data accumulated throughout the years concerning TERRA and SatIII non coding transcripts, many parallels can be made. For example, both are originated from constitutively "silenced" regions of the genome which expression is known to be modulated at very precise physiological conditions (early development, differentiation), particular environmental conditions (heat shock) and interestingly both SatIII and TERRA were detected in human cells in the context of pathologies such as cancer.

Our study clearly demonstrated an HSF1 dependent-TERRA enhanced transcription (~2-fold) upon HS similarly to SatIII 9q12 transcripts (~80-fold) although to a lesser extent. The different stress stimuli capable of activating the HSF1 dependent-SatIII transcription were extensively described (Cotto et al. 1997; Valdardsdottir et al. 2008; Sengupta et al. 2009) and it could be very intriguing to check if TERRAs are parallel activated under similar conditions (Puromycin, MG132, Ibuprofen..). Moreover, TERRA was shown to directly associate with the heterochromatin protein HP1 subunits (Deng et al. 2009; Arnoult et al. 2012) at telomeres and hence to contribute to the heterochromatin state maintenance at telomeres. In parallel, our lab has shown that the HP1 proteins constitutively enriched at pericentric 9q12 loci were significantly dissociated upon HS and preliminary data suggest HP1 may reassociate to the locus under recovery. A tempting hypothesis to test is this SatIII dependent phenomenon. Indeed, one of the proposed functions for SatIII ncRNA is heterochromatin reformation upon stress recovery.

Another interesting point that could be explored in further studies is the fact that SatIII RNAs were shown to be transcribed in a sense and antisense direction, however this occurs at different times after HS induction and both populations coexist in a very restrained time window. Even though antisense SatIII function is not yet clearly elucidated upon HS it could be very interesting to clarify this point for TERRA. Indeed, antisense TERRA were also

detected in the yeast *S. pombe and* known as "ARIA" (exclusively telomeric) and "ARRET" (exclusively subtelomeric), in human cells antisense TERRA was not detected so far. More recently discovered (Azzalin et al; 2007) human TERRA transcription was described as a sens-subtelomeric to telomeric process. However recent RNA-seq publications (Porro et al. 2014) affirm to have detected TERRA transcripts' containing only telomeric-UUAGGG repeats and therefore, that it cannot be excluded transcription may also occur at the chromosome termini independently from the subtelomeric identified TERRA promoters. This recent technical progress allows going dipper into the non-coding genome analysis may reserve some surprises in the near future concerning what we know about the human TERRA.

Together these fascinating parallels may suggest a crosstalk between TERRA and SatIII expression, between telomeres and pericentromeric regions modulation. Therefore, analyzing the structure and partners of satellite and TERRA transcripts in the different contexts where they are expressed is clearly a major issue.

HSF1 and heterochromatin activation in the context of cancer

Inherent to malignant transformation, is the constant proteotoxic stress due to aneuploidy, accumulation of reactive oxygen species (ROS), hypoxia, acidosis, and accumulation of mutated, conformationally aberrant proteins. To overcome these potentially deadly conditions for their survival, cancer cells heavily depend on molecular chaperones, heat shock proteins (HSPs), whose induction in cancer constitutes the powerful adaptive pro-survival mechanism known as the HSR. The essential role of HSF1 in malignant transformation and progression is well documented in literature. Specifically, HSF1 induces a diverse array of HSP-mediated pro-survival mechanisms, including stabilization of oncogenic clients, altered glucose metabolism and signal transduction, and upregulation of protein translation (Mendillo et al. 2012; Dai & Sampson 2016).

Detection of HSF1 elevated protein levels have been observed in several types of human cancers, including a study showing visible nuclear foci in breast cancer, colon, lung and prostate tumor tissues directly isolated from patients (Tang et al. 2005; Khaleque et al.

2008; Mendillo et al. 2012). HSF1 was shown to support malignancy in carcinogenesis-induced mouse model (Dai, Whitesell, Arlin B Rogers, et al. 2007). Strikingly, HSF1 exerts this previously-unidentified function, by triggering a transcriptional program termed the 'HSF1-cancer program', different from HS, to promote cell proliferation and survival mechanisms in cancer cells. The transcriptional program supported by HSF1 was shown to implicate a panel of new HSF1-bound genes (Mendillo et al. 2012). It is noteworthy, that the cited study identified non-canonical HSE binding sequences of HSF1, supporting the idea that its range of action on the cell transcription could be much larger then perilously assessed.

Recent genome-wide sequencing studies have found that major satellite repeat transcripts (human SatIII homologue) were aberrantly overexpressed in various human and mouse epithelial cancers (Ting et al. 2011). In addition, telomere-originated TERRA transcripts can also be found to accumulate in cancer cells and tissues (Zhong Deng, Wang, Xiang, et al. 2012). The exact function of TERRA and SatIII ncRNAs upregulation in the context of cancer is not yet elucidated. However, new functions of TERRA in cancer begin to emerge in the literature. In ALT cancer cells for example, TERRA was shown to form RNA:DNA hybrids called R-loop to favor homologous recombination (HR) events between telomeres and thus, facilitating telomere homeostasis and cell survival. On the other hand, in telomerase positive cancer cells a role for TERRA in regulating telomerase activity is now a controversial topic. While, in yeast subtelomeric TERRA was shown to clearly regulate telomerase activity to short and unprotected telomeres (Moravec et al. 2016), in mammals several mechanisms are proposed in which telomere-bound TERRA or direct TERRA binding to the TERC telomerase subunit could regulate its access to chromosome ends (Azzalin & Lingner 2015). While in various telomerase positive cancer cells TERRA molecules were found to be down-regulated, probably due to high subtelomere DNA-methylation (Ng et al. 2009), TERRA was found to be strongly up-regulated in ALT-dependent cancer cells (Episkopou et al. 2014). Interestingly, parallel studies; support the idea that TERRA expression level correlates with the tumor's grade. In these studies, lower expression levels of TERRA are detected in some of the higher grades of laryngeal cancer, astrocytoma and colon cancer (Sampl et al. 2012; Blasco & Schoeftner 2008).

Altogether these data support the idea that normally compact and silenced heterochromatin regions of the genome, endure major perturbations in the context of cancer. In the light of the cited studies and on the new role of HSF1 in promoting TERRA and SatIII transcription upon HS, it could be very interesting to evaluate if cancer cells and tissues that harbor an HSF1-dependent transcriptional program also show elevated TERRA and SatIII transcripts levels. TERRA and SatIII ncRNAs may appear as new molecular markers in cancer and could then serve to develop innovative therapeutic targets.

In line with this hypothesis, Tutton et al. (Caslini et al. 2009; Tutton et al. 2016) recently demonstrated that the P53 tumor suppressor protein was directly implicated in TERRA transcription under nutrient-deprivation induced stress. Interestingly, the caloric restriction-associated deacetylase Sirtuin-1 (SIRT1) was shown to maintain HSF1 active form (Anckar & Sistonen 2011). Caloric restriction was suggested to delay ageing from yeast to mammals by activating SIRT1 deacetylase. SIRT1 was shown to regulate a number of target proteins including P53 and to inhibit stress-induced apoptotic cell death in the context of caloric restriction (Cohen 2004). In addition, it could be interesting to evaluate if HSF1 and P53 collaborate to ensure TERRA transcription under stress exposure.

MATERIALS AND METHODS

Cell culture, heat stress treatments and siRNA transfection

HeLa Wild Type (WT) cells are derived from cervical cancer cells. HFF2 cells are human foreskin fibroblasts transfected with the catalytic subunit of the telomerase TERT. HT1080 cells are fibrosarcoma cells from ATCC. All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) decomplemented fetal bovine serum, 2% L-glutamine (4mM) and 100 units per ml penicillin and 100mg/ml streptomycin and grown in 5% CO_2 atmosphere at 37°C. Stable HSF1 knock down (KD HSF1) HeLa cells (gently given by Lea Sistonen) were grown in HeLa WT medium supplemented with Geneticine antibiotic at 0.4% final concentration. Stable HSF1 down-regulating cell lines were generated as described (Östling et al. 2007; Sandqvist et al. 2009), briefly, the pSUPER vector (Oligoengine) was used for generating specific hairpin-loop RNA that is processed to functional shRNA in transfected cells. The pSUPER vector was ligated at BglII and HindIII restriction sites with a double-stranded 64-nucleotide oligonucleotide containing the unique 19-nucleotide sequence (GCTCATTCAGTTCCTGATC) specific for HSF1 transcript both in sense and antisense orientation, separated by a 9-nucleotide spacer sequence (TTCAAGAGA) and single clones were established after selection with neomycin. Unless stated HS was performed in a water bath for 1 hour at 43°C followed or not by a recovery period at 37°C. Transient HSF1 depletion was realized using: Lipofectamine-RNAi Max (Invitrogen), siRNA targeting HSF1 (5'-UAUGGACUCCACCUGGAUAA-3'), siRNA control (5'-CGUACGCGGAAUACUUCGATT-3') provided from Eurogentec and prepared according to the manufacturer's instructions. Two consecutive 48h-transfection cycles were performed.

The challenging task of telomeric DNA and TERRA ncRNA analysis

At the projects origin, very little was known about TERRA transcription start sites and made difficult any primer design (Nergadze et al. 2008). During the 3 years period of my PhD project several labs published their sequencing data of human subtelomeric regions (Stong et al. 2014, Porro et al. 2014, Montero et al. 2016) and required revising our data and primers. It is of an importance to consider that telomeres genomic regions similarly to pericentromeic ones clearly challenge the classical biomolecular, biochemical and imaging technics classically used in the case of unique genomic loci. Several points contribute to

complexity telomere and TERRA analysis: TERRA transcripts start in the subtelomeric regions and extend towards the ends of chromosomes made of tandem repeats (UUAGGG) at their 3' ends. In most cell lines, TERRA basal expression is low and was globally detected using sensitive northern blot approach and telomeric (CCCTAA5) probe. Chromosome specific TERRA analysis was recently made possible for a portion of human telomeres thanks to the technical progress made in sequencing repeated regions of the genome (Stong et al. 2014) and relies on primers design at subtelomeric unique regions. Nevertheless, single TERRA subtelomeric sequences also contain repetitive sequences, many of which are shared between different chromosomal subtelomeres rendering primer design and selection a challenging task. One must keep in mind that using RT-Q-PCR approach to analyses unique TERRA molecules is delivering partial information, as the telomeric part of the molecule is excluded from the analysis, like transcription termination problems for example. The same remark is available for global TERRA analysis as only the repeated region of the molecule is usually accounted (5'UUAGGG3'). Indeed in their recent publication, Porro et al (Porro et al. 2014) have proposed an improved human RNAseq experiment renewing TERRA promoter analysis and discuss that "one cannot rule out that transcription at several chromosome termini may initiate within the terminal TTAAGGG-repeat sequences". Also, very few technical solutions exist when it comes to chromosome-specific telomeric DNA analysis, and hence restrain researchers to use global telomere analysis approaches.

Chromatin Immuno-Precipitation (ChIP)

For IgG, RNAPII, TRF2, H3, H3K9me3 and γ-H2AX ChIP analysis, cells were cross-linked with 1% formaldehyde at room temperature (RT) for 10 min before the addition of 125mM glycine (Sigma) at RT for 5 min. After washing, cell nuclei were isolated by suspending cells into cytosol lysis buffer (10mM Hepes (pH=6.5), 0.25% Triton-x, 0.5mM EGTA, 10mM EDTA) at 4°C for 5min. Nuclei were resuspended in nuclei lysis buffer before sonication at 4°C for 19 min (cycles of 30s ON and 30s OFF) with a BioRuptor sonicator (Diagenode) to obtain fragments between 200 and 800 base pairs. Samples were incubated overnight with the antibodies listed in Supplementary Table I and immunoprecipitated using the OneDay ChIP kit (Diagenode), following the manufacturer instructions. Immunoprecipitated DNA was analyzed either by qPCR, using primers listed in Supplementary Table II, or by DNA dot-blot,

using a Telomeric (CCCTAA)4 probe labeled with [γ-32P]ATP (Perkin Elmer) using T4 polynucleotide kinase (New England Biolabs). For RNAPII ChIP, 3 couples of primers were designed for each chromosome arm. For dot blot, alpha satellite sequences, used as controls, were labeled by the Megaprime DNA Labeling System (GE Healthcare) and [α-32P]dCTP (PerkinElmer). Images were captured with a Phosphorimager (BioRad) and signals were quantified using the "Quantity one" software. For HSF1 ChIP analysis, we performed the method described previously (Pernet et al, 2014).

Simultaneous immunofluorescence (IF), DNA or RNA Fluorescence *in situ* Hybridization (FISH): Cells were grown on coverslips and heat-shocked or not as described above. Briefly, cytosol was preextracted with a permeabilization buffer (20mM Tris-HCl (pH=8), 50mM NaCl , 3mM MgCl2, 300mM Sucrose, 0.5% Triton X-100) then all cells were fixed in 4% paraformaldehyde in PBS for 15 min, washed thoroughly and nuclei incubated with a permeabilization buffer. Labeling of HSF1, γ-H2AX and TRF2 were first performed on formaldehyde-fixed cells. The characteristics of the antibodies used are listed in Supplementary Table I. After detection with the secondary antibody, cells were fixed again in 4% formaldehyde and processed for DNA FISH. Briefly prior to *in situ* hybridization, nuclei were successively dehydrated in 70%, 90% and 100% EtOH. For telomeric DNA staining, cells were incubated for 3 min at 80°C with 2ng/µL of PNA TelC-Cy3 (polynucleid acid telomeric C-rich probe coupled with a cyanine 3 fluorochrome from Eurogentec) diluted in hybridization mix (70% Formamide/Tris 20mM, 0.5% blocking reagent (Roche), 5% Mg buffer), followed by 1h hybridization at RT. Finally cells were washed in formamide and in Tris-Tween 0.08%. For TERRA detection, cells were first permeabilized with CSK buffer (10mM Pipes, pH 7.0, 100mM NaCl, 300mM sucrose, 3mM MgCl2, 0.5% Triton X-100, 10mM ribonucleoside vanadyl complex (New England Biolabs). TERRA FISH was performed as described previously (Arnoult et al. 2012), except for hybridization that was performed at 37°C for 2h with 400nM PNA C-rich (Eurogentec), in 50% deionized formamide, 10% dextran sulfate (Millipore), 2XSSC, 2mg ml−1 BSA and 10mM RVC (New England Biolabs). Cells were rinsed with 0.1XSSC at 60°C and with 2XSSC at room temperature. DNA was counterstained with 250 ng/ml 4',6-diamidino-phenylindole (DAPI). Slides were mounted with a Dako fluorescent mounting medium (Dako Invitrogen).

Image acquisition and measurement

Microscopy experiments were performed on a structured illumination (pseudo-confocal) imaging system (ApoTome - AxioImager Z1, Zeiss) equipped with a monochromatic CCD camera (AxioCam MRm, Zeiss) and controlled by the AxioVision software. A minimum of 10 z-planes were acquired with a 63X oil immersion objective (Plan-Apochromat NA 1,4 Oil, Ph3, WD 190 nm) to constitute a 3D image. Z-stacks images (1388 x 1040 pixels per frame using a 12-bit pixel depth for each channel at a constant voxel size of 0.1 x 0.1 x 0.24 μm) were acquired. Segmentation and 3D measurements between objects corresponding to telomeres and γ-H2AX or TRF2 but also to TERRA foci were performed with the Volocity software (Perkin Elmer). The background level was obtained by measuring the mean intensity of each stain outside the cells. Objects were segmented out from the background with a minimal intensity for each channel applied thereafter for all images. Object based colocalization was then analyzed on thresholded images from the red/green intensities by calculating Manders' coefficients based on the co-occurrence of the two probes at the same voxel location (fraction of a total probe that co-localizes with the fluorescence of the second probe) thus representing the fractional overlap. Mander's coefficient can vary between 0 (no colocalization) to 1 (100% colocalization). A value above 0.5 was considered as a positive colocalization. A minimum of 80 cells were analyzed for each condition. The 3D point spread functions were the same for different excitation and emission wavelengths and there was no registration shift between images. This checking was done through imaging 0.5 μm diameter multicolor fluorescent beads. No significant differences were observed in the X, Y or Z directions.

RT Q-PCR

Total RNA was extracted from HeLa cells deficient or not for HSF1, using Trizol reagent (Sigma) in RNase free conditions. RNA was treated with DNase (Ambion) for 30 min at 37°C. 1μg of RNA was reverse transcribed with equal amount of random hexamers and telomere specific (CCCTAA)5 oligonucleotides using First-Strand cDNA Synthesis kit (Roche), according to the manufacturer's instructions. Controls without reverse transcriptase or RNA were performed. For quantification of TERRA transcripts at 2p, 3p, 14q and 10-18p

subtelomeric regions and of HSP70, SYBER green (master mix TAKARA) incorporation-basedreal-time PCR analysis were performed using specific primers (see Supplementary Table II). Q-PCR was performed on a LCR408 (Light-cycler ROCHE) machine.

RNA dot-blot

10 µg of RNA resuspended in SSC and formaldehyde were denatured at 70°C and then dot-blotted on a positively charged nylon transfer membrane (GE Healthcare), and UV crosslinked with a UV-stratalinker (Stratagene). TERRA and U2 were detected using a (CCCTAA)$_5$ or a U2 specific oligonucleotide probe labeled with 32P-γ-ATP by T4 polynucleotide kinase (NEB) and purified with illustra microspin G-25 columns (GE Healthcare). Hybridizations were performed using UltraHyb buffer (Ambion) for 16-18h at 43°C or 50°C. Membranes were washed in 2XSSC/ 0.1% SDS for 10min at room temperature and in 0.2XSSC/ 0.1% SDS for 5min at 50°C. Blots were stripped with 0.1XSSC, 40mM Tris (pH=7.5), and 1% SDS for 10min at 80°C. When indicated, RNA samples were treated with RNaseA (Roche) at a final concentration of 100µg/ml for 30-60 min at 37°C. Images were captured with a Phosphorimager (BioRad) and signals were quantified using "Quantity one" software.

RNA stability

Stressed and unstressed WT and HSF1 KD cells were incubated with 1 µM of triptolide for 2h-8h. After RNA extraction with trizol agent, 3 µg of *S. pombe* RNA was added to each sample as an internal control for reverse-transcription efficiency. Total RNA was reverse-transcribed with a mix of telomeric and *S. pombe* β-actin specific primers and random hexamer primer. TERRA from chromosomes 2p, 3p, 18q, 10p and 14q and U2 cDNA levels were quantified by q-PCR and normalized with *S. pombe* β-actin cDNA levels used as a control.

Western blot

HeLa WT and HSF1-KD cells were submitted to a kinetics of HS (5min to 1h at 43°C) or were submitted or not to a kinetics of recovery (2h to 6h) following a 1 hour HS. Cells were

collected by a 5 min centrifugation at 2500 rpm at 4°C. Cell lysis was performed in NP40 buffer (Tris 20mM (pH=7.5), NaCl 150mM, EDTA 2mM, NP40 1%) on ice and then cells were sonicated for 5min (30sec ON, 30sec OFF) at 4°C. Protein extracts were obtained after 1min centrifugation at 14,000rpm at 4°C. Total protein extracts were quantified by spectrophotometry using a Bradford assay. Equal amounts (25 to 50 µg) of whole protein extracts were loaded and separated on 6%, 8% or 15% Acrylamide gels. Primary antibodies against HSF1, H3, H3K9me3, 53BP1, TRF2 and γ-H2AX listed in Supplementary Table I were used and diluted in PBS1X, BSA 1%. Membranes were washed in PBS1X Tween 0.1% (except for HSF1 staining wash with PBS1X NaCl 0.1M) and then incubated with secondary antibodies anti-rabbit or anti-mouse IgG linked with a fluorochrome. For loading controls, mouse polyclonal anti-Tubulin antibodies were used. Target protein signal was obtained using ECL (GE Healthcare) and revealed using ChemiDoc MP System (Bio Rad). Band intensities were quantified using Image-J software.

In sillico

Bioinformatics databases were explored within 5Kb of subtelomeric regions (vader.wistar.upenn.edu/humansubtel) (Stong et al. 2014). Identification of CpG dinucleotide contents and a prediction of CpG islands were done with the CpGPlot/CpGReport at the European molecular biology open software suite program (EMBOSS: http://www.ebi.ac.uk/Tools/emboss/cpgplot/). The « Genomatix » software was used for the detection of human subtelomeric heat stress elements (HSE). Genomatix software promoter analysis is based on a condensation of published available data plus Genomatix proprietary annotation. All the existing and available HSF1 binding motifs were processed to assess their statistical representation in the human genome, or score (score = degree of conservation for each nucleotide position in the matrix). Sequences with the highest score are then used to analyze the genomic regions of interest (subtelomeric regions up to 5Kb). The presence of CpG islands, transcription activators and other known HSF1-associated factors binding sites in the vicinity (5Kb) of the potential HSE were set as conditions to determine the matrix score. It is noteworthy, that in general transcripts (start sites) identified at the region of interest are taken into account when TF binding sites are searched; however, in our case no such data was available at that time. Our analysis showed

that more than 40 % of sequenced human subtelomeric regions present potential HSE and in 90% of cases binding motifs respecting all conditions correspond to the following sequence nGAAnnTCCnnGAA, also found in heat shock proteins coding genes promoter. The presence and localization of Heat Shock Elements (HSEs) was manually verified using "A plasmid Editor" software (ApE). Retained HSE sequences, are composed of at least three contiguous inverted repeats, nTTCnnGAAnnTTCn. We looked for this specific sequence with different probabilities for n(A, C, G, T).

Promoter CpG methylation

Subtelomeric promoter CpG methylation status was estimated using DNA bisulfite treatment followed by sequencing. DNA bisulfite treatment was performed on approximately 2µg of genomic DNA. After purification (Kit : Geneclear), genomic DNA was submitted to alcalin-desulfonation (NaOH 1N) followed by neutralization (NH4OAc 5M), precipitated in 100% Ethanol and re-dissolved in DNAse free water. The region of interest (h10q: AGGCTTTTCGTTTCCCGCTTTCCACACTAAACCGTTCTAACTGGTCTCTGACCTTGATTATTCA GGGCAGCAAACGGGAAAGATTTTATTCACCGTCGATGCGGCCCCGAGTTATCCCAAAGGCAGGCAG TACCCCCAACGTCTGTGCTGAGAAGAATGCTGCTCCGCCTTTACGGTGCCCCCCACGTCTGTGCTGAA CAGAACGCAGCTCCGCCCTCGCAGTGCCCTCAGCCCGCCCGCCCGGGTCTGACCTGAGAAGAACTCT GCTCCGCCTTCGCAATACCCCCGAAGTCTGTGCAGAGGAGAACGCAGCTCCGCCCTCGCGATGCTCT CCGGGTGTGTGCTAAAGAGAA) was amplified using hemi-nested PCR technique and Dream Taq DNA polymerase (Thermo Scientific). Primers sets (Eurogentec) used were consecutively: Mix1 containing 10qprom-Fext/int (GGTTTTTGATTTTGATTATTTAG), 10qprom-Rint (TTCTCCTCTACACAAACTTC) and 10qprom-Rext: TTCTCTTTAACACACACCC. The PCR conditions used were: -95°C for 3min, 95°C for 30sec, 55°C for 30sec, 72°C for 2min and 72°C for 10min, during 35 cycles. Mix2 containing 10qprom-F (GGTCTCTGACCTTGAT TATTCAG), 10qprom-R CGTTCTCCTCTGCACAGACTTC). The PCR conditions were the same described before. Expected PCR product size 247bp, was controlled on BET-stained 1% agarose gel. Purified (Kit: Quiagen) PCR product was cloned in previously linearized pJET1.2/blunt vector (Fermentas). Purified transformed vectors were electroporated into competent host cells (DH5 alpha E.coli). Isolated selected colonies were grown and collected

to control plasmid integration and integrity by PCR with pJET primers (Eurogentec). Selected colonies were amplified and plasmids were purified and sent to sequencing (GATC).

Quantitative Telomere Repeat Amplification Protocol assay (q-TRAP assay)

Telomerase catalytic activity was assessed using q-TRAP assay (TRAPEZE® XL Kit (Millipore)) following the manufacturer's instructions. Briefly, protein extracts were obtained with 106 cells per condition lysed (CHAPS lysis buffer, 30min on ice) and quantified with Bradford test. 1µg of protein extract containing active telomerase was incubated with furnished TS (telomerase Substrate) primer and ampifluor primer in a Taq polymerase containing reaction mix. TS elongation by active telomerase was assessed using PCR and fluorescent specific primers. An internal control for PCR efficiency was also performed. (PCR conditions used: 1. 94°C, 30sec; 2. 59°C, 30sec; 3. 72°C, 1min; Steps 1 to 3 for 36 cycles; 72°C, 3min extension step; 55°C,25min; 4°C). Fluorescence intensity was estimated with spectrofluorimeter (Roche). The measured fluorescence intensity is directly proportional to the TRAP product PCR amplification, reflecting telomerase activity.

Table 1: Antibodies and dilutions used in this study for ChIP, western blots and immunofluorescence

Target	Species	Application	Dilution	Reference
HSF1	Rabbit	ChIP	5µg/IP	ADI-SPA-901
HSF1	Rabbit	WB	1:1000	Cell Signaling #4356
HSF1	Mouse	IF	1:100	Santa Cruz sc-17757
IgG	Rabbit	ChIP	3µg/IP	Kit Diagenode
γH2AX	Rabbit	IF	1:500	Abcam ab11174
		WB	1:2000	
		ChiP	5µg/IP	
53BP1	Rabbit	IF	1:500	Novous Biologicals
		WB	1:1000	NB100-904
RNA Pol II CTD (pS2)	Rabbit	WB	1 :1000	Abcam ab5095

		ChIP	10µg/IP	
TRF2	Mouse	IF WB ChIP	1:100 1:1000 5µg/IP	Novus Biological NB11057130
H3	Rabbit	ChIP WB	3µg/IP 1 :2000	Abcam ab1791
H3K9me3	Rabbit	ChIP WB	5µg/IP 1 :5000	Abcam ab8898
Tubulin	Mouse	WB	1:5000	Sigma T5168
Mouse IgG (Alexa 488)	Goat	IF	1:500	Invitrogen A-11029
Rabbit IgG (Alexa 488)	Goat	IF	1:500	Invitrogen A-11034
Mouse IgG (HRP)	Sheep	WB	1:5000	GE Healthcare NA931V
Rabbit IgG (HRP)	Goat	WB	1:5000	GE Healthcare NA934V

Table 2: List of oligonucleotides used in this study

primer name	application	sequence
18p-10pV1-F	ChIP HSF1 and RNAPII / q-PCR	CCTGGACACATTCTGGAAAGT
18p-10pV1-R	ChIP HSF1 and RNAPII / q-PCR	ATGGCAAGGTTGTTTGGAGG
18p-10pV2-F	ChIP RNAPII/ q-PCR	ACCTGGCATTCAGCGCGCTC
18p-10pV2-R	ChIP RNAPII/ q-PCR	CAGGTGACCGGTTTGTCCTACCC
18p-10pV3-F	ChIP RNAPII/ q-PCR	TCGTACACTCTCTGGTAGGT
18p-10pV3-R	ChIP RNAPII/ q-PCR	TCATCTCCACCTGCAATCCA

primer name	application	sequence
17q-F	q-PCR	GAAAATAAGGTCGGGATTGCTGTGCTCAC
17q-R	q-PCR	CTATCCCCTCAAATGCCTGTGTTCCTTG
14qV1-F	ChIP HSF1 and RNAPII / q-PCR	ATGGGATCTTGGGTCAGAGT
14qV1-R	ChIP HSF1 and RNAPII / q-PCR	ATTTCCCATGTAGCCGCAAC
14qV2-F	ChIP RNAPII/ q-PCR	TGTTCCCATGCCTACCTTTT
14qV2-R	ChIP RNAPII/ q-PCR	ACCTGTAGAATGTCCTGCCA
14qV3-F	ChIP RNAPII/ q-PCR	TGGCAGGACATTCTACAGGT
14qV3-R	ChIP RNAPII/ q-PCR	AAACCATCGACTTTGTGGCT
11q-F	q-PCR	CCCTGATTATTCAGGGCTGCAAAG
11q-R	q-PCR	ACAGACCTTGGAGGCACGGCCTTCG
3pV1-F	ChIP HSF1 and RNAPII / q-PCR	CATTTAGGTCCATGGGCACA
3pV1-R	ChIP HSF1 and RNAPII / q-PCR	TTCTGGTCCCGGATGATGTT
3pV2-F	ChIP RNAPII/ q-PCR	CCTGGAGGCAAGGGAAGAC
3pV2-R	ChIP RNAPII/ q-PCR	TGCCCATGAAGCTTTTGGTC
primer name	application	sequence
3pV3-F	ChIP HSF1 and RNAPII / q-PCR	GGTTATGGTGAGCCGAGATT
3pV3-R	ChIP HSF1 and RNAPII / q-PCR	GCTTGCTTCAATTCAAAGTGC
2pV1-F	ChIP HSF1 and RNAPII/ q-PCR	CTAAGCCGAAGCCTAACTCGTGT

2pV1-R	ChIP HSF1 and RNAPII/q-PCR	AGCTGCGTTTTGCTGAGCAC
2pV2-F	ChIP RNAPII/ q-PCR	AGTCTTGTGCAGGGAAGTTACT
2pV2-R	ChIP RNAPII/ q-PCR	ATGCCACCATGCCCATCTAA
2pV3-F	ChIP RNAPII/ q-PCR	TCTCCAACCCTGGAACAAAG
2pV3-R	ChIP RNAPII/ q-PCR	TTGCCTTCTCCAGTGTCTCG
1q-F	q-PCR	CCTTGGGAGAATCTCGGTGCGCAGGAT
1q-R	q-PCR	GCATGGCTTTGGGACAACTCGGGGCT
U2-F	q-PCR	GGCTAAGATCAAGTGTAGTATCTGTTC
U2-R	q-PCR	GCTCCTATTCCATCTCCCTGCTC
HSP70-F	ChIP HSF1 and RT/ q-PCR	CCATGGAGACCAACACCCT
HSP70-R	ChIP HSF1 and RT q-PCR	CCCTGGGCTTTTATAAGTCG
yAct1	RT	ACACTTGTGGTGAACGATAG
yAct1-F	q-PCR	ATGTTCCCAGGTATTGCCGA
yAct1-R	q-PCR	ACACTTGTGGTGAACGATAG
oTel	RT	CCCTAACCCTAACCCTAACCCTAACCCTAA
SatIII-F	ChIP RNAPII/q-PCR	TCC-ATT-CCA-TTC-CTG-TAC-TCG-G
SatIII-R	ChIP RNAPII/q-PCR	AAT-CAA-CCC-GAG-TGC-AAT-CGA-A
Actin	RT	AGTCCGCCTAGAAGCATTTG

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BIBLIOGRAPHY

Seyle, H. et al., 1936. A syndrome produced by diverses noccus agents. Nature.

Abad, J.P. et al., 2004. TAHRE, a novel telomeric retrotransposon from Drosophila melanogaster, reveals the origin of Drosophila telomeres. *Molecular Biology and Evolution*, 21(9), p.1620–1624.

Abravaya, K. et al., 1992. The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes and Development*, 6(7), p.1153–1164.

Aguilera, A. & García-Muse, T., 2012. R Loops: From Transcription Byproducts to Threats to Genome Stability. *Molecular Cell*, 46(2), p.115–124.

Akerfelt, M., Morimoto, R.I. & Sistonen, L., 2010. Heat shock factors: integrators of cell stress, development and lifespan. *Nature reviews. Molecular cell biology*, 11(8), p.545–55.

Allis, C.D. et al., 2007. New Nomenclature for Chromatin-Modifying Enzymes. *Cell*, 131(4), p.633–636.

Allshire, R.C., Dempster, M. & Hastie, N.D., 1986. Human telomeres contain at least three types of G-rich repeat distributed non-randomly. 17(2), p.8513–8533.

Amici, C. et al., 1992. Antiproliferative prostaglandins activate heat shock transcription factor. *Proceedings of the National Academy of Sciences of the United States of America*, 89(14), p.6227–31.

Anckar, J. & Sistonen, L., 2011. Regulation of HSF1 function in the heat stress response: implications in aging and disease. *Annual review of biochemistry*, 80, p.1089–115.

Anckar, J. & Sistonen, L., 2007. SUMO: getting it on. *Biochemical Society transactions*, 35(Pt 6), p.1409–1413.

Armanios, M. & Blackburn, E.H., 2012. The telomere syndromes. , 13(3), p.693–704393.

Arneaud, S.L.B. & Douglas, P.M., 2016. The Stress Response Paradox: Fighting Degeneration at the Cost of Cancer. *The FEBS Journal*, p.1–9.

Arnoult, N., Van Beneden, A. & Decottignies, A., 2012. Telomere length regulates TERRA levels through increased trimethylation of telomeric H3K9 and HP1α. *Nature Structural & Molecular Biology*, 19(9), p.948–956.

Arora, R. et al., 2014. RNaseH1 regulates TERRA-telomeric DNA hybrids and telomere maintenance in ALT tumour cells. *Nat Commun*, 5, p.5220.

Arora, R., Brun, C.M. & Azzalin, C.M., 2012. Transcription regulates telomere dynamics in human cancer cells Transcription regulates telomere dynamics in human cancer cells. , p.684–693.

Arsène, F., Tomoyasu, T. & Bukau, B., 2000. The heat shock response of Escherichia coli. *International Journal of Food Microbiology*, 55(1-3), p.3–9.

Artandi, S.E. & DePinho, R.A., 2009. Telomeres and telomerase in cancer. *Carcinogenesis*, 31(1), p.9–18.

Audas, T.E., Jacob, M.D. & Lee, S., 2012. Immobilization of Proteins in the Nucleolus by Ribosomal Intergenic Spacer Noncoding RNA. *Molecular Cell*, 45(2), p.147–157.

Azzalin, C.M. et al., 2007. Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. *Science (New York, N.Y.)*, 318(5851), pp.798–801.

Azzalin, C.M. & Lingner, J., 2015. Telomere functions grounding on TERRA firma. *Trends in Cell Biology*, 25(1), p.29–36.

Azzalin, C.M. & Lingner, J., 2008. Telomeres: The silence is broken. *Cell Cycle*, 7(9), p.1161–1165.

Baler, R., Welch, W.J. & Voellmy, R., 1992. Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. *Journal of Cell Biology*, 117(6), p.1151–1159.

Balk, B. et al., 2013. Telomeric RNA-DNA hybrids affect telomere-length dynamics and senescence. *Nat Struct Mol Biol*, 20(10), p.1199–1205.

Bandaria, J.N. et al., 2016. Shelterin Protects Chromosome Ends by Compacting Telomeric Chromatin. *Cell*, 164(4), p.735–746.

Bannister, A.J. et al., 2001. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature*, 410(6824), p.120–124.

Bannister, A.J. & Kouzarides, T., 2011. Regulation of chromatin by histone modifications. *Cell research*, 21(3), p.381–395.

Batulan, Z. et al., 2003. High threshold for induction of the stress response in motor neurons is associated with failure to activate HSF1. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23(13), p.5789–5798.

Benetti, R., Gonzalo, S., et al., 2007. Suv4-20h deficiency results in telomere elongation and derepression of telomere recombination. *Journal of Cell Biology*, 178(6), p.925–936.

Benetti, R., Blasco, M.A., et al., 2007. Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. *Nature genetics*, 39(2), p.243–50.

Ben-Zvi, A., Miller, E. a & Morimoto, R.I., 2009. Collapse of proteostasis represents an early molecular event in Caenorhabditis elegans aging. *Proceedings of the National Academy of Sciences of the United States of America*, 106(35), p.14914–14919.

Biamonti, G., 2004. Nuclear stress bodies: a heterochromatin affair? *Nature reviews. Molecular cell biology*, 5(6), p.493–498.

Biamonti, G. & Vourc'h, C., 2010. Nuclear stress bodies. *Cold Spring Harbor perspectives in biology*, 2(6), p.1–12.

Bishop, N. a & Guarente, L., 2007. Two neurons mediate diet-restriction-induced longevity in C. elegans. *Nature*, 447(7144), p.545–9.

Blackburn, E.H., 2001. Switching and signaling at the telomere. *Cell*, 106(6), p.661–673.

Blackburn, E.H. & Challoner, P.B., 1984. Identification of a telomeric DNA sequence in Trypanosoma brucei. *Cell*, 36(2), p.447–57.

Blackburn, E.H., Greider, C.W. & Szostak, J.W., 2006. Telomeres and telomerase: the path from maize, Tetrahymena and yeast to human cancer and aging. *Nature Medicine*, 12(10), p.1133–1138.

Blasco, M. & Schoeftner, S., 2009. A "higher order" of telomere regulation: telomere

heterochromatin and telomeric RNAs. *The EMBO journal*, 28(16), p.2323–36.

Blasco, M. & Schoeftner, S., 2008. Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nature cell biology*, 10(2), p.228–36.

Blasco, M.A. et al., 2005. Role of the RB1 family in stabilizing histone methylation at constitutive heterochromatin. *Nature cell biology*, 7(4), p.420–8.

Blasco, M.A., 2007. The epigenetic regulation of mammalian telomeres. *Nat Rev Genet*, 8(4), p.299–309.

Bloomfield, V.A., 1996. DNA condensation. *current Opinion in Structural Biologie*, 6, p.334–341.

Bose, S. et al., 1996. Chaperone function of Hsp90-associated proteins. *Science (New York, N.Y.)*, 274(5293), p.1715–1717.

Boulon, S. et al., 2010. The Nucleolus under Stress. *Molecular Cell*, 40(2), p.216–227.

Boyault, C. et al., 2007. HDAC6 controls major cell response pathways to cytotoxic accumulation of protein aggregates. *Genes and Development*, 21(17), p.2172–2181.

Brock, G.J.R., Charlton, J. & Bird, A., 1999. Densely methylated sequences that are preferentially localized at telomere-proximal regions of human chromosomes. *Gene*, 240(2), p.269–277.

Brown, S.A. et al., 1998. Transcriptional activation domains stimulate initiation and elongation at different times and via different residues. *EMBO Journal*, 17(11), p.3146–3154.

Bryan, T.M. et al., 1995. Telomere elongation in immortal human cells without detectable telomerase activity. *The EMBO journal*, 14(17), p.4240–8.

Budzyński, M.A. et al., 2015. Uncoupling Stress-Inducible Phosphorylation of Heat Shock Factor 1 from Its Activation. *Molecular and cellular biology*, 35(14), p.2530–40.

Bukau, B. & Horwich, A.L., 1998. The Hsp70 and Hsp60 chaperone machines. *Cell*, 92(3), p.351–366.

Buxton, J.L. et al., 2014. Human leukocyte telomere length is associated with DNA methylation levels in multiple subtelomeric and imprinted loci. *Scientific reports*, 4, p.4954.

Carchilan, M. et al., 2007. Transcriptionally active heterochromatin in rye B chromosomes. *The Plant cell*, 19(6), p.1738–1749.

Caslini, C. et al., 2009. MLL associates with telomeres and regulates telomeric repeat-containing RNA transcription. *Molecular and cellular biology*, 29(16), p.4519–4526.

Cesare, A. et al., 2013. The Telomere deprotection response is functionally distinct from the Genomic DNA damage response. *Molecular Cell*, 51(2), p.141–155.

Cesare, A.J. & Reddel, R.R., 2010. Alternative lengthening of telomeres: models, mechanisms and implications. *Nature reviews. Genetics*, 11(5), p.319–30.

Chai, W., Shay, J.W. & Wright, W.E., 2005. Human telomeres maintain their overhang length at senescence. *Molecular and cellular biology*, 25(6), p.2158–2168.

Charmandari, E., Tsigos, C. & Chrousos, G., 2005. Endocrinology of the Stress Response 1.

Physiology, 67(1), p.259–284.

Chiodi, I. et al., 2004. RNA recognition motif 2 directs the recruitment of SF2/ASF to nuclear stress bodies. *Nucleic Acids Research*, 32(14), p.4127–4136.

Choi, D., 2015. Heat Shock RNA 1, Known as a Eukaryotic Temperature-Sensing Noncoding RNA, Is of Bacterial Origin. , 25, p.1234–1240.

Choi, Y., Asada, S. & Uesugi, M., 2000. Divergent hTAF(II)31-binding motifs hidden in activation domains. *Journal of Biological Chemistry*, 275(21), p.15912–15916.

Chu, B. et al., 1996. Sequential Phosphorylation by Mitogen-activated Protein Kinase and Glycogen Synthase Kinase 3Represses Transcriptional Activation by Heat Shock Factor-1. *Journal of Biological Chemistry*, 271(48), p.30847–30857.

Clos, J. et al., 1993. Induction temperature of human heat shock factor is reprogrammed in a Drosophila cell environment. *Nature*, 364(6434), p.252–5.

Cohen, H.Y., 2004. Calorie Restriction Promotes Mammalian Cell Survival by Inducing the SIRT1 Deacetylase. *Science*, 305(5682), p.390–392.

Corey, D.R., 2010. Telomeres and Telomerase: From Discovery to Clinical Trials David. , 16(12), p.1219–1223.

Corey, L.L. et al., 2003. Localized recruitment of a chromatin-remodeling activity by an activator in vivo drives transcriptional elongation. *Genes and Development*, 17(11), p.1392–1401.

Cosgrove, M.S., Boeke, J.D. & Wolberger, C., 2004. Regulated nucleosome mobility and the histone code. *Nature structural & molecular biology*, 11(11), p.1037–43.

Cotto, J., Fox, S. & Morimoto, R., 1997. HSF1 granules: a novel stress-induced nuclear compartment of human cells. *Journal of cell science*, 110, pp.2925–2934.

Cotto, J., Kline, M. & Morimoto, R.I., 1996. Activation of Heat Shock Factor 1 DNA Binding Precedes Stress- induced Serine Phosphorylation. p.3355–3358.

Cusanelli, E. & Chartrand, P., 2015. Telomeric repeat-containing RNA TERRA: A noncoding RNA connecting telomere biology to genome integrity. *Frontiers in Genetics*, 6(MAR), p.1–9.

Cusanelli, E., Romero, C.A.P. & Chartrand, P., 2013. Telomeric noncoding RNA TERRA is induced by telomere shortening to nucleate telomerase molecules at short telomeres. *Molecular cell*, 51(6), p.780–91.

Dai, C., Whitesell, L., Rogers, A.B., et al., 2007. Heat Shock Factor 1 Is a Powerful Multifaceted Modi er of Carcinogenesis. *Cell*, 130(6), p.1005–1018.

Dai, C., Whitesell, L., Rogers, A.B., et al., 2007. Heat Shock Factor 1 Is a Powerful Multifaceted Modifier of Carcinogenesis. *Cell*, 130(6), p.1005–1018.

Dai, C. & Sampson, S.B., 2016. HSF1: Guardian of Proteostasis in Cancer. *Trends in Cell Biology*, 26(1), p.17–28.

Dai, S. et al., 2015. Suppression of the HSF1-mediated proteotoxic stress response by the metabolic stress sensor AMPK. *The EMBO journal*, 34(3), p.275–93.

Damberger, F.F. et al., 1994. Solution structure of the DNA-binding domain of the heat shock

transcription factor determined by multidimensional heteronuclear magnetic resonance spectroscopy. *Protein science : a publication of the Protein Society*, 3(10), p.1806–1821.

Denegri, M. et al., 2001. Stress-induced nuclear bodies are sites of accumulation of pre-mRNA processing factors. *Molecular biology of the cell*, 12(11), p.3502–3514.

Deng, Z., Wang, Z., Stong, N., et al., 2012. A role for CTCF and cohesin in subtelomere chromatin organization, TERRA transcription, and telomere end protection. *Embo J*, 31(21), p.4165–4178.

Deng, Z., Wang, Z., Xiang, C., et al., 2012. Formation of telomeric repeat-containing RNA (TERRA) foci in highly proliferating mouse cerebellar neuronal progenitors and medulloblastoma.

Deng, Z., Wang, Z., Xiang, C., et al., 2012. Formation of telomeric repeat-containing RNA (TERRA) foci in highly proliferating mouse cerebellar neuronal progenitors and medulloblastoma. *J Cell Sci*, 125(Pt 18), p.4383–4394.

Deng, Z. et al., 2009. TERRA RNA Binding to TRF2 Facilitates Heterochromatin Formation and ORC Recruitment at Telomeres. , 35(4), p.403–413.

Deng1, Z., Campbell2, A. & Paul, M.L., 2010. TERRA, CpG methylation and telomere heterochromatin: , 10(1), p.54–56.

Dengeri, M. et al., 2002. Human Chromosomes 9, 12, and 15 Contain the Nucleation Sites of Stress-Induced Nuclear Bodies. *Molecular biology of the cell*, 13(6), p.2170–2179.

Episkopou, H. et al., 2014. Alternative Lengthening of Telomeres is characterized by reduced compaction of telomeric chromatin. *Nucleic Acids Research*, 42(7), p.4391–4405.

Eymery, A. et al., 2009. A transcriptomic analysis of human centromeric and pericentric sequences in normal and tumor cells. *Nucleic Acids Research*, 37(19), p.6340–6354.

Eymery, A. et al., 2010. Heat shock factor 1 binds to and transcribes satellite II and III sequences at several pericentromeric regions in heat-shocked cells. *Experimental Cell Research*, 316(11), p.1845–1855.

Farnung, B.O. et al., 2012. Telomerase efficiently elongates highly transcribing telomeres in human cancer cells. *PLoS ONE*, 7(4).

Feinberg, A.P. & Vogelstein, B., 1983. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*, 301, p.628–631.

Fernandez-capetillo, O. et al., 2002. DNA damage-induced G 2 – M checkpoint activation by histone H2AX and 53BP1. *Nature Cell Biology*, 4(December 2002), p.993–998.

Flick, K.E. et al., 1994. Yeast heat shock transcription factor contains a flexible linker between the DNA-binding and trimerization domains: Implications for DNA binding by trimeric proteins. *Journal of Biological Chemistry*, 269(17), p.12475–12481.

Flynn, R.L. et al., 2011. NIH Public Access. , 471(7339), p.532–536.

Fritah, S. et al., 2009. Heat-Shock Factor 1 Controls Genome-wide Acetylation in Heat-Shocked Cells. *Molecular biology of the cell*, 20(22), p.4976–4984.

Fujimoto, M. et al., 2012. RPA assists HSF1 access to nucleosomal DNA by recruiting histone chaperone FACT. *Molecular Cell*, 48(2), p.182–194.

Fuks, F. et al., 2003. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *Journal of Biological Chemistry*, 278(6), p.4035–4040.

Füllgrabe, J., Kavanagh, E. & Joseph, B., 2011. Histone onco-modifications. *Oncogene*, 30(31), p.3391–3403.

Galati, A. et al., 2012. TRF2 controls telomeric nucleosome organization in a cell cycle phase-dependent manner. *PLoS ONE*, 7(4), p.1–10.

Galati, A., Micheli, E. & Cacchione, S., 2013. Chromatin structure in telomere dynamics. *Frontiers in oncology*, 3(March), p.46.

García-Cao, M. et al., 2003. Epigenetic regulation of telomere length in mammalian cells by the Suv39h1 and Suv39h2 histone methyltransferases. *Nature Genetics*, 36(1), p.94–99.

Geiss-friedlander, R. & Melchior, F., 2007. Concepts in sumoylation: a decade on. *Nature Reviews Molecular Cell Biology*, 8(12), p.947–956.

Gene, D.H.H. & Pelham, H.R.B., 1982. A Regulatory Upstream Promoter Element in the. , 30(September).

Goldberg, A.D. et al., 2010. Distinct factors control histone variant H3.3 localization at specific genomic regions. *Russell The Journal Of The Bertrand Russell Archives*, 140(5), p.678–691.

Gonzalo, S. et al., 2006. DNA methyltransferases control telomere length and telomere recombination in mammalian cells. *Nature cell biology*, 8(4), p.416–24.

Gothard, L.Q. et al., 2003. Lowered temperature set point for activation of the cellular stress response in T-lymphocytes. *Journal of Biological Chemistry*, 278(11), p.9322–9326.

Green, M. et al., 1995. A heat shock-responsive domain of human HSF1 that regulates transcription activation domain function. *Molecular and cellular biology*, 15(6), p.3354–3362.

Guettouche, T. et al., 2005. Analysis of phosphorylation of human heat shock factor 1 in cells experiencing a stress. *BMC biochemistry*, 6(1), p.4.

Guisbert, E. et al., 2004. A chaperone network controls the heat shock response in E. coli. *Genes and Development*, 18(22), p.2812–2821.

Guisbert, E. et al., 2013. Identification of a Tissue-Selective Heat Shock Response Regulatory Network. *PLoS Genetics*, 9(4), p.1–12.

Guo, Y. et al., 2001. Evidence for a Mechanism of Repression of Heat Shock Factor 1 Transcriptional Activity by a Multichaperone Complex. *Journal of Biological Chemistry*, 276(49), p.45791–45799.

Harley, C.B., Futcher, A.B. & Greider, C.W., 1990. Telomere shorten during ageing of human fibroblasts.

Harrison, C.J., Bohm, A.A. & Nelson, H.C., 1994. Crystal structure of the DNA binding domain of the heat shock transcription factor. *Science*, 263(5144), p.224–227.

Hentze, N. et al., 2016. Molecular mechanism of thermosensory function of human heat shock transcription factor Hsf1. , (January), p.1–24.

Hietakangas, V. et al., 2006. PDSM, a motif for phosphorylation-dependent SUMO

modification. *Proceedings of the National Academy of Sciences of the United States of America*, 103(1), p.45–50.

Hietakangas, V. et al., 2003. Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. *Molecular and cellular biology*, 23(8), p.2953–68.

Hirano, K. et al., 2012. How environmental solution conditions determine the compaction velocity of single DNA molecules. *Nucleic Acids Research*, 40(1), p.284–289.

Hirashima, K. & Seimiya, H., 2015. Telomeric repeat-containing RNA/G-quadruplex-forming sequences cause genome-wide alteration of gene expression in human cancer cells in vivo. *Nucleic Acids Research*, 43(4), p.2022–2032.

Hirashima, K. & Seimiya, H., 2015. Telomeric repeat-containing RNA/G-quadruplex-forming sequences cause genome-wide alteration of gene expression in human cancer cells in vivo. *Nucleic Acids Research*, 43(4), p.2022–2032.

Hochstrasser, T., Marksteiner, J. & Humpel, C., 2012. Telomere length is age-dependent and reduced in monocytes of Alzheimer patients. *Experimental Gerontology*, 47(2), p.160–163.

Hofmann, N.R., 2009. The plasma membrane as first responder to heat stress. *The Plant cell*, 21(9), p.2544.

Holmberg, C.I. et al., 2001. Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *EMBO Journal*, 20(14), p.3800–3810.

Hsu, A.-L., Murphy, C.T. & Kenyon, C., 2003. Regulation of Aging and Age-Related Disease by DAF-16 and Heat-Shock Factor. *Science*, 300(5622), p.1142–1145.

Hu, J. et al., 2012. Antitelomerase therapy provokes ALT and mitochondrial adaptive mechanisms in cancer. *Cell*, 148(4), p.651–663.

Jedlicka, P., Mortin, M.A. & Wu, C., 1997. Multiple functions of Drosophila heat shock transcription factor in vivo. , 16(9), p.2452–2462.

Jin, B. & Robertson, K.D., 2013. Epigenetic Alterations in Oncogenesis. *Advances in experimental medicine and biology*, 754, p.3–29.

Jolly, C. et al., 1997. HSF1 transcription factor concentrates in nuclear foci during heat shock: relationship with transcription sites. *Journal of cell science*, 110, p.2935–2941.

Jolly, C. et al., 2004. Stress-induced transcription of satellite III repeats. *Journal of Cell Biology*, 164(1), p.25–33.

Jolly, C. & Morimoto, R.I., 2000. Role of the Heat Shock Response and Molecular Chaperones in Oncogenesis and Cell Death TO. *Journal of the National Cancer Institute*, 92(19), p.1564–1572.

Jurivich, D.A. et al., 1995. Salicylate triggers heat shock factor differently than heat. *Journal of Biological Chemistry*, 270(41), p.24489–24495.

Kampinga, H.H. & Craig, E.A., 2011. The Hsp70 chaperone machinery: J-proteins as drivers of functional specificity. , 4(164), p.579–592.

Khaleque, M. a et al., 2008. Heat shock factor 1 represses estrogen-dependent transcription

through association with MTA1. *Oncogene*, 27, pp.1886–1893.

Khorasanizadeh, S., 2004. The Nucleosome. *Cell*, 116(2), p.259–272.

Kiefhaber, T. et al., 1991. Protein aggregation in vitro and in vivo : a quantitative model of the kinetic competition between folding and aggregation. *Bio/technology*, 9(9), p.825–829.

Kim, Y.E. et al., 2013. *Molecular chaperone functions in protein folding and proteostasis.*

Kline, M.P. & Morimoto, R.I., 1997. Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. *Molecular and cellular biology*, 17(4), p.2107–15.

Kohli, R.M. & Zhang, Y., 2013. TET enzymes, TDG and the dynamics of DNA demethylation. *Nature*, 502(7472), p.472–9.

Krawczyk, P.M. et al., 2011. Mild hyperthermia inhibits homologous recombination, induces BRCA2 degradation, and sensitizes cancer cells to poly (ADP-ribose) polymerase-1 inhibition. *Proceedings of the National Academy of Sciences of the United States of America*, 108(24), p.9851–9856.

Lachner, M. et al., 2001. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature*, 410(6824), p.116–20.

Lai, L.T., Lee, P.J. & Zhang, L.F., 2013. Immunofluorescence protects RNA signals in simultaneous RNA-DNA FISH. *Experimental Cell Research*, 319(3), pp.46–55.

De Lange, T., 2005. Shelterin: The protein complex that shapes and safeguards human telomeres. *Genes and Development*, 19(18), p.2100–2110.

Lehnertz, B. et al., 2003. Suv39h-Mediated Histone H3 Lysine 9 Methylation Directs DNA Methylation to Major Satellite Repeats at Pericentric Heterochromatin Bernhard. *Current Biology*, 13, p.1192–1200.

Levy, M.Z. et al., 1992. Telomere end-replication problem and cell aging. *Journal of Molecular Biology*, 225(4), p.951–960.

Lindquist, S., 1986. The Heat-Shock Response. *Annual Review of Biochemistry*, 55(1), p.1151–1191.

Littlefield, O. & Nelson, H.C., 1999. A new use for the "wing" of the "winged" helix-turn-helix motif in the HSF-DNA cocrystal. *Nature structural biology*, 6(5), p.464–470.

Liu, D. et al., 2004. Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. *Journal of Biological Chemistry*, 279(49), p.51338–51342.

Liu, P.C.C. & Thiele, D.J., 1999. Modulation of human heat shock factor trimerization by the linker domain. *Journal of Biological Chemistry*, 274(24), p.17219–17225.

Lopez de Silanes, I. et al., 2014. Identification of TERRA locus unveils a telomere protection role through association to nearly all chromosomes. *Nat Commun*, 5, p.4723.

Lopez de Silanes, I., Stagno d'Alcontres, M. & Blasco, M.A., 2010. TERRA transcripts are bound by a complex array of RNA-binding proteins. *Nat Commun*, 1, p.33.

Luke, B. et al., 2008. The Rat1p 5' to 3' Exonuclease Degrades Telomeric Repeat-Containing RNA and Promotes Telomere Elongation in Saccharomyces cerevisiae. *Molecular Cell*,

32(4), p.465–477.

Luke, B. & Lingner, J., 2009. TERRA: telomeric repeat-containing RNA. *The EMBO journal*, 28(17), p.2503–10.

Maeda, T. et al., 2012. Parkinson ' s disease er ci al us e om m on e.

Mahat, D.B. et al., 2016. Mammalian Heat Shock Response and Mechanisms Underlying Its Genome-wide Transcriptional Regulation. *Molecular Cell*, 62(1), p.63–78.

Maicher, A., Lockhart, A. & Luke, B., 2014. Breaking new ground: Digging into TERRA function. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, 1839(5), p.387–394.

Makarov, V.L., Hirose, Y. & Langmore, J.P., 1997. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell*, 88(5), p.657–666.

Marion, R.M. et al., 2009. Telomeres Acquire Embryonic Stem Cell Characteristics in Induced Pluripotent Stem Cells. *Cell Stem Cell*, 4(2), p.141–154.

Martínez-Guitarte, J.L., Díez, J.L. & Morcillo, G., 2008. Transcription and activation under environmental stress of the complex telomeric repeats of Chironomus thummi. *Chromosome Research*, 16(8), p.1085–1096.

Masutomi, K. et al., 2003. in Normal Human Cells. , 114, p.241–253.

Mattiussi, M. et al., 2012. Human telomerase represses ROS-dependent cellular responses to Tumor Necrosis Factor- α without affecting NF- κ B activation. *Cellular Signalling*, 24(3), p.708–717.

Mayer, M.P. & Bukau, B., 2005. Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences*, 62(6), p.670–684.

McElligott, R. & Wellinger, R.J., 1997. The terminal DNA structure of mammalian chromosomes. *EMBO Journal*, 16(12), p.3705–3714.

McMillan, D.. et al., 1998. Targeted disruption of heat shock transcription factor 1 abolishes thermotolerance and protection agaisnt heat-inducible apoptosis. *J. Biol. Chem.*, 273(13), p.7523–7528.

Mendillo, M.L. et al., 2012. HSF1 Drives a Transcriptional Program Distinct from Heat Shock to Support Highly Malignant Human Cancers. , p.549–562.

Metchat, A. et al., 2009. Mammalian heat shock factor 1 is essential for oocyte meiosis and directly regulates Hsp90α expression. *Journal of Biological Chemistry*, 284(14), p.9521–9528.

Metz, A. et al., 2004. A key role for stress-induced satellite III transcripts in the relocalization of splicing factors into nuclear stress granules. *Journal of cell science*, 117(Pt 19), p.4551–4558.

Mezger, V., Renard, J.-P., et al., 1994. Detection of heat shock element-binding activities by gel shift assay during mouse preimplantation development.

Mezger, V., Rallu, M., et al., 1994. Heat shock factor 2-like activity in mouse Blastocysts.

Michishita, E. et al., 2009. NIH Public Access. *Gerontology*, 452(7186), p.492–496.

Miozzo, F., Saberan-Djoneidi, D. & Mezger, V., 2015. HSFs, Stress Sensors and Sculptors of Transcription Compartments and Epigenetic Landscapes. *Journal of Molecular Biology*, 427(24), p.3793–3816.

Mivechi, N.F., Murai, T. & Hahn, G.M., 1994. Inhibitors of tyrosine and Ser/Thr phosphatases modulate the heat shock response. *Journal Of Cellular Biochemistry*, 54(2), p.186–197.

Moravec, M. et al., 2016. Article TERRA promotes telomerase-mediated telomere elongation in Schizosaccharomyces pombe, p.1–14.

Morimoto, R.I., 1998. Regulation of the heat-shock transcriptional response: cross talk between a family of heat-shock factors, molecular chaperones, and negative regulators. *Genes Dev*, 12, p.3788–3796.

Morimoto, R.I., Tissieres, A. & Georgopoulous, C., 1990. *Stress Response, Function of the Proteins, and Perspectives*,

Morley, J.F. & Morimoto, 2004. Regulation of Longevity in Caenorhabditis elegans by Heat Shock Factor and Molecular Chaperones. *Molecular biology of the cell*, 15(April), p.3751–3737.

Moyzis, R.K. et al., 1988. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. *Proceedings of the National Academy of Sciences of the United States of America*, 85(18), p.6622–6.

Myohara, M. & Okada, M., 1988. Digitonin treatment activates specific genes including the heat-shock genes in salivary glands of Drosophila melanogaster. *Developmental Biology*, 130(1), pp.348–355.

Nakai, A. et al., 1997. HSF4, a new member of the human heat shock factor family which lacks properties of a transcriptional activator. *Molecular and cellular biology*, 17(1), p.469–81.

Nechaev, S. & Adelman, K., 2011. Pol II waiting in the starting gates: Regulating the transition from transcription initiation into productive elongation. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms*, 1809(1), p.34–45.

Neef, D.W. et al., 2014. A direct regulatory interaction between chaperonin TRiC and stress-responsive transcription factor HSF1. *Cell Reports*, 9(3), p.955–966.

Negishi, Y. et al., 2015. Identification of chromatin marks at TERRA promoter and encoding region. *Biochemical and Biophysical Research Communications*, 467(4), p.1052–1057.

Nergadze, S.G. et al., 2009. CpG-island promoters drive transcription of human telomeres. *RNA (New York, N.Y.)*, 15(12), p.2186–94.

Newton, E.M. et al., 1996. The regulatory domain of human heat shock factor 1 is sufficient to sense heat stress. *Molecular and cellular biology*, 16(3), p.839–46.

Ng, L.J. et al., 2009. Telomerase activity is associated with an increase in DNA methylation at the proximal subtelomere and a reduction in telomeric transcription. *Nucleic Acids Research*, 37(4), p.1152–1159.

Nowak, J. et al., Activity and expression of human telomerase in normal and malignant cells in gastric and colon cancer patients. *European journal of gastroenterology & hepatology*, 15(1), p.75–80.

Oeseburg, H. et al., 2010. Telomere biology in healthy aging and disease. *Pflugers Archiv European Journal of Physiology*, 459(2), p.259–268.

Olins, D.E. & Olins, A.L., 2003. Chromatin history: our view from the bridge. *Nature reviews. Molecular cell biology*, 4(10), p.809–14. Olovnikov, A.M., 1973.

Olovnikov, A.M., 1973. Incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *Journal of Theoretical Biology*, 41(1), p.181–190.

Östling, P. et al., 2007. Heat Shock Factor 2 (HSF2) Contributes to Inducible Expression of hsp Genes through Interplay with HSF1. *The journal of biological chemistry,* 282(10), p.7077–7086.

Palm, W. & de Lange, T., 2008. How Shelterin Protects Mammalian Telomeres. *Annual Review of Genetics*, 42(1), p.301–334.

Palotai, R., Szalay, M.S. & Csermely, P., 2008. Chaperones as integrators of cellular networks: Changes of cellular integrity in stress and diseases. *IUBMB Life*, 60(1), p.10–18.

Pandey, R. et al., 2011. Heat shock factor binding in Alu repeats expands its involvement in stress through an antisense mechanism. *Genome Biol*, 12(11), p.R117.

Passarge, E., 1979. Emil Heitz and the concept of heterochromatin: longitudinal chromosome differentiation was recognized fifty years ago. *American journal of human genetics*, 31(2), p.106–15.

Pauli, D., Arrigo, A. & Tissires, A., 1992. Heat shock response in Drosophila aD ~ partement de Zoologic et Biologic Animale , UniversitO de Genkve , 154 Route de Malagnou , CH-1224 ChFne- Bougeries (Switzerland), b Centre de Gknktique , UMR-106 CNRS , Universitk Claude Bernard , F-69-622 Lyon Hi. *Analysis*, 48, p.623–629.

Pearl, L.H. & Prodromou, C., 2006. Structure and mechanism of the Hsp90 molecular chaperone machinery. *Annual review of biochemistry*, 75, p.271–294.

Perisic, O., Xiao, H. & Lis, J.T., 1989. Stable binding of Drosophila heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. *Cell*, 59(5), p.797–806.

Pernet, L. et al., 2014. HDAC6-ubiquitin interaction controls the duration of HSF1 activation after heat shock. *Molecular biology of the cell*, 25, pp.4187–94. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4263459&tool=pmcentrez&rendertype=abstract.

Petrova, N. V et al., 2014. Heat shock-induced dissociation of TRF2 from telomeres does not initiate a telomere-dependent DNA damage response. *Cell biology international*, 38(5), pp.675–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24474557.

Phosphorylation, T., Sorger, P.K. & Pelham, H.R.B., 1988. Yeast Heat Shock Factor Is an Essential DNA-Binding Protein That Exhibits. , 54, p.855–864.

Pickett, H. a et al., 2009. Control of telomere length by a trimming mechanism that involves generation of t-circles. *The EMBO journal*, 28(7), p.799–809.

Pirkkala, L., Nykanen, P. & Sistonen, L., 2001. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 15(7), p.1118–1131.

Porro, a et al., 2010. Molecular dissection of telomeric repeat-containing RNA biogenesis unveils the presence of distinct and multiple regulatory pathways. *Mol Cell Biol*, 30(20), p.4808–4817.

Porro, A. et al., 2014. Functional characterization of the TERRA transcriptome at damaged telomeres. *Nature communications*, 5, p.5379.

Poulet, A., Pisano, S. & Giraud-Panis, M.-J., 2012. The N-terminal domains of TRF1 and TRF2 regulate their ability to condense telomeric DNA. , 40(6), p.2566–2576.

Powers, E.T. et al., 2009. Biological and chemical approaches to diseases of proteostasis deficiency. *Annual review of biochemistry*, 78(August), p.959–991.

Probst, A. V. et al., 2010. A Strand-specific burst in transcription of pericentric satellites is required for chromocenter formation and early mouse development. *Developmental Cell*, 19(4), p.625–638.

Rabindran, S.K. et al., 1993. Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. *Science (New York, N.Y.)*, 259(5092), p.230–234.

Rasmussen, E.B. & Lis, J.T., 1993. In vivo transcriptional pausing and cap formation on three Drosophila heat shock genes. *Proceedings of the National Academy of Sciences of the United States of America*, 90(17), p.7923–7.

Raychaudhuri, S. et al., 2014. Interplay of acetyltransferase EP300 and the proteasome system in regulating heat shock transcription factor 1. *Cell*, 156(5), p.975–985.

Redon, S., Reichenbach, P. & Lingner, J., 2010. The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase. *Nucleic Acids Research*, 38(17), p.5797–5806.

Redon, S., Zemp, I. & Lingner, J., 2013. A three-state model for the regulation of telomerase by TERRA and hnRNPA1. *Nucleic Acids Research*, 41(19), p.9117–9128.

Reig-Viader, R. et al., 2014. Telomeric repeat-containing RNA (TERRA) and telomerase are components of telomeres during mammalian gametogenesis. *Biol Reprod*, 90(5), p.103.

Reig-Viader, R. et al., 2013. Telomeric repeat-containing RNA and telomerase in human fetal oocytes. *Human Reproduction*, 28(2), p.414–422.

Rice, C. & Skordalakes, E., 2016. Structure and function of the telomeric CST complex. *Computational and Structural Biotechnology Journal*, 14, p.161–167.

Richter, K., Haslbeck, M. & Buchner, J., 2010. The Heat Shock Response: Life on the Verge of Death. *Molecular Cell*, 40(2), p.253–266.

Ritossa, F., 1962. A new puffing pattern induced by temperature shock and DNP in drosophila. *Experientia*, 18(12), p.571–573.

Ritossa, F., 1996. Discovery of the heat shock response. *Cell stress & chaperones*, 1(2), p.97–

98.

Rizzi, N. et al., 2004. Transcriptional Activation of a Constitutive Heterochromatic Domain of the Human Genome in Response to Heat Shock□. *Molecular biology of the cell*, 15(Febl), p.543–551.

Rohlin, L. et al., 2005. Heat Shock Response of Archaeoglobus fulgidus †. *Society*, 187(17), p.6046–6057.

Romano, G.H. et al., 2013. Environmental Stresses Disrupt Telomere Length Homeostasis. *PLoS Genetics*, 9(9).

Rougvie, A.E. & Lis, J.T., 1988. The RNA polymerase II molecule at the 5??? end of the uninduced hsp70 gene of D. melanogaster is transcriptionally engaged. *Cell*, 54(6), p.795–804.

Ruiz-Herrera, A. et al., 2008. Telomeric repeats far from the ends: Mechanisms of origin and role in evolution. *Cytogenetic and Genome Research*, 122(3-4), p.219–228.

Saksouk, N., Simboeck, E. & Déjardin, J., 2015. Constitutive heterochromatin formation and transcription in mammals. *Epigenetics & chromatin*, 8, p.3.

Sampl, S. et al., 2012. Expression of telomeres in astrocytoma WHO grade 2 to 4: TERRA level correlates with telomere length, telomerase activity, and advanced clinical grade. *Transl Oncol*, 5(1), p.56–65.

Sandell, L.L., Gottschling, D.E. & Zakian, V.A., 1994. Transcription of a yeast telomere alleviates telomere position effect without affecting chromosome stability. *Proc Natl Acad Sci U S A*, 91(25), p.12061–12065.

Sandqvist, A. et al., 2009. Heterotrimerization of Heat-Shock Factors 1 and 2 Provides a Transcriptional Switch in Response to Distinct Stimuli. *Seikagaku*, 20(4), pp.1340–1347.

Sarek, G. et al., 2015. TRF2 recruits RTEL1 to telomeres in s phase to promote t-loop unwinding. *Molecular Cell*, 57(4), p.622–635.

Saretzki, G., 2009. Telomerase, mitochondria and oxidative stress. *Experimental Gerontology*, 44(8), p.485–492.

Sarge, K.D., Murphy, S.P. & Morimoto, R.I., 1993. Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. *Molecular and cellular biology*, 13(3), p.1392–407.

Selth, L. a, Sigurdsson, S. & Svejstrup, J.Q., 2010. Transcript Elongation by RNA Polymerase II. *Annual review of biochemistry*, 79, p.271–293.

Sfeir, A., 2012. Telomeres at a glance. *Journal of cell science*, 125(Pt 18), p.4173–8.

Shalgi, R. et al., 2014. Widespread inhibition of posttranscriptional splicing shapes the cellular transcriptome following heat shock. *Cell Reports*, 7(5), p.1362–1370.

Shamovsky, I. et al., 2006. RNA-mediated response to heat shock in mammalian cells. *Nature*, 440(7083), p.556–60.

Shi, Y., Mosser, D.D. & Morimoto, R.I., 1998. Molecular chaperones as HSF1-specific

transcriptional repressors. *Genes and Development*, 12(5), p.654–666.

Simonet, T. et al., 2011. The human TTAGGG repeat factors 1 and 2 bind to a subset of interstitial telomeric sequences and satellite repeats. *Cell research*, 21(7), p.1028–1038.

Sistonen, L., Sarge, K.D. & Morimoto, R.I., 1994. Human heat shock factors 1 and 2 are differentially activated and can synergistically induce hsp70 gene transcription. *Molecular and cellular biology*, 14(3), p.2087–99.

Skene, P.J. & Henikoff, S., 2013. Histone variants in pluripotency and disease. *Development (Cambridge, England)*, 140, p.2513–24.

Slagboom, P.E., Droog, S. & Boomsma, D.I., 1994. Genetic determination of telomere size in humans: a twin study of three age groups. *American journal of human genetics*, 55(5), p.876–82.

Sorger, P.K., 1991. Heat shock factor and the heat shock response. *Cell*, 65(3), p.363–366.

Sorger, P.K. & Nelson, H.C.M., 1989. Trimerization of a yeast transcriptional activator via a coiled-coil motif. *Cell*, 59(5), p.807–813.

Stansel, R.M., De Lange, T. & Griffith, J.D., 2001. T-loop assembly in vitro involves binding of TRF2 near the 3' telomeric overhang. *EMBO Journal*, 20(19), p.5532–5540.

Stanulis, B.M., 1987. Cellular Senescence revisited: A Review. *Mechanisms of ageing and Development*, 38.

Stong, N. et al., 2014. Subtelomeric CTCF/Cohesin binding site organization using improved subtelomere assemblies and novel annotations , p.1039–1050.

Strenkert, D., Schmollinger, S. & Schroda, M., 2013. Heat shock factor 1 counteracts epigenetic silencing of nuclear transgenes in Chlamydomonas reinhardtii. *Nucleic Acids Research*, 41(10), p.5273–5289.

Su, K.-H. et al., 2016. HSF1 critically attunes proteotoxic stress sensing by mTORC1 to combat stress and promote growth. *Nature Cell Biology*, 18(5), p.527–539.

Sullivan, E.K. et al., 2001. Transcriptional activation domains of human heat shock factor 1 recruit human SWI/SNF. *Molecular and cellular biology*, 21(17), p.5826–37.

Sýkorová, E. & Fajkus, J., 2009. Structure-function relationships in telomerase genes. *Biology of the cell / under the auspices of the European Cell Biology Organization*, 101(7), pp.375–92, 1 p following 392.

Szenker, E., Ray-Gallet, D. & Almouzni, G., 2011. The double face of the histone variant H3.3. *Cell Res*, 21(3), p.421–434.

Taipale, M., Jarosz, D.F. & Lindquist, S., 2010. HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nature reviews. Molecular cell biology*, 11(7), p.515–28.

Takai, H., Smogorzewska, A. & de Lange, T., 2003. DNA Damage Foci at Dysfunctional Telomeres. *Medical Engineering & Physics*, 25, p.75–78.

Tang, D. et al., 2005. Expression of heat shock proteins and heat shock protein messenger ribonucleic acid in human prostate carcinoma in vitro and in tumors in vivo. *Cell stress & chaperones*, 10(1), p.46–58.

Tang, Z. et al., 2015. MEK guards proteome stability and inhibits tumor-suppressive amyloidogenesis via HSF1. *Cell*, 160(4), p.729–744.

Tennen, R.I. et al., 2012. SIRT6 is required for maintenance of telomere position effect in human cells. *Nature communications*, 1(1), p.1–16.

Thomson, J.P. et al., 2010. CpG islands influence chromatin structure via the CpG-binding protein Cfp1. *Nature*, 464(7291), p.1082–6.

Tilman, G. et al., 2012. Cancer-linked satellite 2 DNA hypomethylation does not regulate Sat2 non-coding RNA expression and is initiated by heat shock pathway activation. *Epigenetics*, (August), p.903–913.

Ting, D.T. et al., 2011. Aberrant Overexpression of Satellite Epithelial Cancers. , 593(February), p.593–597.

Toivola, D, 2011. Intermediate filaments take the heat as stress proteins. *Cell*, 20(2), pp.79–91.

Tomlinson, R.L. et al., 2006. Cell Cycle-regulated Trafficking of Human Telomerase to Telomeres. *Molecular biology of the cell*, 17(2), pp.955–965.

Tommerup, H., Dousmanis, a & de Lange, T., 1994. Unusual chromatin in human telomeres. *Molecular and cellular biology*, 14(9), p.5777–85.

Trinklein, N.D. et al., 2004. The Role of Heat Shock Transcription Factor 1 in the Genome-wide Regulation of the Mammalian Heat Shock Response□. *Molecular biology of the cell*, 15(March), p.1254–1261.

Tutton, S. et al., 2015. Subtelomeric p53 binding prevents accumulation of DNA damage at human telomeres. *The EMBO journal*, 35(2), p.193–207.

Vaziri, H., Dragowska, W., et al., 1994. Evidence for a mitotic clock in human hematopoietic stem cells: Loss of telomeric DNA with age. *Cell Biology*, 91, p.9857-9860.

Velichko, A.K., Petrova, N., et al., 2012. Dual effect of heat shock on DNA replication and genome integrity. *Molecular Biology of the Cell*, 23(17), p.3450–3460.

Velichko, A.K., Petrova, N. V, et al., 2012. Dual effect of heat shock on DNA replication and genome integrity. *Molecular biology of the cell*, 23(17), p.3450–60.

Velichko, A.K. et al., 2015. Mechanism of heat stress-induced cellular senescence elucidates the exclusive vulnerability of early S-phase cells to mild genotoxic stress. *Nucleic Acids Research*, 43(13), p.6309–6320.

Velichko, A.K. et al., 2013. Mechanisms of heat shock response in mammals. *Cellular and Molecular Life Sciences*, 70(22), p.4229–4241.

Verdun, R.E. & Karlseder, J., 2006. The DNA Damage Machinery and Homologous Recombination Pathway Act Consecutively to Protect Human Telomeres. *Cell*, 127(4), p.709–720.

Vihervaara, A. & Sistonen, L., 2014. HSF1 at a glance. *Journal of cell science*, 127(Pt 2), p.261–6.

Voellmy, R. & Boellmann, F., 2007. Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks. , 594(February), p.89–99.

Vogel, J.L., Parsellt, D.A. & Lindquist, S., 1995. Heat-shock proteins Hspl04 and Hsp70 reactivate mRNA splicing after heat inactivation. , 5(3), p.306–317.

Vuister, G.W. et al., 1994. Solution structure of the DNA-binding domain of Drosophila heat shock transcription factor. *Structure Biology*, 1(9), p.605–614.

Vujanac, M., Fenaroli, A. & Zimarino, V., 2005. Constitutive nuclear import and stress-regulated nucleocytoplasmic shuttling of mammalian heat-shock factor 1. *Traffic*, 6(3), p.214–229.

Wai, L.K., 2004. Telomeres , Telomerase , and Tumorigenesis -A Review, Page 2 of 7. , 6(3), p.1–7.

Walters, R.D., Kugel, J.F. & Goodrich, J.A., 2009. InvAluable junk: The cellular impact and function of Alu and B2 RNAs. *IUBMB Life*, 61(8), p.831–837.

Wang, Z. et al., 2015. Telomeric repeat-containing RNA (TERRA) constitutes a nucleoprotein component of extracellular inflammatory exosomes. *Proceedings of the National Academy of Sciences*, (26), p.201505962.

Welch, W.I. & Suhan, J.P., 1985. Morphological Study of the Mammalian Stress Response " Characterization of Changes in Cytoplasmic Organelles , Cytoskeleton , and Nucleoli , and Appearance of Intranuclear Actin Filaments in Rat Fibroblasts after Heat-Shock Treatment. , 101(October).

Westerheide, S.D. et al., 2009. Stress-Inducible Regulation of Heat Shock Factor 1 by thr Deacetylase SIRT1. *Science*, 323(5917), p.1063–1066.

Westwood, J.T. & Wu, C., 1993. Activation of Drosophila heat shock factor: conformational change associated with a monomer-to-trimer transition. *Molecular and cellular biology*, 13(6), p.3481–3486.

Whatson, J., 1972. Origin of Concatemeric T7 DNA. *Nature*, 239, p.197–201.

Whitesell, L. & Lindquist, S., 2009. Inhibiting the transcription factor HSF1 as an anticancer strategy. *Expert opinion on therapeutic targets*, 13(4), p.469–478.

Wiederrecht, G., SETO, D. & PARKER, C.S., 1988. Isolation of the gene encoding the S. cerevisiae heat shock transcription factor. *Cell*, 54(6), p.841–853.

Wold, M.S., 1997. Replication protein A: A Heterotrimeric, Single-Stranded DNA-Binding Protein Required for Eukaryotic DNA Metabolism. *Annual Review of Biochemistry*, 66, p.61–92.

Wu, B.J., Kingston, R.E. & Morimoto, R.I., 1986. Human HSP70 promoter contains at least two distinct regulatory domains. *Proceedings of the National Academy of Sciences of the United States of America*, 83(February), p.629–33.

Wu, C., 1984. Activating protein factor binds in vitro upstream control sequences in heat shock gene chromatin. *Letters To Nature*, 311, p.81–84.

Wu, C., 1995. HEAT SHOCK TRANSCRIPTION FACTORS : Structure and Regulation translocation , higher order assembly , and protein degradation (Gething &. *Annual Review of Cell and Developmental Biology*, 11, p.441–469.

Xiao, H. & Lis, J., 1988. Germline transformation used to define key features of heat-shock response elements. *Science*, 239(4844), p.1139–1142.

Xiao, H., Perisic, O. & Lis, J.T., 1991. Cooperative binding of drosophila heat shock factor to arrays of a conserved 5 bp unit. *Cell*, 64(3), p.585–593.

Xiao, X. et al., 1999. HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice. *EMBO Journal*, 18(21), p.5943–5952.

Xu, Y. et al., 2012. Post-Translational Modi fi cation of Human Heat Shock Factors and Their Functions: A Recent Update by Proteomic Approach.

Yeager, T.R. et al., 1999. Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. *Cancer Research*, 59(17), pp.4175–4179.

Yehezkel, S. et al., 2013. Characterization and rescue of telomeric abnormalities in ICF syndrome type I fibroblasts. *Frontiers in oncology*, 3(February), p.35.

Yehezkel, S. et al., 2008. Hypomethylation of subtelomeric regions in ICF syndrome is associated with abnormally short telomeres and enhanced transcription from telomeric regions. *Human Molecular Genetics*, 17(18), p.2776–2789.

Yu, G.-L. et al., 1990. In vivo alteration of telomere sequences and senescence caused by mutated tetrahymena telomerase. *Journal of Colloid and Interface Science*, 245(1), p.118–143.

Yu, T.Y., Kao, Y.W. & Lin, J.J., 2014. Telomeric transcripts stimulate telomere recombination to suppress senescence in cells lacking telomerase. *Proc Natl Acad Sci U S A*, 111(9), p.3377–3382.

Zeichner, S.L. et al., 1999. Rapid telomere shortening in children. *Blood*, 93(9), p.2824–2830.

Zou, J. et al., 1998. Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell*, 94(4), p.471–480.