To translate or to degrade? The role of INT6 in histone mRNA translation and Nonsense Mediated mRNA Decay

Julia Neusiedler

THÈSE

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par Mlle Julia NEUSIEDLER

Etude du rôle de la protéine INT6 dans la dégradation des ARN par la voie du "Nonsense Mediated mRNA Decay" (NMD) et dans la traduction et la dégradation des ARN histones.

Directeur de thèse : *Dr. Pierre Jalinot*

Après l'avis de : *M...Prénom NOM*

M...Prénom NOM

Devant la commission d'examen formée de :

Dr. Gilbert Eriani, Institut de Biologie Moléculaire et Cellulaire de Strasbourg, Rapporteur

Dr. Pierre Jalinot, École Normale Supérieure de Lyon, Directeur

Dr. Hervé Le Hir, École Normale Supérieure, Rapporteur

Dr. Theophile Ohlmann, École Normale Supérieure de Lyon, Président

Dr. Stéphane Pyronnet, Centre de Recherche en Cancérologie de Toulouse, Examinateur

Il faut toujours connaître les limites du possible. Pas pour s'arrêter, mais pour tenter l'impossible dans les meilleures conditions.

Romain Gary

To Monika, because I knew you.

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Abbreviations list

3’-UTR	3'-unstranslated region
5’-UTR	5'-untranslated region
AUG	Translation initiation codon
BLV	Bovine Leukaemia virus
CDK9	Cyclin-dependent kinase 9
cDNA	Complementary DNA
C-terminal	Carboxy-terminal
DCP1	Decapping enzyme 1
DCP2	Decapping enzyme 2
DDX1	DEAD polypeptide 1
Env	Envelope protein
eRF	Eukaryotic release factor
G3BP	GTPase activating protein (SH3 domain) binding protein
Gag	Group-specific antigen
GDP	Guanosine diphosphate
GFP	Green Fluorescent Protein
GTP	Guanosine triphosphate
HBP	Hairpin Binding Protein (see SLBP)
HTLV	Human T-cell lymphotropic virus
INT6	Integration Site 6
IRES	Internal ribosome entry site
ITAF	IRES *trans* acting factor
kDa	kilodalton
Lsm	Like Smith antigen protein
LTR	Long terminal repeat
MIF4GD	Middle Domain of eIF4G containing protein
MLV	Murine leukaemia virus
MMTV	Mammary Mouse Tumour Virus
miRNA	micro RNA
mRNA	Messenger RNA
NC	Nucleocapsid
NES	Nuclear export signal
NLS	Nuclear localization signal
NMD	Nonsense mediated mRNA decay
NPC	Nuclear pore complex
N-terminal	Amino-terminal
p300/CBP	CREB binding protein
PABP	Poly(A)-binding protein
P-bodies	Processing bodies
PCI	Proteasome, COP9 Signalosome, eIF3
PR	Protease
P-site	Polypeptide site
RNA	Ribonucleic acid
RT	Reverse transcriptase

siRNA	Small interfering RNA
SL	Steam Loop
SLBP	Steam Loop Binding Protein
SLIP-1	SLBP Interacting Protein 1
tRNA	Transfer RNA
TTP	Tristetraprolin
UPF1(2,3)	Up-frame Shift Protein 1(2,3)
WHO	World Health Organization
XRN1	5'-3' exoribonuclease 1

Truth in science can be defined as the working hypothesis best suited to open the way to the next better one.

Konrad Lorenz

Summary

INT6 has been identified as a tumour suppressor protein. The INT6/EIF3E protein has been implicated in mouse and human breast carcinogenesis. In around 30% of human brest and lung carcinoma *int6* has been found to be under-expressed. Histone mRNAs are unique in that they lack a 3' polyadenosine tail. Their translation and stability has been shown to be dependent on a 3' stem-loop that binds to SLBP and SLIP1. Using a two-hybrid screen with INT6 as bait we report in this report the MIF4GD/SLIP1 protein as one of its interactors. Importantly for this work, SLIP1 bears homology to the middle domain of eIF4G. In mammalian cells, silencing of INT6 seems to marginally affect general translation.This study documents that INT6 binds to SLIP1 and SLBP and is required for efficient translation of histone mRNA. The knockdown of INT6 results in a 2 fold decrease in histone mRNA translation during S phase with.

In another screen, Tax has been identified to bind INT6/eIF3E that is a subunit of the EIF3 translation initiation factor required for efficient degradation of mRNAs by nonsense-mediated mRNA decay (NMD). In line with this association, we show here that Tax inhibits this important cellular pathway and also interacts with the NMD core factor UPF1. Through specific protein-protein interactions Tax alters the normal recycling of UPF1 and morphology of processing bodies (P-bodies), the cytoplasmic structures which concentrate RNA degradation factors. Our data indicate that the effect of Tax on cellular genes expression is not restricted to transcriptional control and that this viral protein can favour production of viral RNAs by impeding their degradation. We observed a significant stabilization of canonical NMD targets and some viral RNAs. This work will contribute to a better understanding of both the basics of NMD and some effects of HTLV-1 infection on cell transformation. Tax, which trans-activates the expression of viral and cellular genes by binding to various enhancer-binding proteins needed for transcription, appears to also interfere with gene regulation at the post-transcriptional level by inactivating or modifying the mechanism of NMD.

Résumé

Différentes observations montrent que la protéine INT6/eIF3E humaine possède une activité suppresseur de tumeurs. Il a été démontré que l'expression d'une forme tronquée de la protéine INT6 dans des lignées épithéliales mammaires humaines ou murines entraîne leur transformation. Des souris « nude » exprimant à l'état hétérozygote INT6 tronquée développent des tumeurs mammaires. Enfin, il a été publié que chez l'homme le gène *int6* était sous-exprimé dans environ 30% des cancers du poumon non à petits cellules et que cette sous-expression était un facteur de mauvais pronostic. Des expériences de criblage double hybride avec INT6 comme appât ont identifié une nouvelle protéine humaine nommée MIF4GD/SLIP1 (SLBP Interacting Protein 1). Un effet de SLIP1 sur la traduction des ARN messager des histones a été montré. Les travaux récents que j'ai menés indiquent aussi qu'INT6 en interagissant avec SLIP intervient dans le contrôle de la stabilité et de la traduction des ARNs codant pour les histones. Un knockdown d'INT6 provoque une baise des niveaux des histones endogènes sans avoir un effet au niveau d'ARN. Mes études, en révélant un nouveau mécanisme de régulation du taux des histones dans lequel INT6 joue un rôle direct, permettent ainsi de faire le lien entre – d'une part – les fonctions connues de cette protéine dans la traduction et son contrôle et – d'autre part – les effets oncogéniques connus de son altération.

Par ailleurs, l'étude de la fonction d'INT6 dans les cellules humaines réalisée au laboratoire d'accueil par ARN interférence montre une inhibition de la dégradation des ARNm possédant un codon stop prématuré par la voie du *Nonsense Mediated mRNA Decay* (NMD). Nous avons étudié son action par rapport aux ARNs HTLV-1. Nous avons observé une stabilisation significative des cibles de NMD. Ceci démontre que la protéine Tax interfère avec cette voie de dégradation des ARN d'une part en empêchant l'interaction entre UPF1 et INT6 et d'autre part en interagissant lui-même avec la protéine UPF1 phosphorylée. En agissant sur le NMD, Tax intervient à un niveau post transcriptionel qui pourrait avantager la réplication virale et aussi permettre la tolérance cellulaire aux mutations liées à l'effet mutagénique établi de Tax.

Part I Introduction

General Introduction.

According to the World Health Organization (WHO) cancer is still one of the main causes of death in Western countries, accounting for 7.9 million deaths (around 13% of all deaths) in 2007 (Fig.1). The most frequent types of cancer differ between men and women but in general lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030.

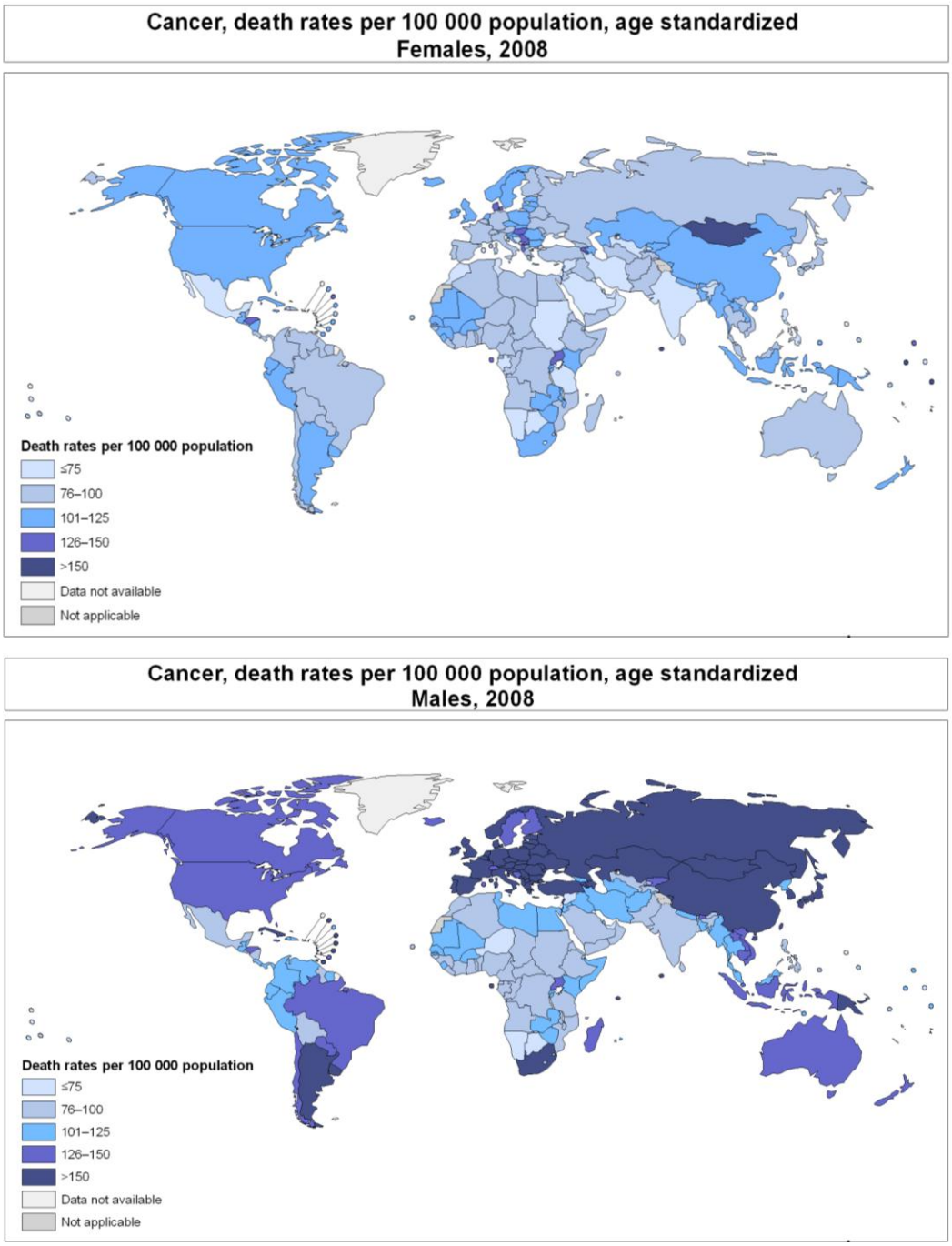

Figure1. Worldwide cancer death distribution. **(adapted from WHO, 2007)**

Cancer is the result of an accumulation of mutations in specific genes, many of them falling under the scope of protooncogenes. Some of them will be presented later. Due to altered gene activity, normal control mechanisms are lost and abnormal cell growth and cell division take place.

The etiology of cancers is one of the main fields of modern biology research. There are more than 100 distinct general types of cancer, and even more different subtypes of tumours which can be found within specific organs. This complexity raises a number of questions. Despite the plethora of cancer studies in the last decades the mechanisms of tumorigenesis remain to be defined with precision. Several theoretical models have been temporarily adapted to address the problem of cancer etiology. At present it is thought that the genome of every living organism contains inactive or weakly active cancer-causing genes called proto-oncogenes. A number of physical, chemical or biological agents can mutate these proto-oncogenes, leading to their activation or over-activation and turning them into cancer causing oncogenes. Symmetrically, other genes – called tumour suppressors – exhibit anti-cancer activities and must be inactivated for cancers to initiate. The functions of the genes involved in oncogenesis are varied, and include cell signalling and signal transduction, cell proliferation, cell death and senescence, immune signalling, DNA repair, cell adherence, etc. Because of this, it is thought that several different mutations affecting both proto-oncogenes and tumour suppressor genes are required to transition a cell from a normal phenotype to the most advanced stages of oncogenesis. Interestingly, some proto-oncogenes and tumour suppressor genes are commonly affected in most cancers (e.g. p53) while others are only affected in some subcategories of tumours.

Among the various agents that can cause cancer, most can be classified as physical or chemical mutagens that directly or indirectly damage DNA. It is then the occasional failures of the DNA repair machinery that result in DNA sequence alterations which, when affecting a gene involved in cancer etiology, may position the cell one step further on the road to cancer. One type of causative agents stands apart for the diversity and complexity of means by which they can induce or facilitate transformation: the cancer causing viruses, or “oncoviruses”.
During the 20th century the realisation that viruses could participate in the oncogenic process was a real breakthrough in the domain of carcinogenesis (reviewed in: (Butel, 2000)). In particular several viruses were revealed to play a role in the etiology of several human cancers: hepatitis B and C viruses infections are involved in hepatocellular carcinoma,

papillomaviruses cause cancer of the cervix, Epstein–Barr virus is involved in lymphoma and carcinoma development. Interestingly many viruses which have not been associated with cancer nonetheless bear genes which when expressed in cells can induce transformation. It is also worth noting that the oncoviruses belong to several different taxonomic groups and include both DNA and RNA viruses. These two apparently unrelated observations can be explained by the hypothesis that in order to successfully replicate in host cells most viruses must deregulate cell cycle, nucleic acid metabolism and immune surveillance mechanisms that play a role in tumour suppression. Asides form this original mechanism of cellular transformation, viruses that accidentally or constitutively integrate their genome in the host cell's DNA can also facilitate cancer progression by either of two mechanisms: (i) inactivation of a gene located at the locus of insertion; (ii) deregulation of the expression of genes around the locus of insertion by the proximity of viral promoters and or enhancers. Luckily, if viruses can cause cancer they can also teach us about it. Indeed, an interesting corollary of the capacity of viruses to induce some cancers is that these viruses make powerful tools for the study of oncogenic mechanisms in living organisms.

The first discovered human retrovirus associated with human cancer was HTLV-1 isolated in 1980. In subsequent years of intense studies, the protein Tax encoded by HTLV-1 was both shown to be responsible for the viral genome's transcriptional activation and identified as the major HTLV-1 oncoprotein, and thus became the subject of a large body of studies.

Although viral infections might be an important cause of cancer, oncogenes may appear spontaneously in cells as a consequence of mutations. In complex organisms the phenotype of each cell is finely controlled and built on two complementary pillars: genome maintenance, itself the result of the quasi-absolute fidelity of DNA replication coupled with extensive DNA lesion repair; and corrects gene expression, which is subject to an extremely precise regulation. One of these is a quality control pathway called the **N**onsense-**M**ediated mRNA **D**ecay (NMD) which removes mRNAs that bear a "**P**remature **T**ermination **C**odon" (PTC). Translation of an abnormal mRNA bearing a PTC would result in premature termination and release of incomplete, generally non-functional polypeptides from the ribosome. PTCs can occur as the result of transcriptional errors or mutations that either directly create a stop codon early in the coding sequence of the mRNA or indirectly create one as the result of a frameshift. It is known that up to 30% of all cancers involve Premature Stop Codons in tumour suppressor genes escaping the NMD and leading to truncated,

potential, dominant negative proteins (Lewis et al, 2003). PTCs can also result from non-functional alternative splicing. Approximately 60–70% of human pre-mRNAs are alternatively spliced. Among these, 45% are predicted to have at least one spliced form that is expected to be targeted by NMD.

An important feature of cancer cells is their intrinsically high genomic instability. Modified total cellular DNA content, chromosome anomalies and abnormal chromatin organization are used by pathologists as diagnostic markers for cancer (He et al, 2008; Khan et al, 2003; Komitowski et al, 1993; Michor et al, 2005). One of the pathways that can lead to genomic instability is altered histone synthesis. Histones govern the correct packaging of DNA and its transcriptional accessibility. Their synthesis is tightly coupled with that of DNA and is like it limited to the S phase. Impeding the fine balance between an excess or a lack of histones can result in chromatin rearrangements and physically weaken the DNA or make the genetic information inaccessible. These defects could in turn result in genomic instability and ultimately in cancer.

The three points I briefly discuss above – viruses, NMD and histone homeostasis – seem to be completely independently connected to tumorigenesis. However from former studies one common feature emerges: the involvement of the INT6 (**Int**egration **S**ite **6** protein), also termed the **e** subunit of **E**ukaryotic **I**nitiation **F**actor **3** (eIF3e). The *int6* (Integration site 6) gene was originally identified as one of several genes that were frequently disrupted by **M**ouse Mammary **T**umor **V**irus (MMTV) integration in MMTV-induced mouse mammary tumours (Marchetti et al, 1995; Miyazaki et al, 1995). As its alternate name of EIF3E indicates INT6 is part of the eukaryotic Initiation Factor 3 (Asano et al, 1997b). This protein is intensely studied in our laboratory. The cDNA coding for INT6 was isolated in a two-hybrid screen using the Tax protein of HTLV- 1 as bait (Desbois et al, 1996). More recently INT6 was identified by Christelle Morris in our group as an important factor of the NMD RNA surveillance pathway (Morris et al, 2007).

It has been reported that a decreased level of INT6 correlates with human breast cancer, the most frequent type of cancer in women according to the WHO. The incidence of this cancer is increasing in the developing world and has been linked with various causes, among which a longer life expectancy, increased urbanization and adoption of a western lifestyle. Unfortunately the only efficient method of breast cancer prevention at present is

early detection. This method permits to reduce the breast cancer rate but cannot eliminate the majority of breast cancers.

Recent published work confirms that in human breast cancer a low level of INT6 protein correlates with a bad prognostic. The aim of this work is to contribute to a better comprehension of the role of INT6 in oncogenesis.

Considering the role of INT6 in the NMD pathway along with its known interaction with the HTLV-1 transactivator Tax, I will address here the question of whether this viral factor is able to act on NMD through INT6. I will show that Tax is indeed able to inhibit the NMD pathway by establishing contacts with both INT6 and the core factor of NMD, UPF1 (**see Part II – Results – Article I**).

In a second part, I will present evidence that links INT6 with histone biogenesis. Because it is but one of the 13 subunits of the human eIF3 complex and, at that, one which does not appear to be essential for translation, INT6/EIF3E is rarely mentioned in relevant studies. Our data suggest that INT6, although dispensable for global translation, is crucial during a unique, non-classical translational mechanism, that of the cell-cycle dependent histone mRNAs. In this manuscript I will present some as yet undescribed functions of INT6 in relationship with the regulation of the synthesis of histones. I will show that INT6-mediated regulation of histone levels intervenes through translational repression. I will also endeavour to demonstrate the implications of these novel activities of INT6 for such catastrophic processes as genomic instability and oncogenesis (**see Part II – Results – Article II**).

Chapter 1. HTLV-1

The term “virus” was first used in the laboratories of Dimitri Ivanovski and Martinus Beijerink in 1890 to describe an “agent that causes infectious disease”. The curiosity caused by these “infectious particles” which were smaller then bacteria grew in the following decades. Interest in retroviruses goes back to 1904 when the first **E**quine **I**nfectious **A**nemia **V**irus (EIAV) was identified (Vallee & Carre, 1904). Since then the Retroviridae family has become one of the most studied, mostly “thanks” to the infamous HIV pandemics that emerged in the 1980s and was estimated to affect 33 million persons in 2009.

Retroviral infections have been found in a vast majority of pluricellular organisms, notably in groups as evolutionarily remote as insects, molluscs and vertebrates (Coffin, 1996).

1. HTLV-1

The **H**uman **T**-cell **L**eukemia **V**irus type **1** (HTLV-1) was the first pathogenic human retrovirus discovered. It was identified in 1980 in Bernard Poiesz’s and Robert Gallo’s laboratory from T-cells isolated from a patient presenting a cutaneous lymphoma (Gallo, 2005; Poiesz et al, 1980). Further studies characterized threes other Human T- lymphotropic viruses (HTLV-2, HTLV-3 and HTLV-4) which were regrouped into the *Deltaretrovirus* genus of the *Orthoretrovirinae* subfamily. In this manuscript I will focus exclusively on HTLV-1.

1.1. HTLV-1 associated diseases and epidemiology.

Shortly after its discovery, HTLV-1 was demonstrated to be a causative agent in **A**dult **T**-Cell **L**eukemia (ATL), although the mechanisms underlying the virus-induced leukemogenesis are still only partially understood as of 2011. This made of HTLV-1 the first human onco-retrovirus ever discovered. Interestingly, HTLV-1 differs from most other known oncoretroviruses in that it does not encode any homologue of cellular proto-oncogenes. (Jeang et al, 1990).

HTLV-1 is also the etiological agent of a chronic progressive neuromyelopathy, tropical spastic paraparesis (TSP)/HTLV-1-associated myelopathy (HAM) (Yoshida et al, 1984). At the present estimations show that up to 2 million people are infected by HTLV-1 in Japan

(which is one of the endemic sources of this virus) and approximately 10 – 15 million worldwide (Proietti et al, 2005) (Fig.2).

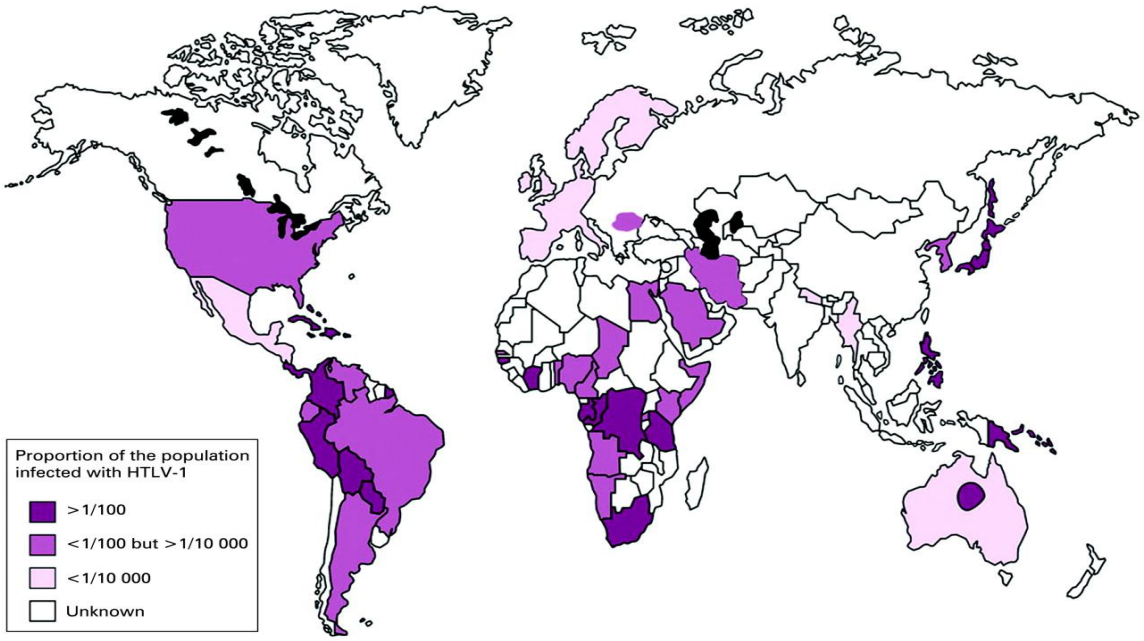

Figure 2. Worldwide distribution of HTLV-I infection. The map specifies the prevalence by region. Colours indicate current prevalence estimates based on population surveys and on studies in pregnant women and blood donors. In some countries, HTLV-1 infection is limited to certain population groups or areas. Changed from: (Verdonck et al, 2007).

Importantly, the vast majority of individuals infected with HTLV-I do not develop any disease: the virus remains in the host throughout its life without causing any harm at all. Only a minority will develop a disease due to HTLV-I but this usually occurs only after several decades of infection. As mentioned briefly above two main types of diseases are caused by HTLV-I:

- Adult T-cell Leukaemia/Lymphoma (ATL) (5% of infected patients, more often males then females).
- tropical spastic paraparesis (TSP)/HTLV-1-associated myelopathy (HAM) (1% of infected patients).

It is extremely rare for one individual to develop both diseases simultaneously. HTLV-I can also cause inflammation of the eye (uveitis), joints (arthritis), muscles (myositis), lung (alveolitis) and skin (dermatitis). These conditions are even less common than ATL and HAM and the skin condition is usually only seen in tropical climates.

1.1.1 ATL

ATL results from monoclonal proliferation of transformed CD4+ T-lymphocytes which possess particular, multi-lobulated ("flower cells") nuclei with condensed chromatin. The individuals present disease-associated symptoms including: hypercalcemia, skin lesions, hepatosplenomegaly and opportunistic infections. ATL patients poorly respond to chemotherapy. ATL appears in four types: acute-, lymphoma-, chronic- and smouldering-type. In general the prognostic is a five-year survival rate of 20%. Recently an antiviral therapy has been shown to significantly increase this survival rate in the early stage patients. In line with the long latency period it is estimated that ATL development involves several phases (Fig.3).

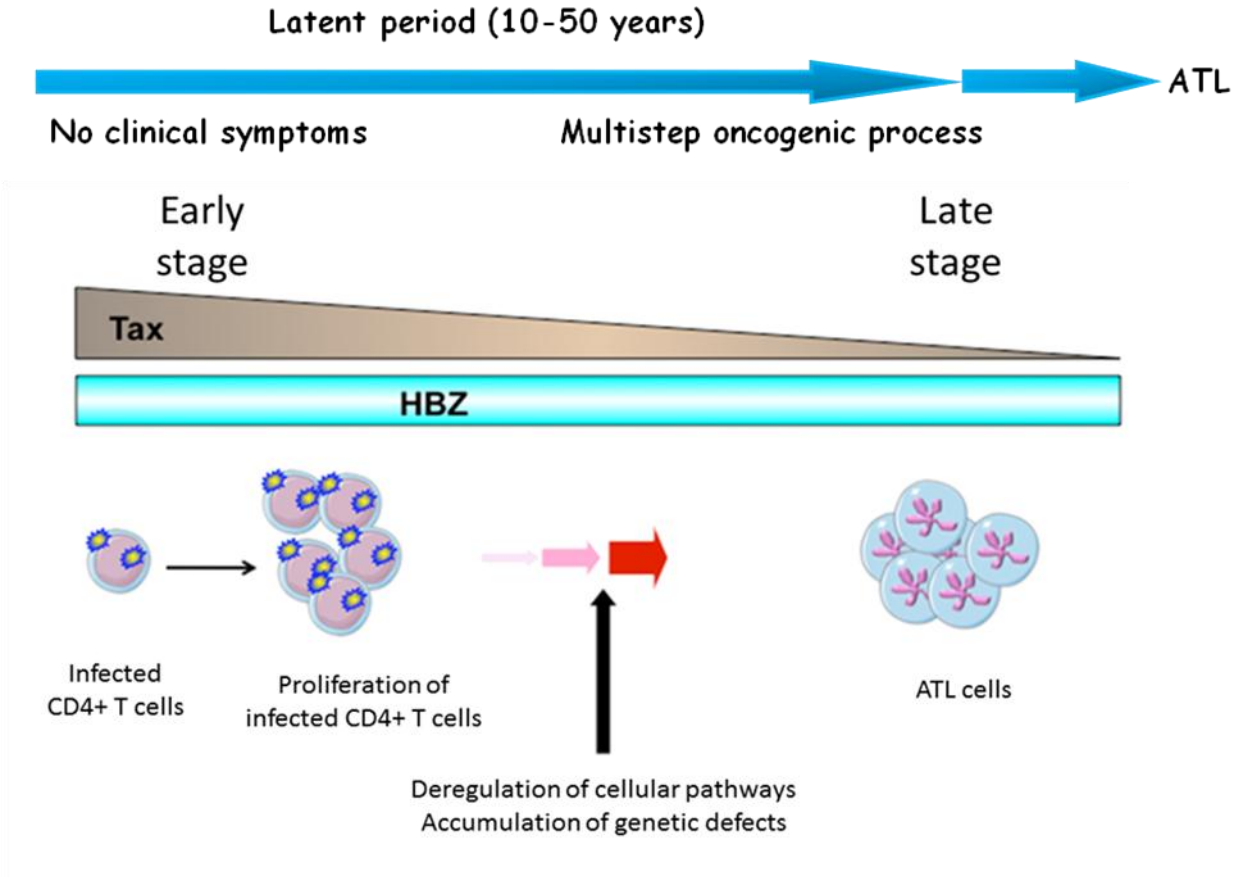

Figure 3. Simplified schematic model showing events from infection with HTLV1 to onset of ATL. The scheme represents the hypothetical flow of events occurring between the initial infection with HTLV-1 and the onset of ATL.
After a long latency period, ATL develops in about 5% of asymptomatic carriers. Tax expression is suppressed by several mechanisms, suggesting that Tax is not necessary at this stage. Although the occurrence of ATL is limited in patients infected with HTLV-1, ATL remains an aggressive form of adult leukaemia with no actual effective treatment

1.1.2 TSP/HTLV-1-associated myelopathy (HAM)

The TSP/HAM is a neurodegenerative disease resulting in chronic inflammation, which affects the central neuronal system (spinal cord). It is characterized clinically by paraparesis associated with spasticity and hyper-reflexia (Höllsberg & Hafler, 1993; McFarlin & Blattner, 1991). Although there is no cure for HAM/TSP a number of treatments are available. There are two approaches to treatment: treatment for the symptoms (e.g. pain or stiffness) and treatment of the cause (i.e. the inflammation in the spinal cord).

1.2 The HTLV-1 particle and genome.

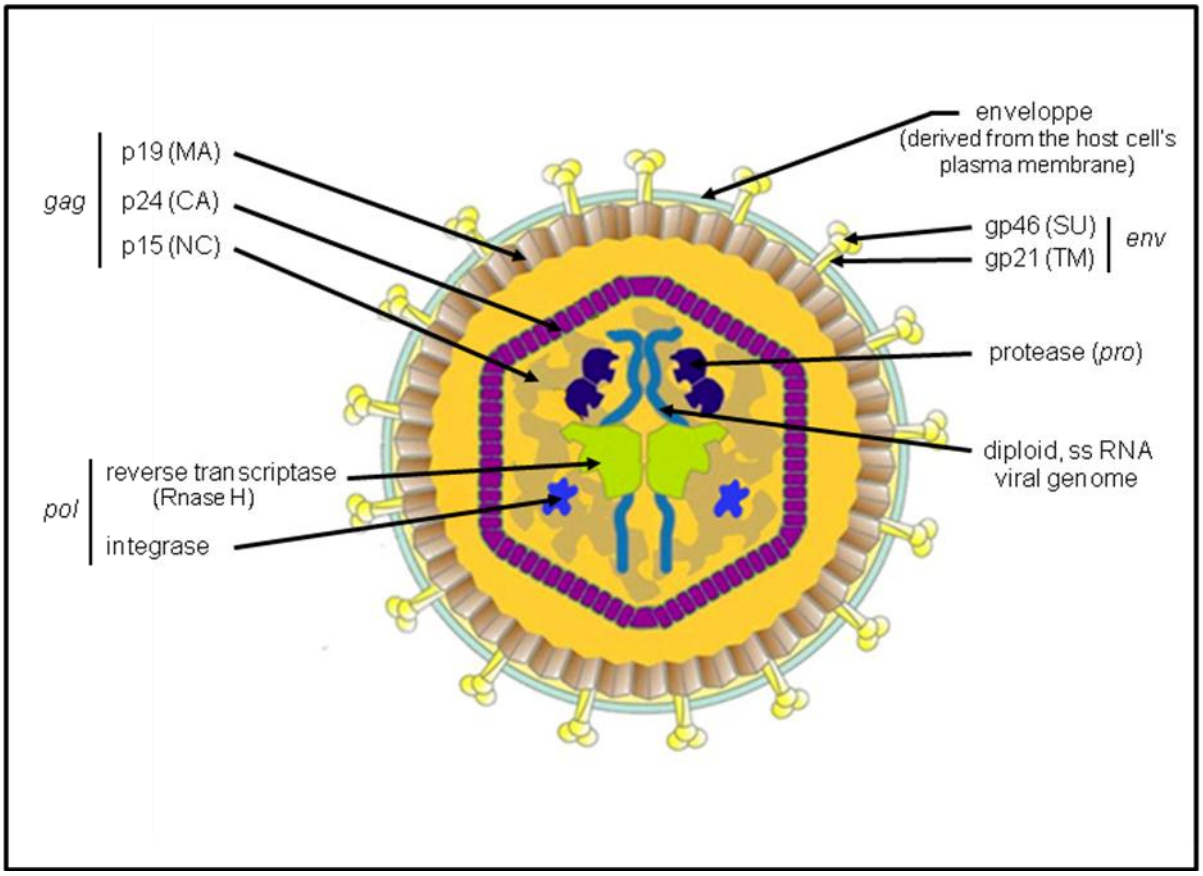

Figure 4. The HTLV viral particle and its structural components. Mature HTLV viral particles consist of a lipidic layer in which are inserted SU and TM, a protein shell composed of MA and the viral core composed of the viral RNA genomic (packaged as a dimer) surrounded by the CA protein. NC, RT, IN are also common components of the viral core.

HTLV-1 presents enveloped virions (Fig. 4) containing a nucleocapsid which protects the viral genome. The typical HTLV-1 particle is between 110 and 140 nm in diameter and contains two copies of a 9 kb ssRNA genome located in a protein capsid. As for most other retroviruses, the HTLV-1 genome contains *gag*, *pol* and *env* genes surrounded by two **L**ong **T**erminal **R**epeat (LTR) sequences (Fig.5). The 5′ LTR functions as the viral promoter. *Env* codes for a 21 kDa transmembrane protein (TM) and a 46 kDa membrane surface glycoprotein (SU). Both *pol* and *gag* encode the so-called structural proteins of the virus. The *pol* gene codes for the reverse transcriptase (RT), integrase (IN) and protease (PR, responsible for the cleavage of HTLV-I structural proteins) enzymes. *Gag* codes for a poly-protein which is cleaved after translation within the Golgi apparatus by PR into the matrix (MA), capsid (CA) and nucleocapsid (NC) structural proteins. The space between the *env* gene and the 3'LTR also contains the "pX" region, which contains four partially overlapping ORFs coding for regulatory factors (the misnamed "accessory" proteins): Tax, Rex, p12, p13, p30, p21 and HBZ on the minus strand of pX (Cavanagh et al, 2006; Gaudray et al, 2002; Matsuoka & Green, 2009; Satou et al, 2006).

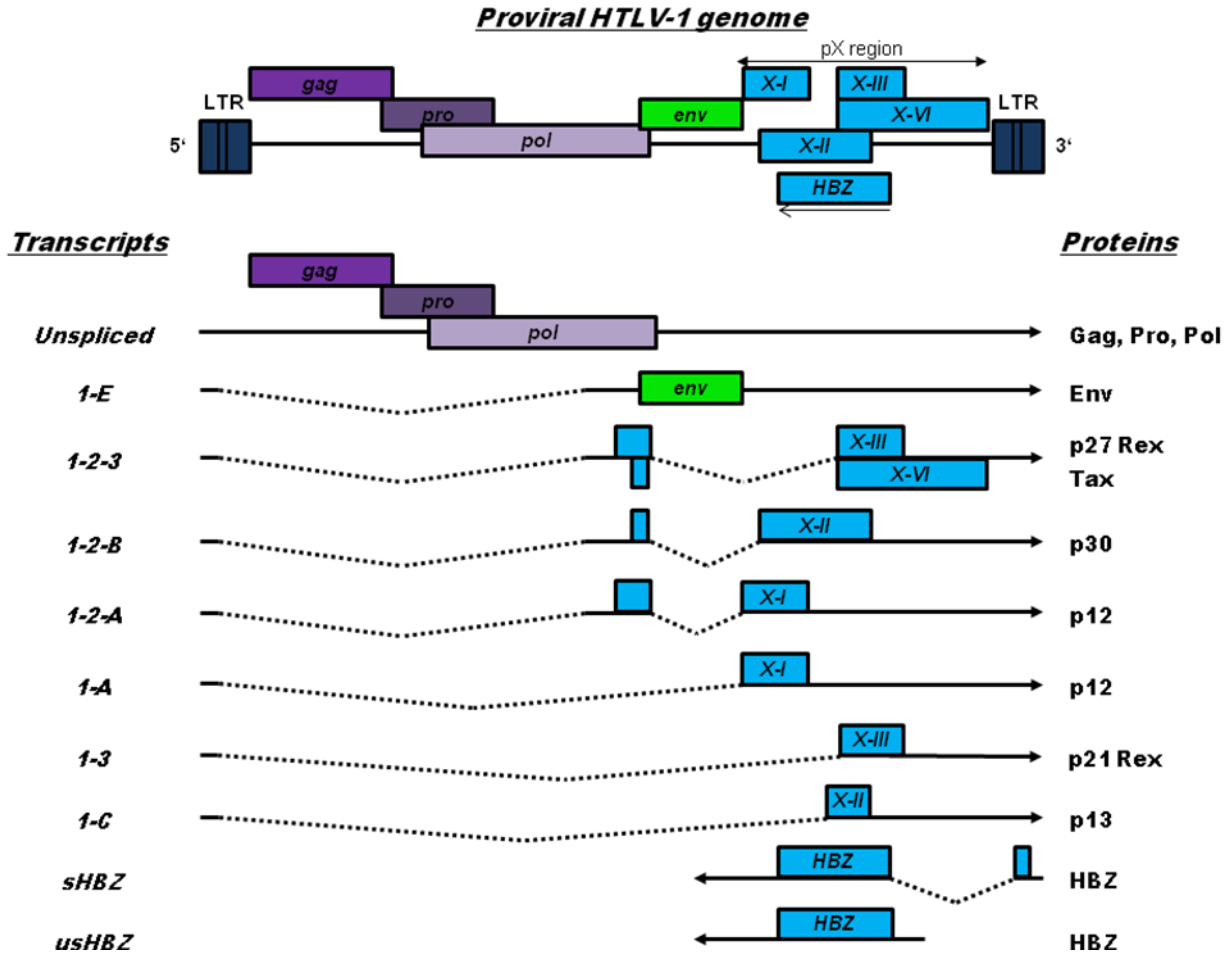

Figure 5. Organization of the HTLV-1 proviral genome. A scheme of the HTLV-1 genome, alternatively spliced mRNAs, and putative proteins encoded by each mRNA is shown. ORFs are indicated by boxes and annotated with roman numerals. The *gag*, *pol*, and *env* structural genes are flanked by 5' and 3' LTRs. In the 3' portion of the genome is a pX region that encodes the Tax, Rex, p21, p12, p13 and p30 proteins in its various open reading frames. HBZ is encoded by a negative strand RNA derived from the 3' LTR.

1.3. HTLV-1 transmission.

HTLV-1 is usually transmitted from mother to infant (breast feeding), by sexual intercourse or by blood transfusion. All three of these methods require cell to cell contact, which means that transmission of a free viral particle from the infected individual to a virus-free individual is not enough: an infected cell must be transmitted for contamination to occur. The contact between the infected and the uninfected cells is established by the formation of the so-called "viralogical synapse" (Fig.6), which is formed at the cell-cell junction by the **M**icro**t**ubule-**O**rganizing **C**enter (MTOC), and where the viral genomic RNA and the Gag complex accumulates. The formation of the synapse is further stimulated by Tax. Three HTLV-1 receptors are known to be important for HTLV-1 entry: the **G**lucose **T**ransporter 1 (Glut1),

the **H**eparan **S**ulfate **P**roteoglycans and the **N**europilin-1 (Jones et al, 2005; Lambert et al, 2009; Manel et al, 2003).

HTLV-1 inserts randomly within the host's genome. Due to an active immune response targeting the virus, proliferation of infected T-lymphocytes mainly occurs by cellular division. It has been shown that after the initial polyclonal stage this leads to an oligoclonal phase which eventually results in selection of a single clone with highly invasive properties. Thus the main proviral load is due to clonal expansion of the virus rather than to *de novo* spread.

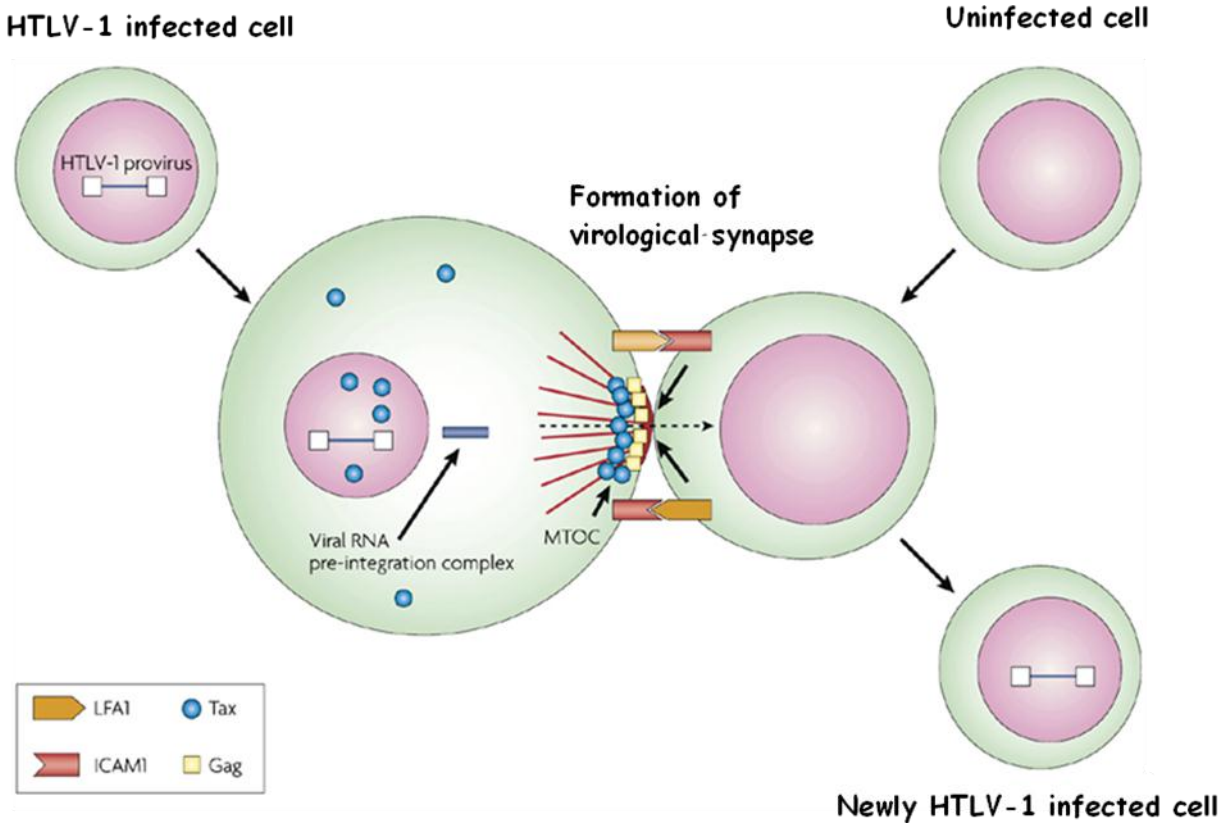

Figure 6. Formation of the viral synapse. Unlike many other viruses, cell-free human T-cell leukaemia virus type 1 (HTLV-1) virions are largely non-infectious. This figure illustrates the cell–cell contact required to create a virological synapse through which the viral genome is transmitted from one cell to another. The roles played by lymphocyte function-associated antigen 1 (LFA1), and intercellular adhesion molecule 1 (ICAM1) in forming cell–cell contact are shown. Tax contributes to the formation of a microtubule organizing centre (MTOC). Adapted from: (Matsuoka & Jeang, 2007)

1.4. The life cycle of HTLV-1.

HTLV-1 has been shown to be able to successfully infect a multitude of cell types, including T and B cells, macrophages and fibroblasts (Jones et al, 2008; Koyanagi et al, 1993). The life cycle of HTLV-I is divided in two main parts. The first includes (i) viral entry, (ii) reverse

transcription of the viral RNA genome into DNA, (iii) nuclear import of the proviral DNA genome, and (iv) integration of the proviral DNA into the host's genome to form a provirus. The second includes (i) viral gene transcription and (ii) protein synthesis necessary for the viral gene expression and virion assembly (Fig.7.).

The initial step of HTLV-1 invasion is the infection of a great number of lymphocytes by viral particles leading to the integration of the provirus and synthesis of viral proteins. HTLV-1 oncogenic progression is extremely slow, and one of the best studied HTLV-1 factors thought to be involved in the process is the viral transactivator protein Tax (the particularities of this protein will be detailed later). Tax has been shown to be necessary and sufficient to transform mice fibroblasts (Tanaka et al, 1990) or to immortalize primary human T lymphocytes (Grassmann et al, 1992; Grassmann et al, 1989). Moreover, it has been reported that transgenic animals treated with a Tax expression vector develop tumours. Experiments with transgenic mice that express the HTLV-1 Tax oncoprotein in thymocytes, even led to the development of lymphoma-like pathologies (Hasegawa et al, 2006). These observations indicate that Tax is likely responsible for at least part of the oncogenic process initiated by HTLV-1 and suggest that studying this protein could be crucial for understanding (and eventually curing) ATL.

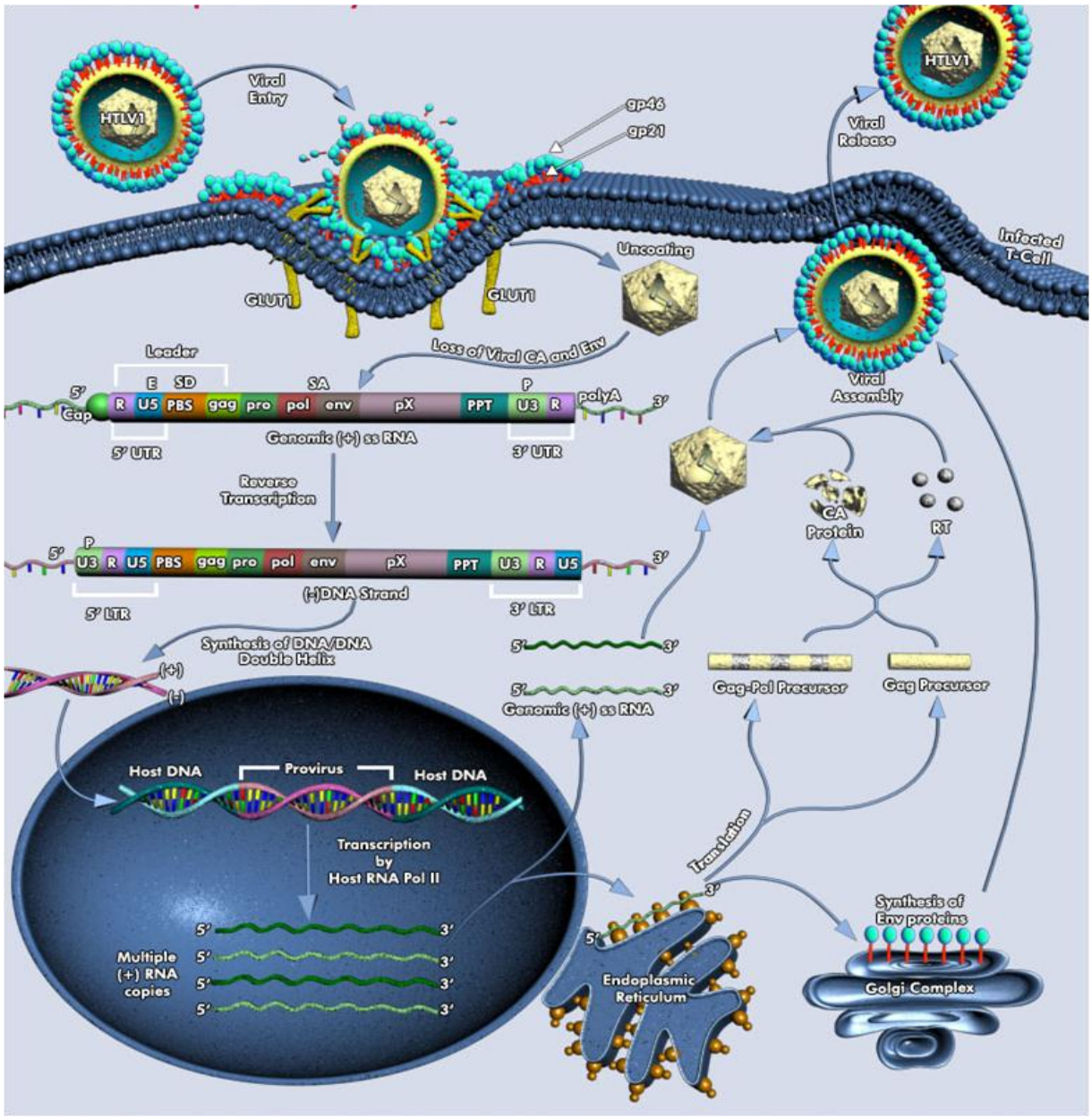

Figure 7. Viral replication in the host and virion formation. HTLV-1 contains the diploid ssRNA and several copies of reverse transcriptase (DNA polymerase). After infecting a cell, the reverse transcriptase is used to make the initial copies of viral DNA from viral RNA. Once a DNA strand has been synthesized, a complementary viral DNA strand is made. These double strand copies of viral DNA are inserted into the host-cell chromosome and host-cell RNA polymerase is used to make virus-related RNA. These RNA strands serve as templates for making new copies of the viral chromosomal RNA and serve also as mRNA. mRNA is translated into viral proteins that are used to make the virus envelope. New viral particles are assembled, bud from the plasma membrane, and are released. Adapted from Qiagen.

1.5 HTLV-1 Accessory proteins.

Under the control of the promoter located in the 5'LTR, HTLV-1 encodes several regulatory proteins that are responsible for HTLV-1 transactivation (Tax / Rex). Another promoter is placed in the 3'LTR of the viral genome and enhances the antisense transcription of the *hbz* gene coding for the **H**TLV-1 **B**asic leucine **Z**ipper (HBZ). This protein is essential for

maintenance of malignant features of HTLV-1 infected T cells. For a long time the proteins p12, p13, p30, p21 were termed "accessory" and known as non-essential.

1.5.1 p12

p12 has been reported to associate with the Golgi Apparatus and Endoplasmic Reticulum and to increase the levels of intracellular Ca^{2+}, thus stimulating cell proliferation and survival. HTLV-1 p12 also participates in viral evasion of the host's immune response, in all likelihood as a result of its associating with immature forms of the **M**ajor **H**istocompatibility **C**omplex class **I** (MHC I). Indeed, p12 has been shown to decrease the surface levels of transfected MHC-I by inducing its degradation by the proteasome (Bindhu et al, 2004; Van Prooyen et al, 2010)

1.5.2 p13

p13 associates with the inner membrane of the mitochondria. Similarly to p12 it modulates the Ca^{2+} homeostasis which might have an impact on lymphocyte T activation via NFAT (Biasiotto et al, 2010). In addition p13 provokes K^{+} entry into mitochondria, which results in membrane depolarisation. The massive changes in ion charge this induces favour the production of **R**eactive **O**xygen **S**pecies (ROS). It has been proposed that p13 might exert distinct functions depending on its intracellular localization. In the nucleus p13 binds Tax and inhibits its transcriptional activity which could lead to turnover of infected cells and the balance between viral latency and productive infection (Silic-Benussi et al, 2010).

1.5.3 P30

The p30 protein acts as a negative regulator. p30 restrains the export of the *tax*/*rex* RNAs which results in slower Tax/Rex protein synthesis and inhibits the viral transcription and general RNA export. Lower Tax/Rex expression results in a decrease of viral particle production. This makes p30 a protein responsible for the induction of a viral latent period within the infected cells (Nicot et al, 2004).

1.5.4 REX

Synthesized from a multi-spliced viral mRNA (ORF III), this 21 kDa protein is responsible for repression of splicing of viral RNA and promotes their nuclear export. It is expressed during the early phase of infection and governs the production of essential proteins during the

late phase of infection. *In vivo* studies demonstrated that Rex is essential for virion production. Rex binds to the **Rex R**esponse **E**lement (RxRE) and coordinates the cytoplasmic accumulation of spliced as well as unspliced forms of the viral mRNAs. Rex synthesis has been shown to be required for viral replication to initiate. Thus Rex is a post-transcriptional regulator and is pivotal for the control of viral latency and gene expression.

1.5.5HBZ

One recently identified HTLV-1 proteins is the **H**TLV-1 **bZ**IP Factor (HBZ). This 30kDa (209 AA) protein contains a bZIP domain in its C-terminal part and a transcription activator domain in its N-terminal part. HBZ is encoded by the antisense strand of the viral RNA and is transcribed from the 3'LTR. It has been found to interact with the factors JunB, JunD, CREB and CBP/p300 to modulate gene transcription. HBZ down regulates Tax functions, which implies an additional level of complexity in the regulation of HTLV-1 gene expression. HBZ hinders the interaction between Tax and CREB on the 5'LTR thereby blocking the recruitment of CBP/p300 on this promoter. In consequence the transcription of other viral genes is stopped. The early phase of infection is characterized by the expression of the genes under the control of the 5'LTR promoter. Proteins participate in infectious virion formation. The natural immune response is directed against Gag, Env and Tax, the outcome being the removal of infected cells. Since HBZ downregulates the synthesis of Gag, Env and Tax, it circumvents the immune response of the host. Of all the viral genes, only the expression of the antisense factor *hbz* is clearly detectable at all stages of the infection at the RNA level (Satou et al, 2006; Suemori et al, 2009). The current hypothesis is that the *hbz* gene has a dual functionality: *hbz* mRNA promotes T-cell proliferation, while the HBZ protein suppresses Tax-mediated viral transcription.

1.5.6 TAX

One of the most studied "accessory proteins" of HTLV-1 is the oncoprotein Tax (Grassmann et al, 2005). Tax (encoded by the ORFIV in the pX region) associates with the **T**ax **R**esponsive **E**lement (TRE or TxRE), an imperfectly conserved (between HTLV subtypes) 21-base-pair repeat sequence containing an octamer motif TGACG(T/A)(C/G)(T/A'). Association of Tax with the TRE stimulates the expression of the HTLV-1 provirus.

Initial studies showed that Tax a 40 kDa (353 AA residues) protein is localized preferentially in the cell nucleus, but it is currently admitted that Tax is able to shuttle between the nucleus

and the cytoplasm (Kashanchi & Brady, 2005). It has been demonstrated that Tax associates with numerous proteins in special Tax-bodies or nuclear speckles (Semmes & Jeang, 1996) and even to localize to centrosomes. The localization and so the interactions are highly dynamic and undergo rapid changes under specific conditions such as stress.

Tax is the first protein expressed after HTLV-1 infection. Its importance seems to be restricted to the initial steps of cellular transformation. Tax presents pleiotropic activities which deregulate numerous cellular pathways. It is known to activate important transcription factors like **N**uclear **F**actor-**K**appa **B** (NF-κB), **A**ctivator **P**rotein **1** (AP-1), **S**erum **R**esponsive **F**actor (SRF) and **c**yclic-AMP-**R**esponse **E**lement-**B**inding protein (CREB) (Li & Gaynor, 1999; Sun & Yamaoka, 2005; Winter & Marriott, 2007). Through these interactions Tax induces aberrant cellular proliferation and genomic instability and inhibits DNA damage repair and apoptosis. Notably all these pathways remain active in ATL cells. At late stages of ATL Tax expression is barely detectable. This means that others factors or cellular events intervene in late ATL stages. Another very important viral protein whose RNAs expression is clearly detectable at this stage is HBZ (described above).

1.5.6.1 Tax interactome summary and pathways deregulated.

It is difficult to make an exhaustive and accurate list of all pathways on which Tax is known or thought to have an impact, but this multiplicity in itself suggests that the protein could be interacting with many different partners. Indeed, a recent review describes around 100 interaction partners for Tax (Boxus et al, 2008). Tax presents several features of a multiple interaction protein such as presence in both the cytoplasm and the nucleus, a poorly structured tridimensional conformation and susceptibility to multiple posttranscriptional modifications – notably phosphorylation, acetylation, ubiquitinylation and SUMOylation (Boxus et al, 2008). Many of the known interactions between Tax and cellular factors are high affinity, suggesting that these interactions play an important role in the viral life cycle. However, it should be noted that because many of these interactions have been described *in vitro* in absence of other HTLV-1 proteins, some of them may not actually occur during wt HTLV-1 infection, as other viral partners of Tax or the virus-induced modifications of the cell may prevent their occurrence. As for the many interactions which have been validated *in vivo*, a study of their relevance to the viral life cycle in the context of a living organism is often lacking and would present a great interest. In this respect, a recent work opens interesting possibilities by proposing a new tool which mimics HTLV-1 infection in a non-human model: the “Human

Immune System" (HIS) Rag2$^{-/-}\gamma_c^{-/-}$ mouse model (Villaudy et al, 2011). This animal model is derived from newborn mice by intrahepatic transplantation of human CD34^{+} cord blood cells, which results in the development of the major functional components of the human adaptive immune system in the host mouse (Chicha et al, 2005). Analysis of the partners of Tax in this model would give a more physiological picture of the real Tax interactome and thus yield more precise insights in the complex way Tax acts upon a cell.

In this manuscript I will concentrate on the subset of factors and/or pathways known or thought to be targeted by Tax that are the most relevant to our study of the links between Tax and the NMD pathway (Table 1). Tax acts as a stimulator of proliferation, but also as a mutagen and as an activator of genomic instability. In fact, Tax deregulates directly or indirectly (by associating with proteins involved in post-transcriptional control) the expression of more than one hundred genes (reviewed in: (Boxus et al, 2008)).

Some of the main pathways activated by Tax are summarized in the table below:

Mechanism	Factor	Effect
Transcription	AP-1 CREB (CBP/p300) NF-κB, SRF, TBP	Increased cellular proliferation; impaired histone synthesis, octamer disassembly; chromatin transition
Cell-cycle progression	CDK2 and 4 Cyclin D2 and 3 E2F1, p16, p21/WAF1	Accelerated G1-S phase progression and "hyper-replication"
Chromatin remodelling	SWI/SNF family	Activation of specific cellular promoters
Cellular checkpoint and DNA repair	CHK1 and 2 DNA Topoisomerase I Ku80, MAD1, Rad51,p53 Telomerase	Genomic instability, aneuploidy
Signal transduction, regulation	- Nuclear receptors - SMAD1 - ARE-containing genes (TNFα, GM-CSF) - TTP	- Inhibition of the transcription of target genes - Cellular resistance to TGFβ; interaction with immune surveillance; stimulation of expression of proteins involved in viral binding and fusion (Glut1) - Inhibition of cell proliferation, differentiation and death, immune response - Stabilization of TNFα mRNA favouring cell transformation

Table.1 Some of the interaction partners of Tax and the effect of the interaction on cellular biology.

One particular interaction partner is of high interest to me: it has been demonstrated via a two hybrid screen that Tax interacts with the e subunit of the eIF3 translation complex, a protein also termed INT6. So far no function has been accredited to this interaction (Desbois et al, 1996).

Chapter 2 INT6/EIF3E

2. Integration Site 6, the gene (*int6*) and the protein (INT6/eIF3E).

The gene *int6*, coding for the INT6 protein, was first identified in 1995. Having then been independently re-discovered by several groups over the years, the gene is also known as *EIF3E, INT6, EIF3S6, EIF3-P48, eIF3-p46, LOC3646* or *moyshoy, PCI.3*.

The *int6* gene is located on chromosome 8, spans a respectable 254 kb and contains 25 distinct introns. The *int6* ("Integration site 6 ") gene has been conserved during evolution from yeast to mammals, which points it out as an essential cellular factor. *Int6* codes for a 52 kDa proto-oncoprotein ubiquitously present in the cell. In 1997 INT6 was identified to correspond to the "e" subunit of the eIF3 initiation factor (Asano et al, 1997b). This shuffling protein holds a **N**uclear **E**xport **S**equence (NES) as well as a **N**uclear **L**ocalisation **S**ignal (NLS) (Guo & Sen, 2000) (Fig.8). In mammals, *int6* is widely expressed. A transcript of about 1.6 kb is found in the brain, lung, heart, liver, spleen, pancreas, skeletal muscle, mammary glands, lymph nodes, and thymus (Desbois et al, 1996). However Northern Blot analysis revealed variable expression levels. The highest has been found in pancreas, muscles, and lymphoid tissues.

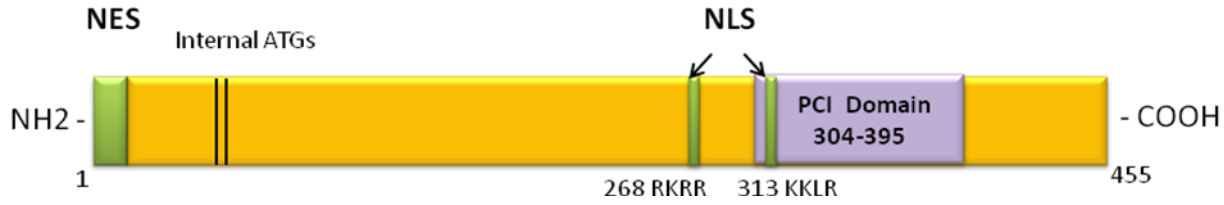

Figure 8. INT6 protein functional domains. This 445 AA protein has a Nuclear Export Signal on it N-terminal, internal ATGs (denoting alternative start sites located approximately 40 amino acids downstream of the predominant start site); NLS, bipartite Nuclear Localization Sequences (at amino acid 268 and 310); PCI domain (proteasome, COP9 signalosome, initiation factor 3/proteasome subunits).

2.1 Identifiction of INT6

In the 90ties of the last century three independent studies have led to the identification of INT6:

- The *int6* gene was demonstrated to be a frequent site of **M**ouse Mammary **T**umor **V**irus (MMTV) integration (Marchetti et al, 1995; Miyazaki et al, 1995).
- The human cDNA coding for INT6 was isolated in a two-hybrid screen using the Tax protein of HTLV- 1 as bait (Desbois et al, 1996).
- The INT6 protein was characterized as a subunit of translation initiation factor eIF3 (Asano et al, 1997b).

2.2 MMTV and the *int6* genes.

The **M**ouse **M**ammary **T**umor **V**irus **(**MMTV) is a retrovirus that induces carcinomas in mice. Unlike most transforming retroviruses, it causes malignant transformation by insertion and clonal expansion of cells, rather than by directly encoding an oncogene. MMTV (similarly to avian leukosis virus (ALV) and murine leukemia virus (MLV)) elicits tumorigenesis trough insertional mutagenesis. This means that tumour formation caused by these viruses depends foremost on the host cellular proto-oncogenes, mutated as a consequence of proviral integration. Viral integration into the *int6* gene occurs at any of several introns and leads to the synthesis of truncated mRNAs, expression of a truncated form of INT6 and tumour development in mice (Marchetti et al, 1995). Both loss of expression of the modified allele or production of shortened forms of the protein can deregulate cell growth.

In MMTV infected mice six different loci of *int* genes have been identified. The name "*int"* for "integration site" mirrors the preferences of MMTV to integrate within these genes. Most of them, like the: int1/wnt1, wnt3, wnt 10B, int2/fgf3, fgf4, int3/notch and int6 have been identified as affected while mammary tumours occurred (Tekmal & Keshava, 1997).

In this manuscript I will focus on the *int6* (Integration site 6) gene, the sixth member of the int genes family which has been demonstrated to be a frequent site were the MMTV integrates. The integration of the provirus has been studied in preneoplastic and neoplastic mammary lesions.

2.3 INT6 as a subunit of eIF3.

The eukaryotic translation initiation factor eIF3 consists of 6 proteins in *Saccharomyces cerevisiae* and 13 protein subunits in human cells – consecutively named eIF3a - l)(Browning

et al, 2001; Zhou et al, 2008). With a weight of around 800kDa it is the largest initiation factor in mammals. The eIF3 directs the interactions between mRNA the 40S ribosomal subunit and initiator Met-tRNA, promotes translation re-initiation and functions as a platform for interactions with other regulatory eIFs (Hinnebusch, 2006). INT6 has been shown to be an eIF3 component which is dispensable for general translation and forms together with subunits a, b, c, f, and h the functional core of eIF3. From fission yeast studies it is known that INT6 mutants present a very slight inhibition of the translation rate and a poor decrease in global polysome content (Akiyoshi et al, 2001; Bandyopadhyay et al, 2000; Zhou et al, 2005). Intriguingly a study reported the presence of two distinct eIF3 complexes in fission yeast. One of those, defined by the Csn7Bp/eIF3m subunit, seems to be responsible for the translation of an important part of mRNAs, whereas the other, containing INT6, is associated with the regulation of a more restricted mRNA subset (Zhou et al, 2005). Thus in contrast to other eIF3 subunits, INT6 is not essential for global translation but seems to regulate the translation of specific classes of mRNAs. INT6 was reported to act positively or negatively on the presence of several mRNAs in polysomes. (Grzmil et al, 2010). INT6 positively regulates a group of mRNAs encoding proteins involved in coagulation, taxis and endocytosis, and negatively regulates genes controlling cell division and adhesion.

2.4 HTLV-1 Tax INT6.

Human INT6 has been identified in our laboratory as an interacting partner of the **H**uman **T**-cell **L**eukemia **V**irus type **1** (HTLV-1) protein Tax in a two-hybrid screen (Desbois et al, 1996). A cDNA library of immortalized human B lymphocytes transformed by the Epstein-Barr Virus was screened using the entire Tax protein as bait and led to the isolation of human INT6. The interaction between Tax and INT6 was further confirmed by immunoprecipitation experiments and was confirmed to be strong and specific. The complex between both proteins was found to be cytoplasmic, whereas in normal cells INT6 is present both in the cytoplasm and the nucleus (Desbois et al, 1996; Watkins & Norbury, 2004b). INT6 has been demonstrated to be a component of specific nuclear foci called ND10 (nuclear domain 10), or PML bodies after their defining component, the **P**romyelocytic **L**eukemia **P**rotein (PML). Between 5 and 50 of these bodies are found in different cell types, and they have been proposed to participate – among other roles – in tumour suppression. Interestingly localisation of Tax to PML bodies is lost in Tax-transfected cells (Desbois et al, 1996).

2.5 INT6 in the NMD.

Recent studies in our laboratory led to the demonstration that INT6 interacts with UPF2, UPF1 and CBP80, three important Nonsense mRNA Mediated Decay (NMD) players, and revealed INT6 as a new NMD-factor (Morris et al, 2007). NMD is a mRNA quality control mechanism which leads to degradation of mRNAs which include a **P**remature **T**ermination Codon (PTC) and necessitates a first translation of the mRNA (reviewed in: (Chang et al, 2007; Ishigaki et al, 2001; Muhlemann, 2008))

The NMD mechanism will be described in details in chapter 3 of this manuscript.

2.6 INT6 and the proteasome.

Other studies identified INT6 as a component of the 26S Proteasome-COP9 signalosome. (Hoareau Alves et al, 2002; Karniol et al, 1998; Yahalom et al, 2001; Yen & Chang, 2003).

INT6 contains a **P**roteasome-**C**OP9 (constitutive photomorphogenesis 9, (Lee et al, 2011)) signalosome (CSN)-**I**nitiation of translation (PCI) domain (Hofmann & Bucher, 1998). Co-purification studies performed in diverse species (*Arabidopsis thaliana*, human) indicate that INT6 associates with several subunits of the CSN. The CSN a target for kinase activity and has been to coordinate activity of kinases (Harari-Steinberg & Chamovitz, 2004). It is involved in regulation of the degradation of cellular proteins in mammals and in photomorphogenesis in plants. Intriguingly the truncated INT6 proteins, induced by MMTV integration, (INT6ΔC) lack the PCI domain, suggesting that its loss disrupts INT6 functions. It is probable that INT6 plays a role of a regulatory protein to coordinate activity of all three complexes (Proteasome – CSN – eIF3).

Such a domain exists in several subunits of the lid of the 19S proteasome regulatory particle, of the CSN and of eIF3 (Hoareau Alves et al, 2002; Karniol et al, 1998; Yahalom et al, 2001). PCI-domain bearing subunits of the 19S proteasome, CSN are involved in protein degradation, SCF E3 ubiquitin ligase regulation and mRNA translation, respectively. It has been hypothesized that they may play a scaffold role (Pick et al, 2009). The 26S proteasome degrades poly-ubiquitinylated cellular proteins (Baumeister et al, 1998). It contains two main parts: the 19S regulatory complex and the 20S core complex. INT6 binds to the Rpt4 protein which is one of the six AAAs forming the base of the 19S regulatory particle. These ATPases are believed to play an important role by unfolding the protein prior to degradation by the 20S core proteasome subunits.

2.7 Other INT6-interaction factors

INT6 has been identified in other numerous studies summarized below (Table 2). Some of them are associated with specific functions in the cell, whilst others need to be explored.

Mechanism/Pathway	Interaction Factors	INT6 Function/Effect	References
INT6			
NMD	UPF1, UPF2	Stimulation of mRNA decay.	(Morris et al, 2007)
NMD	CBP80	Pioneer translation round	(Morris et al, 2007)
DNA damage and replication control	MCM7	Regulation of MCM7 stability during S phase.	(Buchsbaum et al, 2007)
Degradation of cellular proteins	CSN: CSN3, CSN6 and CSN7a	Not Defined	(Hoareau Alves et al, 2002)
Polyubiqutinilation (degrades poly-ubiquitinylated cellular proteins)	26S Proteasome: Rpt4 in the 19S HC3 in the 20S	INT6 stabilizes the complex	(Asano et al, 1997a; Hoareau Alves et al, 2002; Karniol & Chamovitz, 2000; Yen & Chang, 2003; Zhou et al, 2005)
HIF2α dependent angiogenesis	HIF2α	Degradation	(Chen et al, 2007)
RING-finger domain, thus it is a presumed E3 ligase for polyubiquitination.	Rfp (RING-finger protein)	PML bodies localization Degradation E3 ligase	(Desbois et al, 1996; Hoareau Alves et al, 2002)
Translation initiation (Formation of the 43S PIC)	EIF4G-1	eIF4G binds to eIF3 through INT6	(LeFebvre et al, 2006; Morris-Desbois et al, 2001)
Translation initiation (Further studies required)	HSPC021 eIF3-associated protein that can be phosphorylated on tyrosine	Directly interacts with Int-6.	(Morris-Desbois et al, 2001)

Table 2. Overview of the many interactions and roles of INT6 in mammalian cells.

2.8. INT6 in tumorigenesis: a double edged protein?

Different subunits of the eIF3 complex have been described to participate in tumour development and malignant transformation. Evidence comes from experiments performed in immortal fibroblasts. The expression of any of the following five subunits of eIF3: a, b, c, h or i, resulted in malignant transformation (Ahlemann et al, 2006; Savinainen et al, 2006; Zhang et al, 2009). The implication of INT6 (EIF3E) in tumorigenesis is more complex. Various studies suggested contradictory roles of INT6 in either tumour suppression or oncogenesis (Buttitta et al, 2005; Chen et al, 2007; Marchetti et al, 2001b; Rasmussen et al, 2001).

The expression of INT6 appears to be altered in human cancers. INT6 levels are downregulated in 27% of non-small cell lung cancer, (Buttitta et al, 2005) and in 30% of human breast cancer in both the tumours and in the stroma surrounding the tumour (Finak et al, 2008; Marchetti et al, 2001a; Umar et al, 2009; van 't Veer et al, 2002). However it is difficult to study more deeply the role of INT6 disruption during tumorigenesis since a knock-down animal model does not exist: attempts have been done for both Drosophila and mice and both these organisms presented a lethal phenotype (Rencus-Lazar et al, 2008).

MMTV insertions into one or more introns of the *int6* gene result in synthesis of a C-terminally truncated INT6 protein (Int6ΔC). Its synthesis in mouse mammary glands has been shown to induce hyperplasia and tumour formation (Mack et al, 2007). Furthermore, Int6ΔC can transform human cells in tissue culture, and these transformed cells produce tumours when injected into immunodeficient mice (Mack et al, 2007; Mayeur & Hershey, 2002; Rasmussen et al, 2001).

The oncogenic effect of INT6 has been reported although the mechanisms underlying its involvement in tumorigenesis remain obscure. Recent data suggest that INT6 plays a direct role in the promotion of breast cancer cell proliferation and invasion. The authors have shown that overexpression of INT6 acts on one hand as an enhancer of the translation of mRNAs coding for factors involved in cancer growth (mRNAs encoding proteins involved in coagulation) invasion (taxis and endocytosis) and apoptosis inhibition, and on the other hand as an inhibitor of the translation of mRNA coding for factors that control cell division and adhesion (Grzmil et al, 2010).

Chapter 3 Nonsense mRNA Mediated Decay

3. The quality control pathways in ekuaryotes.

After transcription messenger RNAs are likely to accumulate errors. Such fidelity mistakes can have fatal effects for single cells or the whole organism. It is thus no surprise that several surveillance mechanisms evolved in order to ensure the high reliability of mRNA molecules. Currently four basic mRNA surveillance pathways are known: the **N**onsense **M**ediated mRNA **D**ecay (NMD); the **N**onstop **M**ediated mRNA **D**ecay (NSD); the **N**o-**G**o Mediated mRNA **D**ecay (NGD); and the **R**ibosome **E**xtension-**M**ediated **D**ecay (REMD). In this manuscript I will focus on the most studied of these four mechanisms: the NMD. The NMD regulates mRNAs containing premature STOP codons (PTC). The NSD as it name indicates targets mRNAs with lacking stop codons (Frischmeyer et al, 2002). When mRNAs have strong pauses in elongation, and the ribosome remains stalled on the mRNA, it is targeted for endonucleolytic cleavage in a process referred to as No-Go decay (NGD) (Doma & Parker, 2006). Finally when ribosomes inappropriately translate and then terminate within the 3′ UTR at least some mRNAs are destabilized in a process referred to as ribosome extension-mediated decay (REMD, (Inada & Aiba, 2005)).

3.1. The Nonsense Mediated mRNA Decay.

The NMD pathway has been found in all organisms studied so far, starting from *Saccharomyces cerevisiae, Caenorhabditis elegans, Arabidopsis thaliana* to *Homo sapiens* (Amrani et al, 2004; Brogna, 1999; Gatfield et al, 2003; He et al, 1993; Le Hir et al, 2001b; Pulak & Anderson, 1993), for reviews, see (Hentze & Kulozik, 1999; Jacobson & Peltz, 1999; Maquat, 1995a; Maquat, 2004). The main function of NMD is to remove mRNAs that contain a **P**remature **T**ermination **C**odon (PTC) (Fig. 9). The need for a reliable mRNA proofreading mechanism seems to be obvious: fast degradation of aberrant mRNAs protects the cells from potentially harmful C-terminally truncated proteins that may function in a dominant-negative manner. It has been demonstrated that abolishment of the NMD in mice is lethal in an embryonic state (Medghalchi et al, 2001).

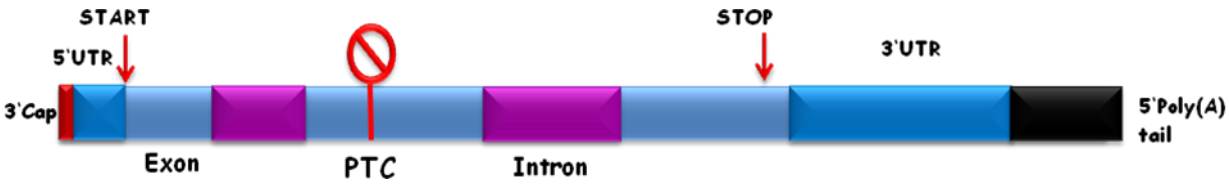

Figure 9. Scheme of a typical NMD target mRNA. The PTC is located in the second exon.

3.2. Sources of NMD substrates

NMD was originally discovered in studies of β-thalassemias where it was triggered by a PTC occurring at the position 39 of the β-globin gene. The mRNA encoded by the mutated β-globin allele was found to be subjected to rapid degradation. During the next 30 years of intense studies, the picture of NMD evolved a lot. From a "simple" surveillance pathway it changed to a complex and ubiquitous post-transcriptional, translation-dependent mechanism of gene expression regulation in eukaryotes. Indeed, the NMD pathway not only degrades PTC-containing mRNAs but also influences the expression of transgenes and of dozens of endogenous genes during development (we will see below that NMD can also affect mRNAs that do not contain PTCs) (Table 3.B.). Still abnormally spliced mRNAs are probably the most frequent NMD substrates in cells. Computational analysis of the human EST database showed that around 64% of human transcripts are alternatively spliced (AS), and about 45% of the transcripts generate at least one splice isoform encoding a PTC (Green et al, 2003) (Table 3. A.). Small insertions or deletions which cause frame-shifts, often also generate downstream PTCs in the coding region. In addition, PTCs can be generated by transcriptional errors and by abnormal pre-mRNA processing. Mutations that alter splicing signals generate nonsense mutations, frequently due to the retention of intronic sequences (Mendell & Dietz, 2001). The **Drosophila** NMD pathway has critical cellular and developmental roles beyond the classical surveillance function of eliminating mutant transcript (Metzstein & Krasnow, 2006).

Aberrant mRNAs subjected to NMD		References
Aberrant mRNA generated by DNA alterations.		
Nonsense mutations	Base substitutions that directly generate PTCs.	(Chang & Kan, 1979)
Insertions and deletions	Random nucleotide insertions and deletions shift the reading frame in two of three cases, resulting in a PTC.	(Culbertson et al, 1980)
Mutations changing splicing signals	Mutations leading to aberrant splicing often result in a frameshift or in intron-encoded PTCs	(Mendell & Dietz, 2001) (Holbrook et al, 2004)
Somatic DNA rearrangement	The immunoglobulin superfamily represents a special class of NMD targets that undergo very efficient NMD. During programmed somatic maturation of the immunoglobulin and T-cell receptor genes. Two of three rearrangements of the V, D, and J segments result in a frameshift, often producing PTCs	(Carter et al, 1995; Li & Wilkinson, 1998; Weischenfeldt et al, 2008)
Problem at RNA level		
Unproductive alternative splicing	45% of alternatively spliced mRNAs are predicted to be an NMD target.	(Green et al, 2003; Lewis et al, 2003; Sayani et al, 2008)
Problem at translation level		
Leaky scanning	Observed only in yeast. Ribosomes scan beyond the initiator AUG and initiate at a downstream AUG in a reading frame with a PTC.	(Welch & Jacobson, 1999)[1]

Physiological mRNAs		References
Programmed translational frameshifting	Programmed + 1 or − 1 frameshifts lead into a PTC, if the ribosome fails to shift the reading frame properly.	(Culbertson et al, 1980; Lee et al, 1995)
mRNAs with introns in the 3′ UTR	Observed in yeast and mammals.	(Mendell et al, 2004)
mRNAs with long 3' UTRs	observed in yeast, fly, plant and human	(He et al, 2003; Kebaara & Atkin, 2009; Mitrovich & Anderson, 2005; Schwartz et al, 2006)
mRNA of transposons and retroviruses Transcribed pseudogenes	Observed in *S.cerevisiae* and mammals. NMD limits the ''transcriptional noise'' of supposedly non-functional RNAs such as transcribed pseudogenes, ancient transposons or mRNA-like non-protein coding RNAs from intergenic regions	(He et al, 2003; Kurihara et al, 2009; LeBlanc & Beemon, 2004; Mendell et al, 2004; Mitrovich & Anderson, 2005)
Bicistronic mRNAs	Observed only in *S. cerevisiae.*	(He et al., 2003)
mRNAs with upstream ORFs (uORF)	The termination codon of the uORF is likely to be interpreted as PTC, unless the mRNA harbors stabilizing elements nearby	(He et al, 2003; Mehta et al, 2006; Mitrovich & Anderson, 2005)
mRNAs encoding selenoproteins	The UGA is recognized as a PTC at low selenium concentrations	(Moriarty et al, 1998; Sun & Maquat, 2002)

Table 3. Features and origins of NMD targets. Modified from Mühlemann 2008 (A) mRNAs presenting aberrant features and recognized by the NMD via the PTC. (B) Physiologically regulated mRNAs subjected to NMD, often not presenting any PTC, but instead sequences recognized as a PTC.

3.3. NMD model

Transcriptomes of Saccharomyces *cerevisiae*, *D. melanogaster* and human cells have been analysed and have revealed that 3–10% of all mRNAs are affected by NMD. The more contemporary view is that NMD has been conserved trough evolution rather as a gene expression regulatory mechanism than a degradation pathway for abnormal mRNAs.
Although NMD and its core factors have evolutionary conserved features the mechanism importantly differs among species. I decided to devote this manuscript to mammalian NMD.

The two main questions to ask about the NMD are: (i) how the cell decides that an mRNA should be degraded? and (ii) when is this decision made?

For a long time the "Holy Grail" in NMD studies was to determine how a cell can distinguish between a regular stop codon and a PTC. Over the past decade it became clear that the NMD is tightly coupled to (i) splicing and (ii) translation (Carter et al, 1996; Le Hir et al, 2000a; Le Hir et al, 2000c; Thermann et al, 1998). During mRNA splicing, a complex called the **E**xon-**J**unction **C**omplex (EJC) is placed at each splice site. The EJC is a 350 kDa multi-protein complex containing about ten diverse proteins that remains associated with the mRNA in the immediate vicinity of an exon-exon junction (20 - 24nt upstream) after the splicing reaction. In normal conditions the EJC is disassembled shortly after nuclear export, however some EJC-components remain on the RNA until the ribosome chases them during the first round of translation (Dostie & Dreyfuss, 2002). The presence of an EJC 50-55 nt downstream of a termination codon triggers NMD (Carter et al, 1996; Thermann et al, 1998).
Importantly NMD is also clearly linked to translation termination. The distance between the termination codon and the poly(A) tail, depending on the length of the 3' UTR seems to be determinant for the classification of a stop codon as premature. If the distance is too short NMD is inhibited.

3.3.1 The EJC-dependent model of NMD.

The core EJC factors consist of: the heterodimer MAGOH–Y14, the DEAD-box RNA helicase eIF4AIII and MLN51 (Le Hir et al, 2000b). In association with an mRNA molecule, the EJC complex has been shown to contain also important NMD factors such as UPF2 and UPF3b (Le Hir et al, 2001b). Up to now more than a dozen proteins have been identified as

components of the EJC, including splicing coactivators and alternative splicing factors (SRm160, RNPS1 and Pinin) and mRNA export proteins (UAP56, REF/Aly and NXF1/TAP: p15) (Kataoka et al, 2000; Le Hir et al, 2001a; Le Hir et al, 2000a; Lykke-Andersen et al, 2000). A conserved domain in UPF3a and UPF3b associates with Y14 and RNPS1, and this interaction is essential for NMD. Thus, the EJC has been proposed to provide a direct link between splicing and NMD by serving as the anchor site for UPF2 and UPF3 (Le Hir et al, 2001b).

According to the EJC-dependent model of NMD, normal termination codons can only be found in the last exon of an mRNA molecule. This also means (and has been experimentally confirmed) that RNAs originating from intronless genes (and thus devoid of EJC) are immune to at least this mechanism of NMD induction (Maquat & Li, 2001).

However the group of Lykke-Andersen recently reported that the introduction of a PTC in an intron-deprived **G**lutathione **P**ero**x**idase 1 (GPx1) mRNA triggered NMD, although the decay observed was less efficient than when a GPx1 mRNA containing an intron has been used. Moreover the study failed to identify natural human intron-less mRNAs that are subjected to NMD. Thus the statement that naturally intron-less genes are immune to NMD seems to be correct at least for a vast majority of examined genes (Singh et al, 2008).

3.3.2 The faux 3' UTR model.

The second model of PTC detection proposed was first discovered in yeast. Recently it has been found that mammalian mRNAs regulated by NMD can be also eliminated due to a signal coming from the translation termination. As mentioned in the previous paragraph mutated/abnormal mRNAs that contain a PTC tend to have longer 3'UTRs than their wild type analogs. A longer 3'UTR results in turn in the stimulation of one of the core NMD factors (UPF1) and in the reduction of the efficiency of translation termination (Rodriguez-Gabriel et al, 2006). This discovery suggested that the important distinction between normal and premature terminations might simply rely on the distance between the stop codon and the 3' end of the mRNA. This model – called the *faux* 3' UTR model – claims that efficient termination requires an interaction with factors associated with the 3' UTR. The original *faux* 3' UTR model proposed that inefficient termination is due to lack of interaction between the terminating ribosome and PABPC (Poly(A) Binding Protein Complex) (Amrani et al, 2004). Further studies indicated that the distinction of a PTC from a normal stop codon depends on

its distance from the poly(A) tail and upon the competition between UPF1 and PABP for binding to eRF3 on the terminating ribosome.

In agreement with the *faux* 3' UTR model, any extension or shortening of the distance between PTCs and the poly(A) tail affected NMD in different experimental systems (Behm-Ansmant et al, 2007; Buhler et al, 2006; Eberle et al, 2008). Moreover PTC-bearing mRNA that lack a poly(A) tail are not subject to NMD, and depletion of PABPC strongly inhibits NMD in *Drosophila* S2 cells, suggesting that both the poly(A) tail and PABPC are crucial for NMD (Behm-Ansmant et al, 2007).

3.3.3 A unified model?

Despite the intense study of NMD, the PTC-recognition mechanism remains incomplete. It is interesting to imagine a coherent PTC-recognition model (Fig.10). What emerges is the multitude of important signals influencing the NMD. Those signals might be for instance the nature of the stop codons, the nucleotide immediately following the stop codon, and the sequences, length, and associated proteins of 3′UTR and ending with specific factors triggering the NMD e.g., EJC, PABP; the list is long and continues growing. These features might be working in opposing or orchestra fashions (e.g., inhibit or stimulate normal or aberrant termination). One can suppose that depending on the transcript, cell conditions, model organism, and/or experimental setup, some of these features may appear to be more important than others.

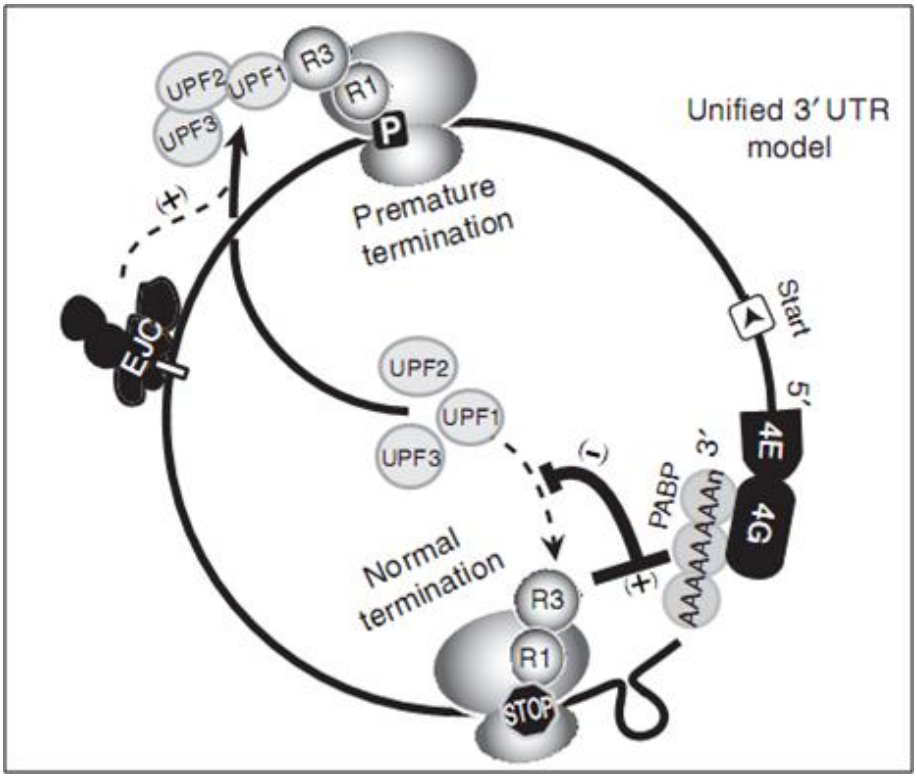

Figure 10. The unified 3' UTR model for NMD. NMD is mainly determined by the flanking 3 UTR. PABP plays an antagonistic role in NMD. NMD is elicited if the PTC is placed in a long distance from the poly(A) tail. When termination occurs near the poly(A) tail, PABP prevents the recruitment of UPF1,and so preventing the NMD. The presence of a downstream intron deposits an EJC which can further stimulate the recruitment of UPF proteins and NMD execution.

3.4. The Pioneer Translation Round – to degrade or not to degrade?

In mammalian cells the fate of an mRNA molecule is sealed during the so-called Pioneer Round of Translation (Ishigaki et al, 2001). During this first ribosomal scanning aberrant transcripts are detected according to the features presented previously. The NMD machinery has been shown to target mRNA bound by the **C**ap-**B**inding **P**roteins (CBP80–CBP20) (Ishigaki et al, 2001; Lejeune et al, 2002).

The initiation of the pioneer round engages at least one ribosome (this depends on the length of the translational open reading frame and general initiation efficiency) (Isken et al, 2008). The pioneer round of translation occurs before the bulk translation and involves mRNA that is bound by the cap-binding protein (CBC a heterodimer of CBP80 and CBP20) (Fig. 10). In higher eukaryotes intron containing pre-mRNA involves the deposition of an EJC upstream of

each exon-exon junction which along with other NMD factors and the poly(A) tail are required for NMD. After the pre-scanning of mRNA, the pioneer round translation complex is transformed into a steady-state initiation complex. Remarkably, the NMD factors along with EJC components are removed and so the RNP is reconfigured allowing the eIF4E binding to the cap (Lejeune et al, 2002). These rearrangements support the idea that NMD is triggered by the pioneer round of translation.

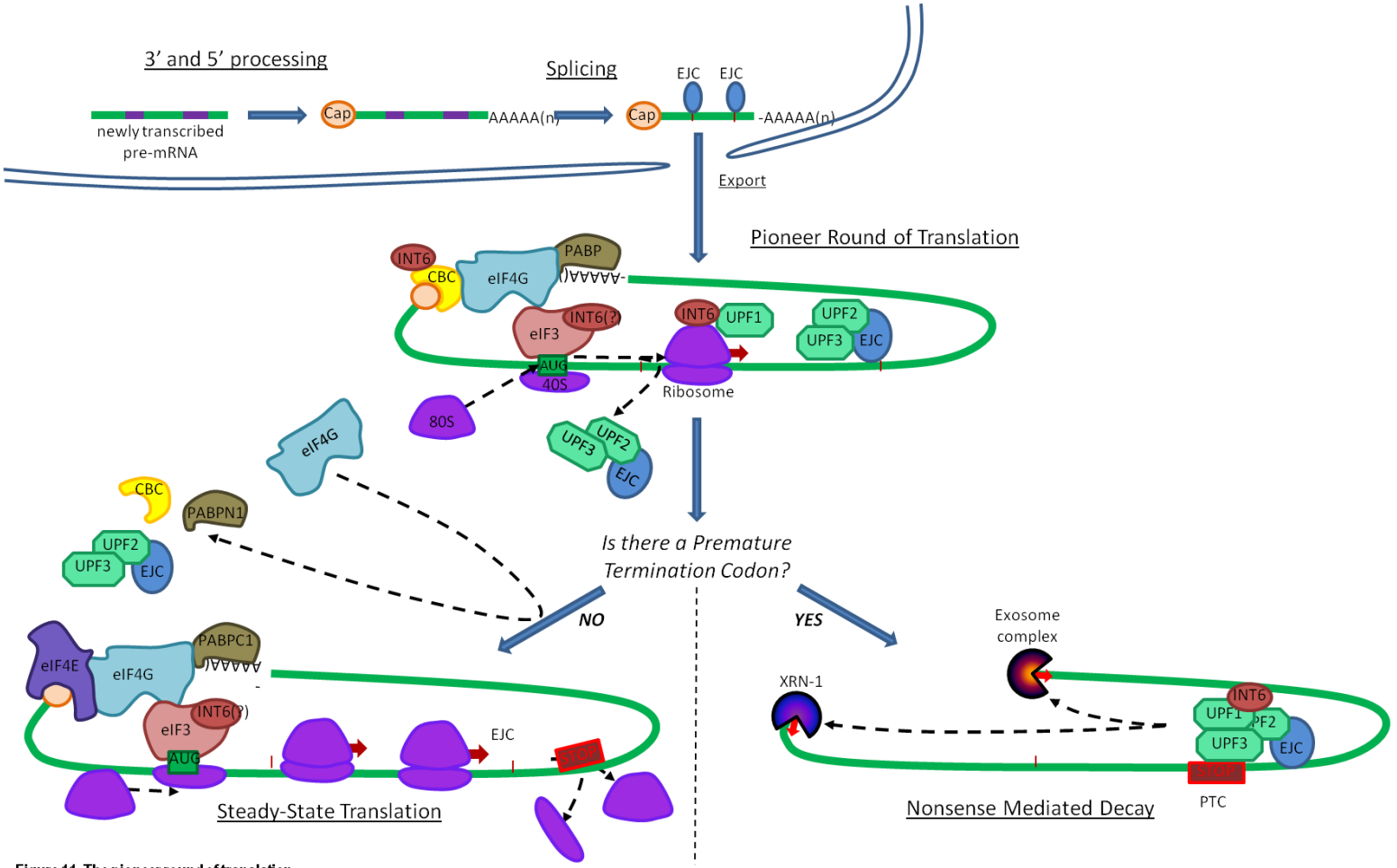

Figure 11. The pioneer round of translation.
The scheme illustrates the pre-scanning of an mRNA and it's two possible fates. If the termination codon is correct the mRNA undergoes the steady-state translation (left part). If on the contrary a PTC is detected the NMD will lead to rapid mRNA degradation (right part).

3.5 NMD Factors

The discovery of NMD factors started in *S. cerevisiae* and *C. elegans* (Culbertson et al, 1980; Hodgkin et al, 1989) and homologs were found later in higher eukaryotes. One can distinguish three main trans-acting factors, the so called **U**p-**F**rameshift (UPFs) proteins (UPF1, UPF2 and UPF3 which exists in two isoforms UPF3a and UPF3b also termed UPF3X). A very important class of proteins regulates the function of the UPFs, the **S**uppressor of **M**orphological defects on **G**enitalia (SMG) proteins (SMG-1, SMG-5, SMG-6, and SMG-7). They are known to mediate the phosphorylation and dephosphorylation cycle of UPF1.

3.5.1 UPFs

In mammalian cells, UPF proteins are very abundant, up to millions of copies per cell can be detected, much more than the protein levels in budding yeast (Culbertson & Neeno-Eckwall, 2005). Interestingly UPF1 is almost 5-10 times more abundant than UPF2 and UPF3. This does not only suggests a pivotal role in the NMD but supports the data of many studies in which it was shown that UPF1 has additional non-NMD roles. Those are independent of UPF2 and UPF3 (Azzalin & Lingner, 2006; Isken et al, 2008; Kaygun & Marzluff, 2005a).

3.5.2 UPF1

The UPF1 (also known as RENT1 in human) is a RNA binding protein with, ATPase-dependent, helicase activity (Czaplinski et al, 1995). Both in yeast and human UPF1 associates with the ribosome via the interaction with the eukaryotic translation release factors eRF1 and eRF3 during premature translation termination (Czaplinski et al, 1995; Ivanov et al, 2008; Kashima et al, 2006; Weng et al, 1998). UPF1 has two separate functional domains: a cysteine-histidine rich (CH) domain at the N terminus and an ATPase/helicase domain at the C terminus.

3.5.3 UPF2

UPF2 contains three continuous domains which resemble middle domain of the eukaryotic initiation factor 4G motifs (MIF4G) (Rehwinkel et al, 2005b). MIF4G domains are found in several proteins involved in mRNA processing and translation, such as eIF4G and CBP80 (Marcotrigiano et al, 2001; Mazza et al, 2001).

3.5.4. UPF3

In humans two UPF3 homologues are known, UPF3a and UPF3b (Lykke-Andersen et al, 2000). Both proteins contain a putative RNA recognition motif (RRM) at the N terminus, which is involved in RNA binding.

A number of observations indicate that UPF1, UPF2 and UPF3 form a complex (He et al, 1997; Weng et al, 1996). In human cells, the UPFs form a trimeric complex where UPF3 contacts via its N-terminal region UPF2, which in turn interacts with the CH domain of UPF1 (Chamieh et al, 2008; Kadlec et al, 2006; Kadlec et al, 2004; Lykke-Andersen et al, 2000; Mendell et al, 2000).

3.5.5 SMGs

As mentioned previously the SMG proteins are regulatory proteins. During NMD, UPF1 undergoes sequential dephosphorylations and phosphorylations. The phosporylation state of UPF1 is critical for proper NMD function in metazoan and human cells (Grimson et al, 2004; Isken & Maquat, 2008; Ohnishi et al, 2003).

SMG1 is a phoshatidylinositol kinase-related kinase that phosphorylates UPF1 and activates it for NMD (Grimson et al, 2004; Yamashita et al, 2001). SMG5 and SMG7 are involved in the dephosphorylation of UPF1 and inhibit the UPF1-driven NMD (Anders et al, 2003; Chiu et al, 2004; Fukuhara et al, 2005; Ohnishi et al, 2003). Two additional proteins, SMG-8 and SMG-9, have also been shown to bind SMG1 (Yamashita et al, 2009). Both of them suppress the kinase activity of SMG1 and are components of the NMD inducing complex. The so called SURF complex contains the NMD factors, SMG1, UPF1, and the release factor eRF1 and eRF3 (Kashima et al, 2006; Yamashita et al, 2009). SMG-8 also seems to play an important role in the interaction between the complex and EJC (Yamashita et al, 2009).

3.6 The Core NMD Machinery

Following PTC recognition a cascade of events triggers fast degradation of aberrant mRNA. The process starts by the joining of UPF3 to the EJC in the nucleus.
During the pioneer round of translation it comes to the formation of the **S**MG1-e**R**F1-e**RF**3-**U**PF1 (SURF) complex with the mRNA. SURF is recruited by the ribosome stalled on a PTC containing mRNA. Then UPF2 bridges the interaction between the ribosomal SURF (via UPF1) and the downstream EJC (via UPF3).In this complex SMG1 phosphorylates UPF1 and elicits the dissociation of the two release factors (eRF1 and eRF3). As a result, a complex containing the EJC, UPF2, UPF3, phosphorylated UPF1 and SMG1, triggers the inhibition of translation and degradation of the mRNA.
In parallel with mRNA degradation, UPF1 is dephosphorylated by SMG5 and PP2A and can be recycled (Chiu et al, 2004; Ohnishi et al, 2003).

3.6.1 Alternative NMD pathways.

It has been reported that there are important variations from the "standard" NMD mechanism. The linear model of mammalian NMD is likely oversimplified because examples have been described where NMD can be UPF2 or UPF3b independent, or even occur independently of splicing (Buhler et al, 2006; Chan et al, 2007; Gehring et al, 2005).

3.6.1.1 The INT6-dependent NMD.

In order to complete the current NMD-core model it has to be mentioned that not all NMD targets require exactly the same set of factors. Additionally to the UPFs some other factors and alternative NMD pathways have been identified. In a previous work of our laboratory it has been established that INT6 was able to interact with both UPF1 and UPF2 (Morris et al, 2007). Moreover in INT6 silenced cells NMD was inhibited. This observation along with the fact that INT6 was able to bind CBP80 was in agreement with the fact that INT6 is not necessary for steady translation but rather for a pre-translational proofreading process. Although this possibility needs to be further investigated its interaction with the CBP80 subunit of the CAP Binding Complex would support this notion (Morris et al, 2007). Such an interaction could facilitate further loading of the remaining eIF3 complex and lead to the

initiation of the pioneer round of translation. It was shown that the composition of eIF3 is highly dynamic so it does not seem aberrant that a specific sub-complex of eIF3 might function in the pioneer round of translation. The formation of an INT6-mediated complex can lead to lead to different patterns. The first possibility is to complete translation normally and replace the EJCs and the CBC by eIF4E (Chiu et al, 2004; Lejeune et al, 2002). The remodelled mRNP can then be routed towards active translation which might use an eIF3 complex lacking INT6. Alternatively, after detection of a premature termination signal the SURF complex is recruited triggering SMG1-mediated phosphorylation of UPF1. This event leads to translation initiation inhibition and allows the recruitment of degradation factors such as DCP1, XRN1 and EXOSC2 (Isken et al, 2008; Lejeune et al, 2003b).

3.7 The way to decay.

The degradation can pursue a "canonical" mRNA decay pathway that destroys "normal" mRNAs by a 3'-5' or 5'-3' decay (He & Jacobson, 2001; Lejeune et al, 2003b; Sheth & Parker, 2003). More recent studies have reported that in *D. melanogaster* and human cells, NMD substrates appear to be degraded by an alternative pathway, involving an endonucleolytic cleavage (Behm-Ansmant et al, 2007; Eberle et al, 2009; Gatfield & Izaurralde, 2004).

3.7.1 The 3' – 5' decay.

Eukaryotic mRNAs have two stability elements, the 7–methylguanosine cap at the 5' end and poly(A) tail at the 3' end. Deadenylation typically precedes the breakdown of the mRNA. In higher eukaryotes, the **P**oly(**A**) **R**ibonuclease (PARN) is the main poly(A) nuclease of 5' capped mRNA (Garneau et al, 2007). Following the shortening of the poly(A) tail, mRNAs are the target of the cytoplasmic exosome. This 3'-5' exonucleolytic complex is composed of 10-12 subunits and associated with the Ski complex (consisting of Ski2, Ski3, Ski7 and Ski8) (Garneau et al, 2007; Ibrahim et al, 2008; Schmid & Jensen, 2008).

3.7.2 The 5’ – 3’ decay.

Meanwhile, the 5’ends are decapped by Dcp1 and Dcp2 enzymes. Decapped mRNAs are subjected to 5’-3’ degradation by the exoribonuclease XRN1 (Anderson & Parker, 1998; Decker & Parker, 2002; Lejeune et al, 2003a; Mangus et al, 2003). The rapid decapping of PTC-containing mRNAs is probably mediated by the interaction of UPF1 with Dcp1:Dcp2 (Lykke-Andersen, 2002).

3.7.3 Endonucleolytic Cleavage

Evidence of the involvement of the endonucleolytic cleavage was reported already in the No-Go mRNA decay (NGD) in *S. cerevisiae* where a stem loop prevents translation elongation and also triggers mRNA decay (Doma & Parker, 2006). In human cells the endonucleolytic cleavage has been reported to occur at multiple sites close to a PTC. Surprisingly the cleavage reaction can take place both before and after the stop codon. Studies of *D. melanogaster* and human cells reveal that this cleavage depends on a functional SMG6 protein (Eberle et al, 2009; Huntzinger et al, 2008). This last observation suggests a possible link between the stalling of the ribosome and the SMG6-dependent endonucleolytic cleavage.

In summary, NMD substrates are likely to be degraded by a number of pathways, requiring both endo- and exonucleases (Fig.12).

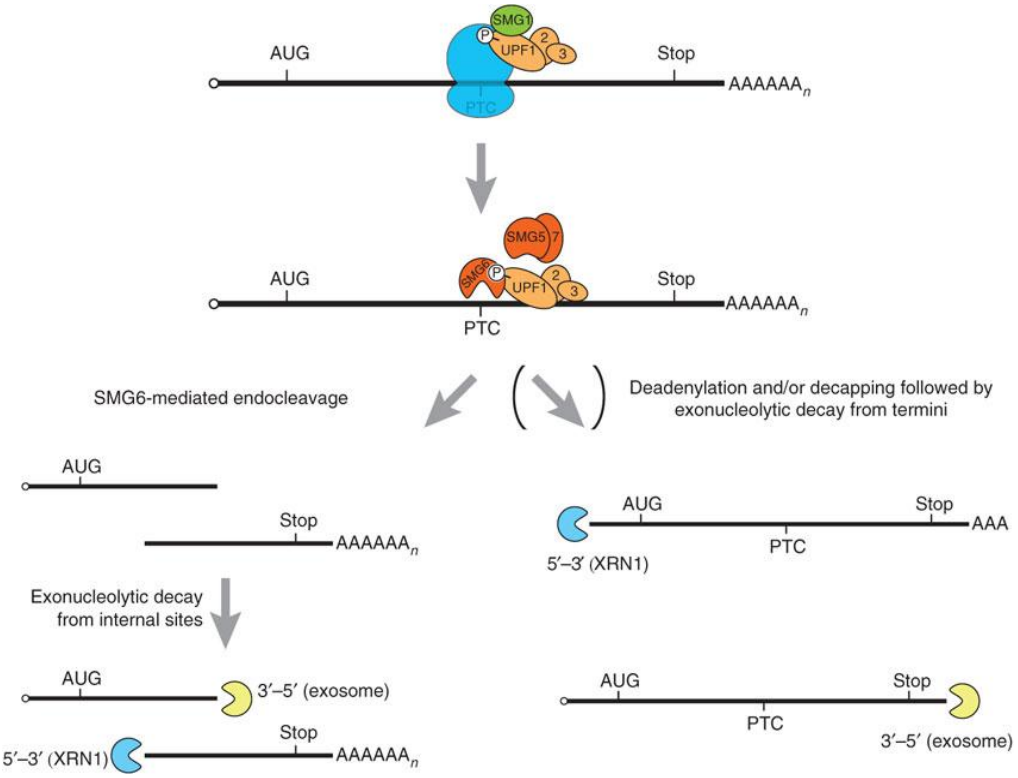

Figure12.**Three possible pathways leading to RNA degradation**. Adapted from (Eberle et al, 2009)

3.8 Localization of mRNAs degradation.

Can translation take place in the nucleus? Very controversial studies.

The localization of mRNA degradation is a subject of animated discussion. Some studies in human cells indicate that NMD occurs while the mRNA is still associated with the nuclear cap binding complex (CBC), but not with eIF4E. Early studies of NMD of the β-glogin mRNA and **T**riose**p**hosphate **I**somerase (TPI) mRNA in human cells indicated that NMD might target mRNAs still associated with the nucleus. These studies reported that there was no change in the stability of cytoplasmic mRNAs containing or not nonsense mutations (Baserga & Benz, 1992; Cheng & Maquat, 1993). Further studies with human TPI mRNA also reported that nucleus associated NMD takes place after splicing (Belgrader et al, 1994; Cheng et al, 1994). It appears that the level of nucleus-associated mRNA was always reduced to almost the same amount as the cytoplasmic mRNA containing a PTC, in human cells (Maquat, 1995b). Furthermore, several studies reported that there is no obvious difference in stability between mRNA carrying PTCs and the corresponding wild-type mRNA, in spite of the low steady state level of the PTC-containing mRNAs (Baserga & Benz, 1992; Cheng & Maquat, 1993). These observations have led to a suggestion that the PTC-containing

transcripts are degraded in the pioneer round translation while they are still associated with the nucleus (Hentze & Kulozik, 1999). It was also proposed that mRNA escaping this first round of translation escape NMD as well. Nucleus associated NMD in mammalian cells presents a paradox, because it is believed that only cytoplasmic ribosomes recognize and scan the open reading frames.

3.8.1 Cytoplasmic NMD.

A great amount of studies was performed demonstrating that it takes place in particular cytoplasmic compartments known as **P**rocessing **B**odies (P-bodies). These structures include various proteins intervening in degradation as the decapping enzymes DCP1, DCP2 and also exonucleases as XRN1. (Couttet & Grange, 2004; Rehwinkel et al, 2005a). Recently it has been shown that degradation can still occur if formation of the processing bodies is inhibited. (Stalder & Muhlemann, 2009). This suggests that P-bodies and their components function as enhancer for correct and efficient NMD but are not absolutely indispensible for the process to occur.

3.8.1.1 Processing bodies (P-bodies)

Initial studies on the subcellular localization of the main 5' to 3' exoribonuclease involved in mRNA decay in eukaryotic cells (XRN1), revealed that it was localized in small granular structures enriched in cytoplasmic foci (Bashkirov et al, 1997) (Fig.13). However, the biological significance of this discovery was only appreciated some years later when the decapping enzyme DCP2 and some of its cofactors were also shown to co-localize with XRN1 in the same cytoplasmic foci (Ingelfinger et al, 2002; van Dijk et al, 2002). Due to this kind of protein component (decapping and 5' to 3' mRNA decay machineries), these DCP/XRN1 cytoplasmic foci were suspected to function in cytoplasmic mRNA decay and were referred as processing (p)-bodies, DCP bodies or mRNA-decay bodies. Interestingly, similar cytoplasmic foci were discovered at the same time by using a serum that recognized GW182, a human protein rich in GW (Gly-Thr) repeats of 182-kDa with unknown function (Figure 47) (Eystathioy et al, 2002). Only one year later it was demonstrated that GW182, DCP2 and XRN1 were present in the same cytoplasmic structure (Eystathioy et al, 2003). An important point for P-bodies formation was their dependency on RNA further suggesting that these cytoplasmic foci could be a place for mRNA decay (Andrei et al, 2005; Cougot et al, 2004; Teixeira et al, 2005).

3.8.1.2 P-bodies and mRNA Surveillance

P-bodies have been shown to contain proteins involved in the NMD such as UPF1, UPF2, UPF3, SMG-5 and SMG-7 (Sheth & Parker, 2006; Unterholzner & Izaurralde, 2004). There are several lines of evidence showing that NMD-targeted mRNAs along with NMD factors are shipped to P-bodies both in yeast and humans cells (Durand et al, 2007b; Sheth & Parker, 2006). However, more recent studies demonstrate that the formation of P-bodies is not essential for NMD at least in human cells (Stalder & Muhlemann, 2009). Nevertheless, downstream steps following NMD involve deadenylation, decapping and the 5' to 3' degradation (Chen & Shyu, 1995; Couttet et al, 1997; Lejeune et al, 2003b). Since proteins like DCP1 and 2, Xrn-1 are essential components of P-bodies it could suggest that they are eventually involved at the end of the NMD process.

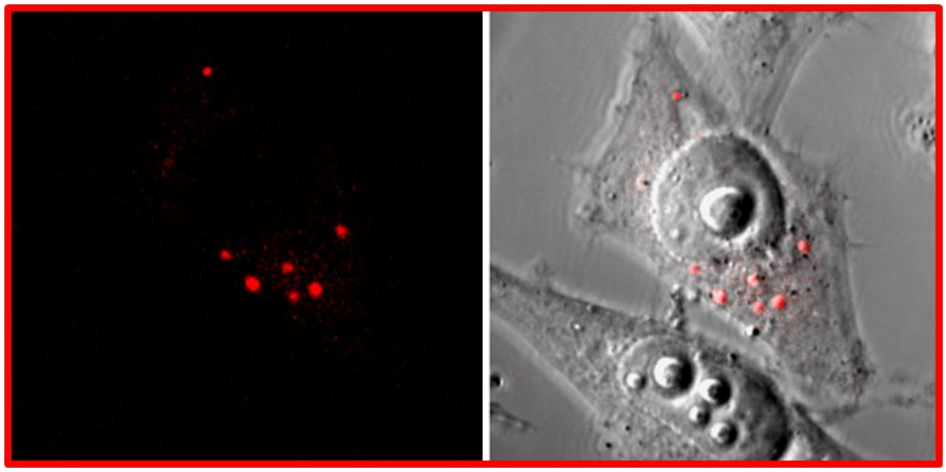

Figure 13. P-bodies in Hela cells. The P-bodies (visualized by red spots) have been observed with a confocal upright microscope. One of the P-bodies components contains and RFP-tag (DCP1-RFP) and is transitionally overexpressed.

3.9 The ability to escape NMD.

According to the present vision of NMD, a PTC-containing transcript should be subjected to NMD recognition and further degradation, but what would be a rule without any exception? Few cases were reported in which NMD seem not to detect mutated transcripts. This may occur when (i) the PTC locates in the last exon of an mRNA (Perrin-Vidoz et al, 2002) (ii) at the beginning of the coding sequence (so that translation re-initiation can be induced) (Puel et al, 2006), or (iii) when the PTC is absent because of exon skipping event (Disset et al, 2006; Zetoune et al, 2008).

Chapter 4 Histones – from discovery to functional insights.

4. The discovery of histones; at the origins of the chromatin research.

Histones, which were discovered at the end of the 19th century, are chief proteins around which DNA is packaged in the cell nucleus. They are thus essential components of eukaryotic chromosomes. In metazoans, they are encoded by the so-called replication-dependent histone genes. The biogenesis of histones is tightly coupled to DNA replication. DNA replication is controlled in the context of the cell cycle. As the cell grows and divides, it progresses through stages in the cell cycle; DNA replication occurs during the synthesis phase (S phase).The stoichiometric manner of control is extremely important because an excess of histones is highly toxic for the cell. Since histones present a highly basic character any excess is extremely hazardous resulting in non-specific binding to nucleic acids and non-nucleosomal aggregate forming. Therefore, a strict cell cycle-regulation of the critical factors involved in histone expression ensures exclusive S phase expression.

4.1. The history of chromatin research.

The history of chromatin and histone research is a long one, starting with Walter Flemming's original studies of mitosis performed in the late 19th century (Fig. 14). In 1880 this remarkable researcher proposed the name "chromatin" explaining that: "...in view of its refractile nature, its reactions, and above all its affinity to dyes is a substance which I have named chromatin." Thus, the term chromatin has been derived from the Greek "khroma" meaning coloured and "soma" meaning body, based on its stain ability with basic dyes.
Later biochemical studies followed and two students of E. Hoppe-Seyler, F.Miescher and Albrecht Kossel, performed crucial work for the characterization of chromatin components. In 1871 Miescher described a strong phosphorus-rich acid and baptised it "nuclein". In 1884 Albrecht Kossel identified histones in acidic extracts from avian erythrocyte nuclei (Olins & Olins, 2003).

The impact of Miescher's work was overshadowed by the more hyped discovery of the rules of genetic inheritance by Austrian monk Gregor Mendel and the theory of evolution by British scientist Charles Darwin. More and more progress was done in the field of genetics. In 1900 Hugo Marie de Vries rediscovered Mendel's laws, introduced the concept of genes and the term "mutation". He also developed the mutation theory of evolution (Lenay, 2000), followed by the development of the gene theory and principles of linkage by T. H. Morgan in

1910 (Olins & Olins, 2003). While these revolutionary discoveries were made in the field of genetics, progress in the adjacent field of chromatin and histones remained in the shade of genetics. Indeed, histones were still considered to be the bearers of genetic information until the early 40s. Then another big achievement was made by Franklin Griffith in 1928 (Olins & Olins, 2003) by describing the principle of transformation which led Oswald Avery, Colin Macleod and Maclyn McCarty in 1944 (Avery et al, 1944) to demonstrate that DNA was actually the molecule encoding the genetic information. The early discovery of polytene chromosomes in *Drosophila* and gene localization studies inspired D. Mazia to use proteases and nucleases to study salivary gland polytene chromosomes (Mazia & Jaeger, 1939). The use of nucleases in particular revolutionized the chromatin field. Then came the famous April 1953 issue of Nature which contained the historic three papers by Watson & Crick, Wilkins, Stokes & Wilson and Franklin & Gosling describing the structure of the DNA double helix. This "Rosetta stone" discovery lay down the foundations of modern molecular biology. Solving the complementary DNA structure allowed the understanding of the still abstract genetics and the function deprived chromatin study. In 1959 G. Zubay`s laboratory used the first time biophysical approaches to study the chromatin structure preparing soluble chromatin. In the meantime, histone proteins were fractionated by the group of E. Johns.

The next big step was the development of electron micrography, introduced in the chromatin field in 1970 by H. Davies who had observed 30 nm chromatin threads in chicken erythrocyte nuclei. Similar fibres were observed subsequently in purified chromatin preparations by the group of Klug in 1976. The "beads on the thread" were visualized by two independent groups: first Olins and Olins in 1974 who named them "v (nu) bodies", then C.L.F. Woodcock in 1976 (Olins & Olins, 1974; Woodcock et al, 1976).

In 1974 R. Kornberg and J. Thomas postulated a model for the structure of chromatin, describing it as a repeat of ~200 base pairs of DNA in complex with an octamer of core histone, itself made of a tetramer of histones H3 and H4 and two dimers of histones H2A and H2B. This chromatin subunit was christened "Nucleosome" by P. Chambon in 1975 (Oudet et al., 1975). Linker histones were subsequently proposed to link the nucleosome core particles in chromatin (Kornberg & Thomas, 1974; Noll & Kornberg, 1977).

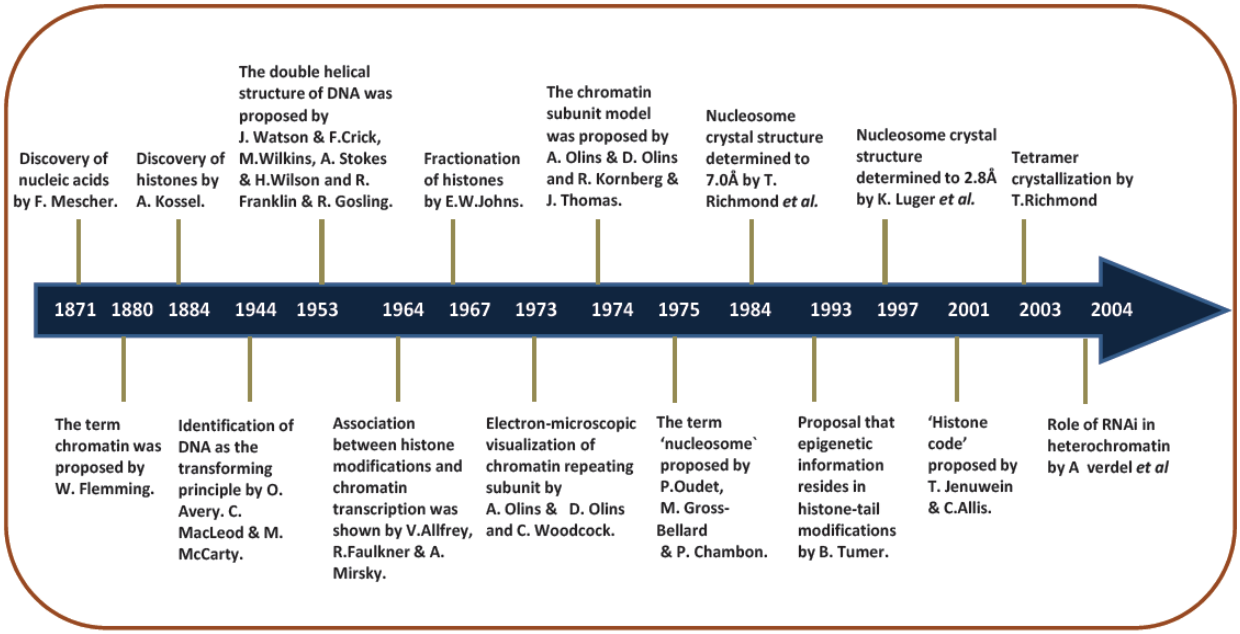

Figure 14. Diagram representing the milestones in chromatin studies. From (Olins & Olins, 2003)

The concept of the organization of DNA and histones in nucleosomes was derived from X-ray crystallography studies of the histone octamer and the core particle by the groups of E.N. Moudrianakis (Arents et al, 1991) and T.J. Richmond (Luger et al, 1997), respectively. However, there have been many controversial reports about the precise location of linker histones in chromatin (Kornberg & Lorch, 1999; Rando & Chang, 2009).

In vitro studies of the chromatin fibre have led to the proposal of two main, competing models of the higher order (30 nm scale) structure of chromatin, namely the "zigzag model" (Williams et al, 1986; Worcel et al, 1981) and the "solenoid model" (Dorigo et al, 2003). Despite many refined and compelling studies, a consensus has yet to emerge.

4.2. Chromatin and Core histones

In human cells roughly two meters of DNA (6 x 10^9 bp) have to be compacted in a micron sized (5-10μm) nucleus (Fig. 15) and at the same time must be rapidly accessible to allow the various transactions – replication, transcription, repair and recombination – that affect the DNA of a cell to be efficiently mediated. The structure of chromatin and its dynamic re-organization can thereby potentially influences all the functions of the genome.

In the nuclear genome, within chromatin the DNA molecule is bound tightly to an almost equal mass of histones, so that about 60 million histone molecules may be found per human cell. Histones are small 12-20 kD proteins that possess an open, unfolded structure and

attached to the DNA molecule by ionic linkages. Up to now 5 major histone classes have been identified. Four of them fall under the core histone category (H2A, H2B, H3, H4) and form the nucleosome core particle, while the fifth class, linker histones (LH; H1/H5) regroups proteins that connect nucleosomes one to another. Despite the H1 linker histone, core histones are deeply evolutionarily conserved and can be found in the nuclei of Euryarchaeota but not in Bacteria. Their conserved character emphasizes the important role they play in the biology of the nucleus. However the conservation of core histone sequences is not uniform and mainly affects the main, central domain, which contains a "histone fold motif" averaging about 65-70 amino acids. This motif consists of three alpha helices connected by short loops and is responsible for DNA-histone and histone-histone interactions (Arents & Moudrianakis, 1995; Bolognese et al, 2000). The two termini extend outside the nucleosome core (Fischle et al, 2003). Both the C-terminal and the N-terminal histone tail are the major sites of post-translational modifications affecting histones, and those in turn play a crucial role in the regulation of gene expression. More about this subject will be described later in this manuscript.

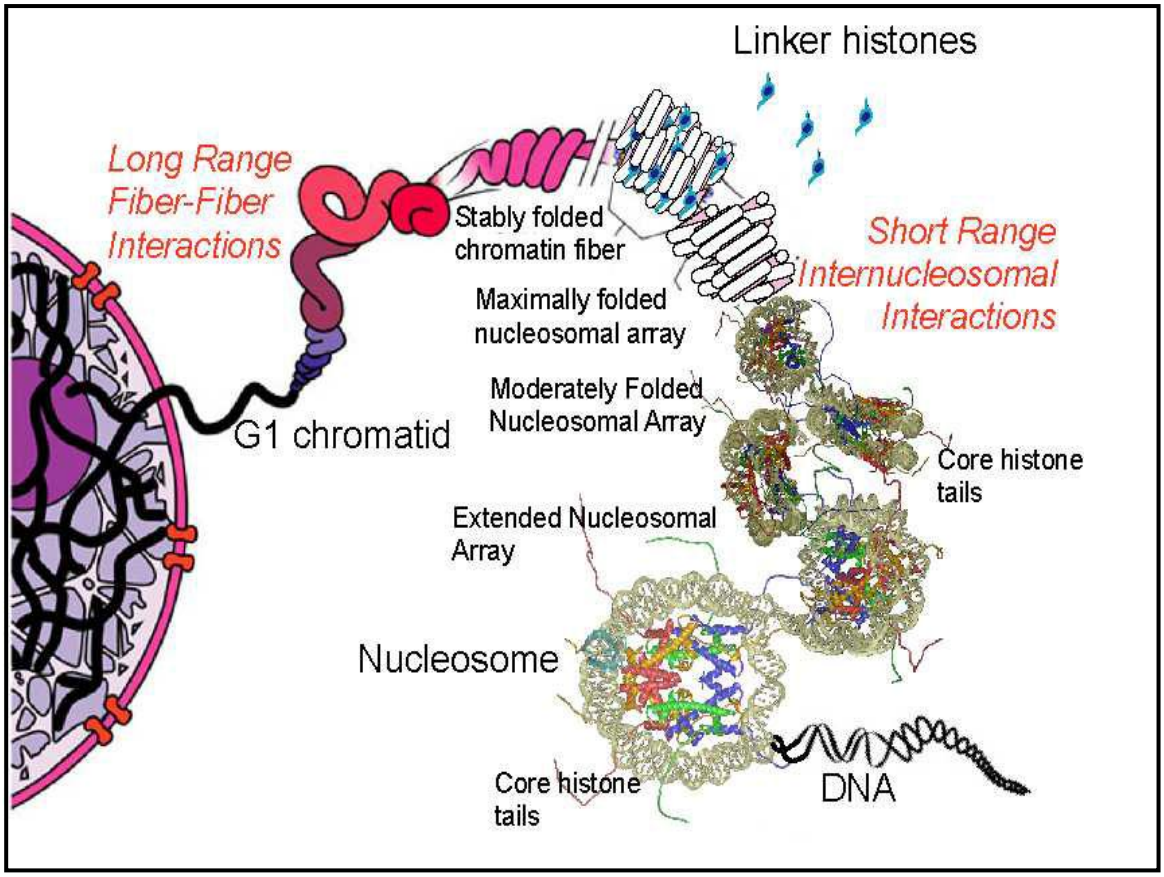

Figure 15 Organization of eukaryotic chromatin fibers. The fundamental unit of chromatin is defined as nucleosome that forms the "beads-on-a-string" chromatin structure. Internucleosomal interactions, linker histones and non-histone proteins mediate the further condensation of chromatin into 30nm fibers and higher order structures. Adapted from (Hansen, 2002)

4.3. The nucleosome.

H2A and H2B form two heterodimers flanking a heterotetramer composed of H3 and H4. Together they make up an octamer around which 147 bp ± 1bp of DNA is wrapped, forming the nucleosome core particle (NCP) – the basic particle of chromatin (Fig. 16). Adjacent nucleosomes are connected one to another by a double-stranded linker DNA (20-80bp). The average size of the linker DNA segment varies depending on species and tissue. This linker DNA is stabilized by the fifth histone, H1. Different models of H1 binding are adopted. The most common is representing the "H1siting" on the nucleosome core and binding the DNA entry/exit points, thus protecting around 20 bp of linker DNA. Former studies have shown that LHs exhibit a general preference for AT-rich DNA. The globular domain can interact with the minor groove located in the centre of each nucleosome. The LH is believed to be responsible for the 30 nm chromatin fibre formation.

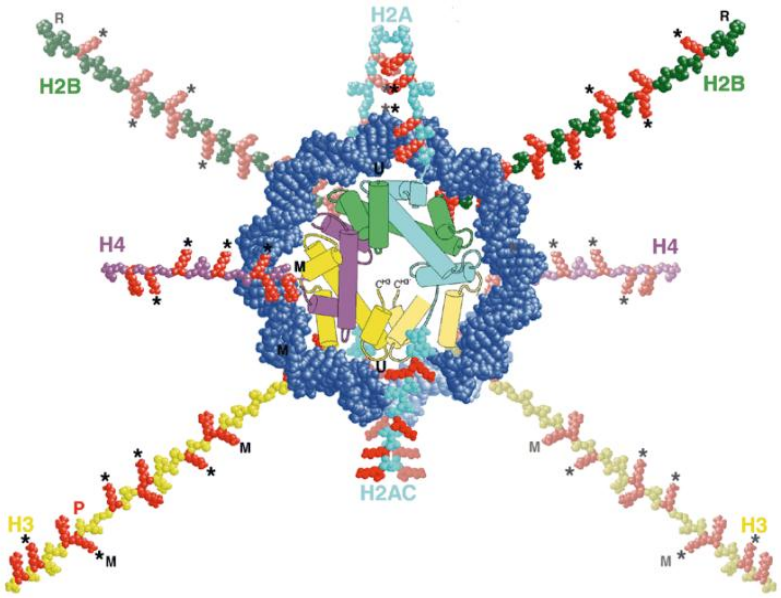

Figure 16 Model of a nucleosome: From: (Wolffe & Hayes, 1999)

4.3.1. Histone Chaperones.

It has been shown that correct DNA condensation is ensured by interactions between H1 and so called "histone chaperones" which modify chromatin dynamics in an ATP-dependent manner (Table 4). The one responsible for correct H3-H4 tetramers deposition on the DNA is called **C**hromatin **A**ssembly **F**actor 1 (CAF-1). Targeting the complex to the newly replicated DNA is enhanced by the **P**roliferating **C**ell **N**uclear **A**ntigen (PCNA), a ring-shaped protein known to encircle DNA and to serve as a processivity factor for the DNA polymerase II (Akey & Luger, 2003; Elsasser & D'Arcy, 2011; Gunjan et al, 2005) The next factor required for efficient nucleosome assembly is the **R**eplication-**C**oupled **A**ssembly **F**actor (RCAF), which comprises the **A**ntis**i**lencing **F**unction **1** protein (ASF1) and acetylated $(H3–H4)_2$ tetramers. An additional histone chaperone is required for deposition of two H2A–H2B dimers, and this role is thought to be fulfilled by **N**ucleosome **A**ssembly **P**rotein-**1** (NAP-1) (Campos & Reinberg, 2010; Green et al, 2005; Mello et al, 2002; Tagami et al, 2004; Tyler et al, 1999; Tyler et al, 2001).

The regulated deposition of histones onto DNA arises not only during the replication of DNA in S phase, but also during DNA damage outside S phase or in non- dividing cells undergoing gene expression. Most organisms encode histone variants that are constitutively expressed in small quantities during the cell cycle. These variants are incorporated in the nucleosome by distinct pathways. In higher eukaryotes, the **H**istone **R**egulatory **H**omolog **A** (HIRA), is known to promote the assembly of histone H3.3 into chromatin independently of DNA replication .Similar to replication dependent nucleosome assembly mediated by CAF-1, Asf1 is proposed to deliver H3–H4 to HIRA for subsequent nucleosome assembly (Li et al, 2011)

Histone chaperone	Replication coupled (RC)	Replication independent (RI)
Asf1	+	+
CAF-1	+	+
RCAF	+	–
NAP-1	+	–
HIRA(HIR1)	–	+
Daxx	–	+

Table 4. Main histone chaperones, modified from (Li et al, 2011)

4.3.2. Histone replacement variants

As mentioned before in addition to each of the five major histone types (H1, H2A, H2B, H3 and H4), whose genes are expressed during the S phase of the cell cycle, some minor variant forms have been discovered over the years of chromatin studies (Table 5). Many of the replacement variants are replication-independent. They are classified on a structural basis into homomorphous and heteromorphous families, depending on the extent of their amino acid sequence departure from the main canonical isoforms. The homomorphous variants involve only a few amino acid changes (i.e. H2A.1 and H2A.2; H3.1, H3.2 and H3.3) whilst heteromorphous variants involve changes that affect larger portions of the histone molecule [i.e. H2A.X, H2A.Z, macroH2A (mH2A), H2A Barr body-deficient (H2A.Bbd) and centromeric protein A (CENP-A) (Ausio, 2006).

Core histone variants with potential unique functions			
Variant	**Variant species**	**Mutant phenotype**	**Function**
CENP-A	Ubiquitous	Lethal (mouse embryos)	Kinetochore assembly
MacroH2A	Vertebrate	Severe malformations in the brain (Zebrafish)	X-chromosome inactivation; gene expression
H2A-Bbd	Vertebrate	Not Determined	Transcriptional Activation
H2A.Z	Ubiquitous	Lethal (mouse, flies, *Tetrahymena*)	Unclear; altered higher-order chromatin structure
H2AX	Ubiquitous	Tumor formation in mice T- and B-cell lymphomas. Reduced fecundity (females); sterility (males)	DNA repair/recombination/transcription repression
H3.3	Ubiquitous	Embryonicly lethal	Transcription

Table 5 Histone variants Modified from (Kamakaka & Biggins, 2005)

4.3.3. Modification of histone proteins

It is well known that both canonical histones and their variants are often subjected to specific **P**ost **T**ranscriptional **M**odifications (PTMs) i.e. acetylation, methylation, phosphorylation, ubiquitination and poly-ADP-ribosylation (Fig. 17). The evolution from the theory that histones provide only a backbone for DNA to the discovery of the PTMs affecting the deciphering of DNA information surely revolutionized the way chromatin is perceived. Since histone modifications were found to be implicated in a plethora of cellular and developmental processes as well as in diseases the field has developed incredibly fast in the last few years (Bodai et al, 2003; Cress & Seto, 2000). Histone modifications as well as chromatin remodelling facilitate the binding of transcription factors to gene regulatory regions, which allows assembling of the RNA polymerase complex to activate transcription reviewed in (Felsenfeld & Groudine, 2003; Narlikar et al, 2002)).

The combinations of PTMs determine the so-called ‘‘histone code’’ (Fischle et al, 2003).This histone code dictates, on a global scale, the state of the chromatin (euchromatin versus heterochromatin) and, on a smaller scale, the transcriptional activity of specific genes (Bradbury, 1992; Jenuwein & Allis, 2001; Peterson & Laniel, 2004).

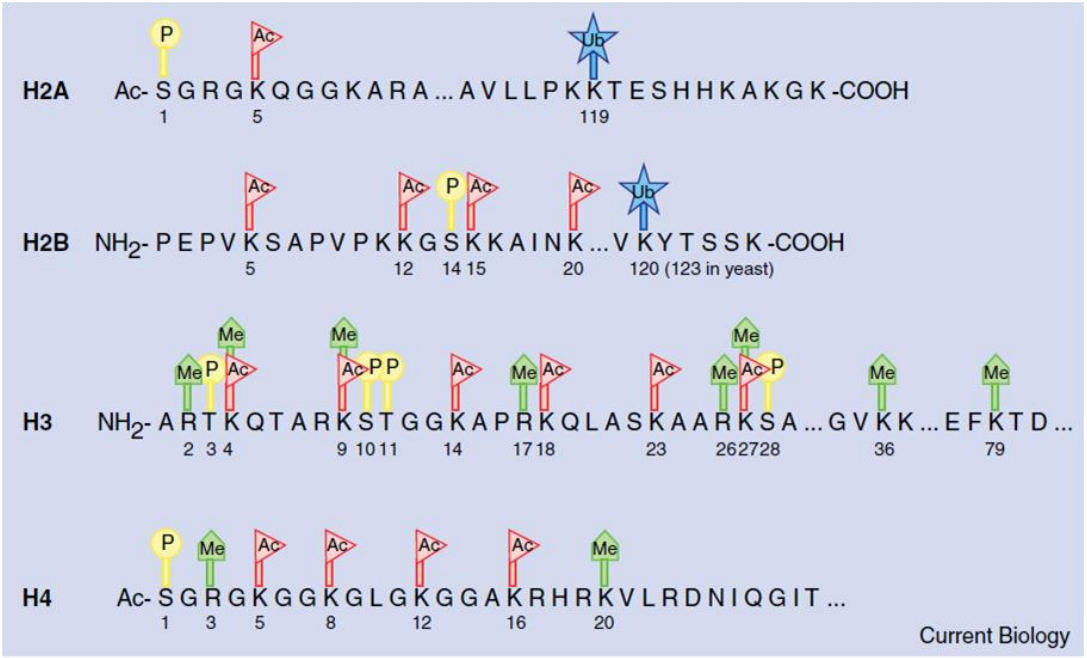

Figure 17. Post-translational modifications of the core histones.The colored shapes represent known post-translational modifications of the core histones. The histone tails can be methylated at lysines and arginines (green pentagons), phosphorylated at serines or threonines (yellow circles), ubiquitylated (blue stars) and acetylated (red triangles) at lysines (Peterson & Laniel, 2004).

4.4. Gene expression

In living organisms five major steps of gene expression can be distinguished. These are the transcription of DNA into pre-mRNA, maturation of pre-mRNA, mRNA translation, mRNA degradation and finally protein degradation. Even though similarities in the main parts of all these mechanisms can be found between different organisms and their various genes, it is clear that each of them is made up of an individually controlled cascade of events.

4.4.1. The origin of histone genes.

Classically one speaks about a unique histone gene family present only in metazoans. As mentioned before this statement lacks precision because these histone genes were identified as well in organisms which classification is not completely clear such as *Volvox carteri* and *Chlamydomonas reinhardtii*. Previous estimation of late evolutionary emergence of histone genes turns out to be incorrect. Samuelssons studies shed new light on this issue. In 2008 they discovered several components of the 3'histone mRNA translational machinery in numerous protozoa such as *Dictyostelium, alveolates*, *Trypanosoma*, and *Trichomonas.* After astute analysis it turned out that these organisms present a potential stem–loop structure in the 3′ UTR (Dávila López & Samuelsson, 2008).

Moreover their data highlight the fact that some histone mRNAs, in meta-, and protozoa, possess a poly(A) tail as well as a 3' SL. This makes up an exciting and unique gene regulation model in which the processing of the 3' end can generate either a poly(A) mRNA or the one characteristic of replication-dependent histone mRNAs with a SL-mediated processing of the 3'end.

Taken together the specific SL and its binding protein SLBP appeared rather early in the eukaryotic evolution process. Probably SLBP was even involved in histone mRNA metabolism regulation, which could include pre-mRNA processing. Although this specificity was lost in numerous species several representative remnants are known in most phyla. In metazoans SLBP acquired additional functions regarding the histone gene expression (Dávila López & Samuelsson, 2008; Marzluff et al, 2008).

4.4.2. Coordinate Regulation of Histone Genes.

One can define the canonical histone proteins, which make up the vast majority of the histones found in a cell at any given time, as those encoded by a family of replication-

dependent histone genes. These genes code for all the four core histones (H2A, H2B, H3 and H4) and for the linker histone H1.

4.4.2.1. Organization of canonical histone genes.

In all metazoans each of the five classes of canonical histones is encoded by between 10 and 20 different genes (this number varies depending on the canonical histone and the species). These canonical histone genes present the unusual feature that they are clustered together. This is interesting as coordinately regulated genes are only rarely physically linked – especially in mammals. This feature, when conserved at a large evolutionary scale, is usually considered to be a mark of a positive selection pressure during evolution, itself resulting from the existence of local factors critical for the correct regulation of the expression of all genes in the cluster. Other famous examples of clusters include the Hox gene cluster, the Major Histocompatibility Complex and the Alpha and Beta Haemoglobin clusters.

Importantly, although the final protein products of the canonical histone genes within a given class are almost exactly identical, each has a distinct promoter sequence and gives rise to an exclusive mRNA, with specific 5'- and 3' UTRs and some slight changes in the nucleotide composition within the coding sequence. In yeast the genes encoding histone proteins are associated as dimers: H2A and H2B, and H3 and H4, respectively are present in the genome on opposite DNA strands as pairs that are transcribed divergently from a common promoter (Hereford et al, 1979). In *Drosophila* single copies of the five genes coding for each of the five histones are associated within a 5 kb unit on the long arm of chromosome 2 which is repeated about a hundred times. Here again within each repeat the couples H2A-H2B and H3-H4 are divergently transcribed from opposite strands with only very short distances between their transcription start sites (Pardue et al, 1977). The histone H1 is encoded by a single gene in *Drosophila*.

In mammals more than 75 canonical histone genes have been identified, but although they are clustered they are not organized in a repeating structure, nor do they share common promoters. Human histone genes are located in three clusters on chromosomes 1 and 6 (Marzluff et al, 2002). The main cluster, HIST1, is located on chromosome 6 (6p21-p22) and contains 55 (80%) histone genes. The two other, smaller clusters, HIST2 and HIST3, are located chromosome 1 (at1q21 and at1q42) and contain 6 and 3 histone genes, respectively

(Marzluff et al, 2002). Each of the 14 H4 genes encodes the same protein whereas there are 3 H3 variants encoded by the 12 H3 histone genes and up to 12 variants for both H2A and H2B. So far there are only two canonical histone genes known which do not fall into any cluster and are expressed during the S phase of the cell cycle. One is the gene coding for the H2AX histone variant whose phosphorylation in response to DNA damage is thought to induce a modified chromatin state favouring the assembly of repair factors at the lesion and the repair reaction itself. The other encodes an H4 variant with an as yet unknown function.

In addition to the replication-dependent histones genes providing the matrix for non polyadenylated histone mRNAs, some histone genes code for polyadenylated mRNAs. Those are non cell cycle dependent and encode so-called "replacement histone variants". Those include CENP-A, MacroH2A, H2AZ, H2A-Bbd, H3.3, $H1^0$, H1oo. Until now no polyadenylated forms of histones H2B or H4 have been detected in mammals.

4.4.2.2. Cajal bodies

In higher eukaryotes histone genes are known to accumulate in or close to specific subnuclear bodies containing proteins required for processing and transcription processes. In mammals their known under the name **C**ajal **B**odies (CBs) characterized by the presence of coilin (Barcaroli et al, 2006b). Typically around 10 of these structures are distinguishable in mammalian cells. CBs are sites of spliceosomal snRNP assembly (Carmo-Fonseca et al., 1992). CBs contain among other factors the U7 snRNP required for the endonucleic cleavage (Shopland et al, 2001) and NPAT required for transcription (Ma et al., 2000). CBs also contain the Flice-associated huge protein (FLASH) which seems to be required for histone mRNA processing (Barcaroli et al, 2006a).

In *Drosophila* the histone genes associate with the Histone Locus Bodies (HLBs) which contain the U7 snRNP as well as components of the cleavage factor and are distinct structures from CBs. Mammalian HLBs may be defined as structures containing U7 snRNP and NPAT that are adjacent to histone genes (Ghule et al, 2008). In Xenopus oocytes they are known as "spheres" contain all the U7snRNPs in the oocyte and a small amount of SLBP. Spheres were found to be located next to the histone gene clusters in Xenopus oocytes (Marzluff, 2005).

4.4.3. Transcription

4.4.3.1. Histone biogenesis and metabolism.

Histone genes are unique and so is the regulation of their expression. Most of the histone genes are cell-cycle regulated. These replication dependent histone genes code for the so-called canonical histones, whose expression must be tightly coupled with the S phase to allow correct packaging of newly replicated DNA. To carry out this highly challenging task properly the histone genes and their mRNAs evolved very specific features and unique mechanisms to make the whole gene expression process as efficient as possible.

Proliferating eukaryotic cells double their DNA content during the S phase, and histone proteins are concomitantly incorporated into newly synthesized DNA. The beginning of S phase is associated with a 3-5 fold increase of histone genes transcription by RNA polymerase II (Marzluff & Duronio, 2002); a 30-50 fold global increase of histone mRNA levels and a 10 fold increase of the efficiency of histone mRNA processing.

The replication dependent histone mRNAs are the only known mRNAs where the 3'UTR poly(A) tail (50-300 nt in length) is replaced by a conserved **S**tem-**L**oop (SL). This structure is followed by an AC-rich element where the adenosines at the second and third position present the highest conservation and are essential for function. This 16nt long secondary structure is found five nucleotides upstream of the 3' end of the histone mRNA. It consists of a 6 bases stem and a 4 bases invariant loop. Moreover the 5 nucleotides lying directly before and after the SL are highly conserved as well. This 25-26-nucleotide sequence present a binding site for the **S**tem **L**oop **B**inding **P**rotein (SLBP) (Dominski & Marzluff, 1999). The SL conformation is stabilized by base stacking interactions and backbone hydrogen bonds.

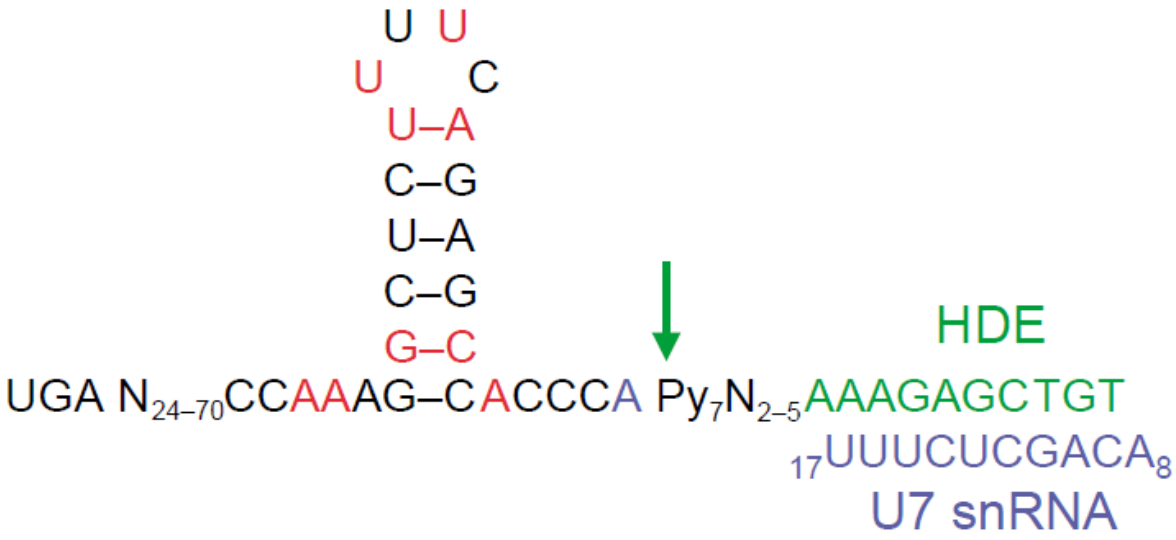

Figure 18. The 3'UTR of mammalian histone pre- mRNAs.

This specific 3′ end is generated by an endo-nucleolytic cleavage during mRNA maturation. It replaces classical pre-mRNA processing consisting of splicing (necessary for intron-containing genes) and polyadenylation. Instead the formation of the SL results in the requirement of a distinct set of factors required for metabolism and regulation of these histone mRNAs. Crucial is the fact that the processing and mRNA translation take place during the S phase what means that histone mRNAs are subject to a rapid decay at the conclusion of S phase or if DNA replication is halted.

4.4.3.2 Transcription regulation.

The mammalian canonical histone genes are transcribed by RNA polymerase II. The observation that histone genes are clustered together originally suggested a common transcription mechanism or common promoters, but astonishingly no unified transcription model has been identified. Regulation patterns seem lineage specific and mainly regulated by gain or loss of transcription factors on their respective binding sites. Interestingly, while the strong correlation between the expression patterns of all canonical histone genes would have suggested the existence of conserved promoter sequences, this does not seem to be the case. Indeed, canonical histone gene promoters only share weak sequence conservation. However,

some conserved sequence elements, located outside of the promoter region, have been identified, and they have been shown to affect the regulation of canonical histone gene transcription (Table 7).

4.4.3.2.1. The cis-regulatory elements and their trans-acting factors.

Years of studies of histone gene transcription led to the identification of two types of conserved sequences acting in cis. Historically the first one was called **S**ubtype **S**pecific **C**onsensus **S**equences (SSCS) while the second is known as "**C**oding **R**egion **A**ctivating **S**equence" (CRAS). They bind a distinct set of proteins responsible for the enhancement of inhibition of histone gene transcription.

4.4.3.2.1.1. CRAS

One of the conserved regions which have been identified a decade ago, was the so-called "**C**oding **R**egion **A**ctivating **S**equence" (CRAS). This element, which is about 110 nucleotides long, is common to the four families of canonical core histone genes (H2A, H2B, H3 and H4) and is located within the coding region (Bowman et al, 1996; Hurt et al, 1991). More recently in rat cells analysis of a H1 linker histone variant gene led to the identification of an incomplete CRAS (Horvath et al, 2003). CRAS contain two 7 bp long conserved DNA elements: histone alpha α (CATGGCG) and histone ω omega (CGAGATC). The histone α element has been found to bind a ubiquitously expressed transcription factor, the Ying-Yang 1 (YY1) protein (Bowman et al, 1996; Eliassen et al, 1998; Hurt et al, 1989). When bound to the CRAS, YY1 enhances the transcription of histone genes during the S phase of the cell cycle.

4.4.3.2.1.2. SSCS

Prior to the discovery of CRAS elements it had been shown that mammalian histone gene promoters are also regulated by distinct cis-acting sequences termed **S**ubtype **S**pecific **C**onsensus **S**equences (SSCS) (La Bella & Heintz, 1991; La Bella et al, 1988). Different transcriptional regulators are known to associate with the SSCSs depending on the histone subtype. For example the HINF-P factor has been demonstrated to associate with the SSCS

specific for H4. Another transcription factor the **Oc**tamer-Binding **P**rotein-**1** (OCT-1) together with its co-activator OCA-S (OCT-1 co-activator in S phase, a protein complex) is specific for the H2B gene promoter. The transcription factor E2F binds the H2A.1 promoter and activates its transcription (Oswald et al, 1996). The H1 proximal SSCS associates with the transcription factor TF2 in a cell-cycle regulated manner (La Bella & Heintz, 1991). This factor recruits a global regulator of histone transcription, the **N**uclear **P**rotein **A**taxia-**T**elangiectasia (NPAT) (Zhao et al, 2000). Interestingly, immunofluorescence experiments have shown that NPAT localises within/or close to Cajal Bodies (CB), nuclear foci that are themselves found in close proximity with histone gene clusters (see below). NPAT is phosphorylated at the G1/S phase border by the Cyclin-Dependent Kinase 2 E-CDK2 in CBs. Only the phosphorylated form endures throughout S phase resulting in an canonical histone gene expression increase. Thus NPAT establishes a link between the cell-cycle regulation machinery and the replication-dependent histone gene transcription (Zhao, 2004)

4.4.3.2.1.3. HIRA

HIRA (**Hi**stone **R**egulatory **H**omolog **A**) was discovered as a transcriptional repressor active during the whole cell cycle, except the S phase (Gunjan et al, 2005). Its activity stales the cells in S phase by blocking the DNA synthesis. Alike NPATs, HIRAs activity is kinase-related. In contrast to NPAT phosphorylation by cyclin E-CDK2 at the G1/S phase transition (Zhao et al, 2000), phosphorylation of HIRA occurs during S phase by the cyclin A-CDK2 and E-CDK2) suggesting that this repression factor is inactive in its phosphorylated state. Intriguingly HIRA acts both as a transcriptional repressor and as a histone chaperone (incorporating the H3.3 histone variant in the nucleosome) (Gunjan et al, 2005). This double function remains unexplained.

It remains obscure how all this factors collaborate in order to ensure a coordinate histone gene transcription. As the cells progress through the cell cycle and enter S phase, cyclin E/cdk2 dependent phosphorylation of NPAT allows its association with histone promoter sequences. NPAT and YY1 then cooperate to promote histone gene transcription, while HIRA becomes inactive due to phosphorylation. However, it is thought that stimulation of histone gene transcription only makes a minor contribution to the massive augmentation of histone mRNA levels and histone protein synthesis expression around the G1/S transition (Baumbach et al,

1987; Harris et al, 1991; Sive et al, 1984), indicating that at least one more layer of regulation is involved – in all probability at the levels of mRNA stability and/or transcription.

Histone	Common cis-acting sequences	Specific trans acting factors	Global trans-activator	Global trans-repressor
H2A	CRAS SSCS	E2F	NPAT YY1	HIRA
H2B		Oct-1, OCA-S		
H3		Unknown		
H4		HINF-P		
H1		TF2		

Table 7. Histone gene transcription regulating sequences and factors found in mammalian cells.

3.4.4. Histone pre-mRNA processing.

The transcription results typically in a long 3'extension which requires pre-mRNA maturation. Typically a human gene contains 8 exons. In average internal exons are 145 nucleotides long and introns average more than 10 times this size (Lander et al, 2001). The nascent mRNA has to undergoes splicing, 5'capping and 3'polyadenylation. Concomitant with increased transcription is an elevated level of histone pre-mRNA processing which stabilizes the mRNAs and results in a 35-fold increase of histone mRNA levels. This increase is accomplished via upregulation of SLBP levels (Whitfield et al, 2000). Thus, throughout S phase histone genes are highly transcribed, their pre-mRNAs efficiently processed and packaged into mature messenger ribonucleoproteins (mRNPs) with SLBP, at which point they are ready for export to the cytoplasm and translation.

Although in metazoans replication-dependent histone pre-mRNA processing is quite limited compared to canonical pre-mRNAs it remained unexplored for a long time. It is only recently that we started getting more understanding about the complexity of the endonuclolytic cleavage reaction important for histone pre-mRNA maturation.

Since core histone gene transcripts lack introns and are the only known eukaryotic RNAs lacking the poly(A) tail, the synthesis of a mature histone mRNA requires only one RNA-processing reaction. To form a proper 3´end an endonucleolytic cleavage is carried out. Analogously to the poly(A)-pre-mRNAs, the nascent histone mRNA presents two typical structures on its 3' end. One is the conserved 26-nucleotide sequence that contains a 16-nucleotide **S**tem-**L**oop (SL) (Fig. 19), the other a purine-rich region, the **H**istone **D**ownstream **E**lement (HDE). The cleavage takes place between these two sequences.

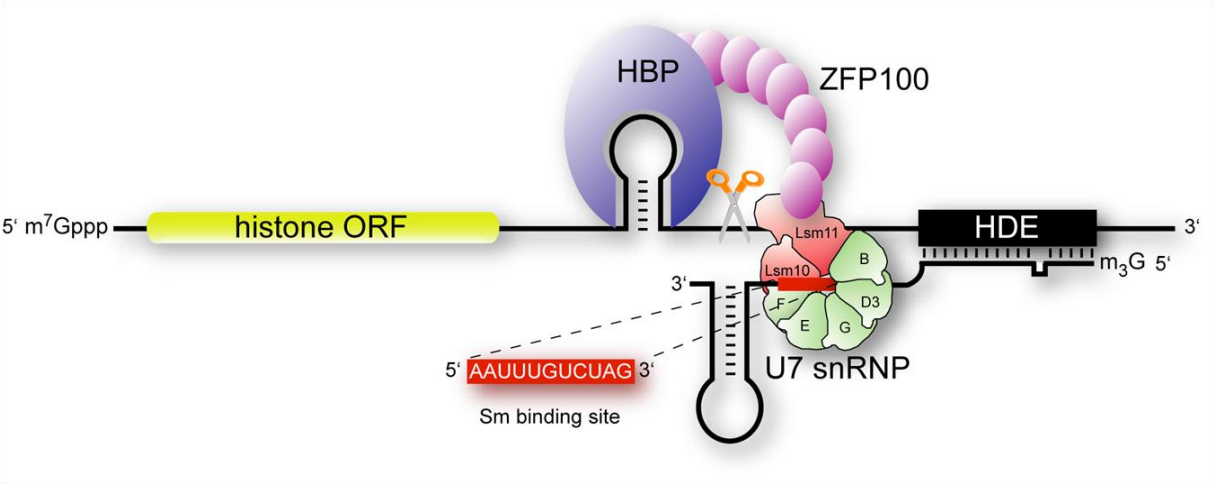

Figure. 19 The histone mRNA processing. Several factors are recruited via SLBP and the HDE downstream of SLBP to perform a cleavage reaction several nucleotides downstream of the SL.

4.4.4.1 SLBP

The histone mRNA processing is ensured by the **S**tem **L**oop **B**inding **P**rotein (SLBP) bound to the stem-loop. This factor is also involved in other steps of histone mRNA metabolism, and has his part in the recruitment of the U7 snRNP complex by the HDE (Sullivan et al, 2009b). SLBP is a 32 kDa protein with a unique 73 AA **R**NA **B**inding **D**omain (RBD). In somatic cells, SLBP is detectable only during S phase of the cell cycle (Whitfield et al, 2000). SLBP acts by stabilizing the U7 snRNP on the histone pre-mRNA. It has been demonstrated both *in vitro* and *in vivo* that the presence of SLBP, though not absolutely necessary, is critical for histone mRNA processing which in the absence of SLBP is only half that efficient. Pandey et al. reported that mutations in the SL preventing SLBP binding resulted in the inhibition of histone mRNA expression *in vivo* (Pandey et al, 1994).

4.4.4.2. The mRNA processing complex

The U7 snRNA is measuring only 57-70nt depending on species. Its 5'end contains a conserved CUCUU sequence complementary to the AAGAG sequence in the core of HDE which is followed by the Sm binding side. The Sm complex is a homolog of the spliceosomal snRNPs. Its Sm heteroheptameric ring is composed of Sm Lsm10 and Lsm11 which replace the spliceosomal SmD1 and SmD2 (Pillai et al, 2003). SmB and SmD3, interact with the region lying in-between the cleavage site and HDE. SmG, E and F complete the structure of the ring.

Another stabilizing factor is a Zinc-finger protein, ZFP100 that interacts with both SLBP bound to the SL (Dominski et al, 2002) and the Lsm11 (Azzouz et al, 2005; Wagner & Marzluff, 2006). This factor is required for entry into the S phase and is limiting for histone pre-RNA processing *in vivo* (Wagner & Marzluff, 2006).

Cleavage is catalyzed by CPSF73 (Dominski et al, 2005a) Symplekin and CPSF100. CPSF73 has been identified as the endonuclease for both polyadenylated (Mandel et al, 2006) and histone mRNAs (Dominski et al, 2005b). Symplekin has been originally described as a tight junction protein in mammalian cells (Keon et al, 1996). Last but not least, CPSF-100 is like CPSF73 a member of the metallo-6-lactamase family of zinc-dependent endonucleases and required for cleavage, though it lacks residues critical for catalysis (Kolev et al, 2008).

The cleavage efficiency requires the interaction between Lsm11 and a recently characterized protein FLASH (Yang et al, 2009) that might act as a recruitment factor for CPSF73, CPSF-100 and symplekin (Yang et al, 2011).

Once the processing is completed a **D**ownstream **C**leavage **P**roduct (DCP) is released and quickly degraded in order to recycle the U7 snRNP.

4.4.5. Nuclear export

The more a cell's nucleus is active the more its envelope will present **N**uclear **P**ore **C**omplexes (NPCs). In mammals their number can vary between 3000 and 4000 per nucleus. During DNA synthesis the cell needs to deal with the complex task of importing about 10^6 histone molecules every 3 minutes from the cytosol. This means that 100 histone molecules must pass through each NPC every minute.

Simultaneously to transcription mRNA associates with members of the hnRNP family. They are believed to modulate many aspects of the nuclear fate of pre-mRNAs, including their appropriate processing and folding, and remain associated with nuclear mRNAs after splicing is completed. During mRNA nuclear export, some of the hnRNPs, such as hnRNP C, are selectively removed at the NPC while others, such as hnRNPs A1 and K, accompany the mature mRNA into the cell cytoplasm and are only then released and returned to the nucleus. Different macromolecules and complexes are subject to a distinct export mechanism. Many are exported by the importin β-like receptors (for review, see (Fried & Kutay, 2003)).Those receptors require Ran, a small GTPase. Some RNAs like tRNA and snRNA are shipped by Ran-dependent mechanism. On the contrary most of the canonical PolyA mRNAs require a conserved export receptor Nxf1/ TAP (Mex67 in yeast) (Gruter et al, 1998; Segref et al, 1997).

4.4.5.1. General mRNA export mechanisms.

Most mRNAs are derived from genes containing introns, which are specifically removed during the splicing process prior to nuclear export and translation. Additionally, an m^7G cap and a polyadenylation signal are added at the 5' and 3’ ends, respectively, but neither is necessary or sufficient by itself for mRNA export, although both can enhance it (Cullen, 2003).

The different pathways for RNA export from the nucleus are listed below:

- TAP-mediated export
- The transcription/export or TREX complex that associates with pre-mRNAs during transcription elongation (Lei et al, 2001; Strasser et al, 2002; Zenklusen et al, 2002).
- Aly/REF splicing-dependent mechanism as part of a complex of proteins, the exon junction complex (EJC) (Kataoka et al, 2001; Kataoka et al, 2000; Le Hir et al, 2001a; Le Hir et al, 2000a).
- Aly/REF splicing-independent mechanism (Masuyama et al, 2004). Unstructured RNA itself can support export factor recruitment and hence serves as an mRNA export identity element (Ohno et al, 2002).

None of these mechanisms are mutually exclusive, and it is not clear which mechanism for mRNA export adapter recruitment predominates in the cell.

4.4.5.2. Histone mRNA export.

Only 8% of human genes are known to be intronless. This suggests a strong evolutionary pressure driving the maintenance of at least one intron per gene. Some data reveal the importance of new intron insertions in several cellular genes (Fedorova & Fedorov, 2003).
Most of the intronless genes are expressed transitionally and in extreme conditions like stress-response. RNAs encoded by those genes are not exported from the nucleus by the same mechanism as intron-containing genes. Thus it is likely that cells have evolved an alternative export mechanism for non-canonical RNAs.

Some of the non-canonical mRNAs share specific structures/features that have been shown to be required for their specific maturation and export pattern. However several reports show that intronless genes recruit the same export factors used by the classical RNAs. Among the "alternative RNAs" the ones whose export has been most extensively described are those coding for (i) histones, (ii) IFN-α1, (iii) Jun and (iv) HSPA1. In the case of histone mRNAs the bio-molecular procedures that take place following processing in the nucleus and prior to translation are still ill-understood. The nuclear export is thought to be rapid, taking around 5 min. (Schochetman & Perry, 1972). Evidence coming from mammalian tissue culture cells suggests that the histone mRNA 3' end is necessary for the accumulation of histone mRNAs in the cytoplasm and that U7 snRNP-mediated mature 3'end formation facilitates export (Eckner et al, 1991). In addition, replication-dependent histone mRNAs have been shown to contain specific transport elements in the protein coding region capable of redistributing a heterologous mRNA to the cytoplasm (Huang & Carmichael, 1997). These transport elements recruit specific adaptors to the mRNAs. There is other evidence for the ability of SR ASF/SF2 proteins to associate with H2A mRNA in human cells (Lai & Tarn, 2004). A 22nt sequence lying in the middle of the H2A mRNA was observed to be the binding site for SR (Huang & Steitz, 2001).
The splicing factors 9G8 and SRp20 can be cross-linked to histone mRNA transport elements (Huang & Steitz, 2001) and 9G8 interacts with the mRNA export receptor TAP (Huang et al, 2003). These observations, and work performed by others (Braun et al, 1999; Huang et al, 2003), suggest that TAP mediates the export of replication-dependent histone mRNAs from the nucleus. Studies from both *Drosophila* and *Xenopus* cells (Erkmann et al, 2005; Huang & Steitz, 2001) confirmed that nuclear export of histone mRNA is TAP-dependent. Depletion by

RNA interference of the export factor TAP leads to nuclear export arrest (Erkmann et al, 2005). The authors claim as well that the histone mRNA export is SLBP independent since mutants of the SL 3' sequence unable to bind SLBP, failed to affect the nuclear export. It was suggested by this group that it is rather the length of the region upstream of the SL, and not the sequence of the region itself, which is important for efficient export. However more recent studies have shown that deletion of the SL as well as SLBP knockdown result in nuclear RNA retention. (Ghule et al, 2008; Sullivan et al, 2009a). Thus, besides its role in the 3' end formation, SLBP is also important for histone mRNA export towards the cytoplasm.

4.4.6. Translation

In 1956 Francis Crick laid out in the "**Ideas on Protein Synthesis**" the Doctrine of the Triad: *"Once information has got into a protein it can't get out again*. *Information here means the sequence of the amino acid residues, or other sequences related to it"* (CRICK, 1958). He re-stated his idea in 1970 in Nature as follows: *"The central dogma of molecular biology deals with the detailed residue-by-residue transfer of sequential information. It states that information cannot be transferred back from protein to either protein or nucleic acid"* (Crick, 1970; CRICK, 1958).

In other words, the process of producing proteins is irreversible: a protein cannot be used to create DNA information. This unique chance to obtain the right polypeptide in the living cell occurs immediately after processing and export of the mRNA to the cytoplasm, when the molecule is ready to be translated into proteins. In eukaryotes translation is an extremely complex process which can be divided in three main stages: initiation, elongation and termination. Although the speed of elongation and termination are also important for the rate of translation (Cannarozzi et al; Pisarev et al, 2007; Pisarev et al; Tuller et al), the initiation of translation is the most controlled step (Jackson et al; Sonenberg & Hinnebusch, 2009).

4.4.6.1. Translation initiation

During peptide synthesis an mRNA is scanned and translated by ribosomes in a 5' to 3' direction until it reaches a stop codon. The basic structure ensuring translation in a cell is the ribosome. This Ribo-nuclear Particle contains an almost equal amount of ribosomal RNA (rRNA) and proteins. Ribosomes are divided into two subunits. The smaller subunit (40S) binds to the mRNA, while the larger subunit (60S) binds to the tRNA and the amino acids.

Together they form a mature and translationally active ribosome (80S). When a ribosome finishes reading the mRNA, these two subunits split apart and are recycled.

Translation initiation begins with the recruitment of the small 40S ribosomal subunit onto the mRNA and ends when the elongation-competent 80S ribosome is assembled and the initiator codon is base-paired with the initiator tRNA in the ribosome P-site. It takes up to 13 **e**ukaryotic **I**nitiation **F**actors (eIFs) (Fabian et al, 2010; LeFebvre et al, 2006) to ensure proper binding of the 40S ribosomal subunit to the RNA and its assembly with the 60S subunit into a functional 80S ribosome placed at the correct initiation codon. Therefore, translation initiation rates play key roles in many fundamental biological processes such as cell growth, differentiation or responses to biological and environmental stress conditions. In eukaryotes two different initiation models are to be listed. Firstly the "classical" cap-dependent model, secondly the cap-independent one (IRES-driven). In this manuscript I would focus on a novel translational model that might be a fusion of these two, providing an extremely efficient translational mechanism. This last one evolved in histone mRNAs.

4.4.6.1.1. Cap-dependent translation initiation

Classical, nuclear-encoded, poly(A) mRNA translation involves the 5'-terminal m^7GpppN cap (where N is any nucleotide) (Shatkin, 1976) that enhances initiation by a cap-dependent ribosomal scanning mechanism (Kozak, 1978; Kozak, 1989). Cap enhances translation trough binding with the eIF4F complex. The cap-dependent translation initiation is comprised of the following steps:

(i) 43S **P**re-**I**nitiation **C**omplex (PIC) formation,
(ii) PIC loading onto the 5' end of mRNA,
(iii) mRNA circularization
(iv)ribosomal scanning of the 5'-UTR,
(v) initiation codon recognition,
(vi)ribosomal subunits joining and
(vii) ribosomal subunits and initiation factors recycling and re-initiation.

4.4.6.1.1.1. Formation of a 43S pre-initiation complex

Former to 43S complex formation the 40S subunit is activated by IFs and the initiator tRNA. Then $tRNA_i^{Met}$ and eIF2-GTP building a ternary complex (TC), bind to the recycled 40S small ribosomal subunit, to form the 43S complex stabilized by eIF3, eIF1, eIF1A and

probably eIF5. (Benne & Hershey, 1978; Chaudhuri et al, 1999; Chaudhuri et al, 1997; Majumdar et al, 2003; Passmore et al, 2007; Peterson et al, 1979; Thomas et al, 1980; Trachsel et al, 1977) These factors play further important roles in the succeeding steps of translation initiation namely in 43S pre-initiation complex loading onto the mRNA and start of codon recognition.

4.4.6.1.1.2. PIC loading onto the 5' end of mRNA and mRNA circularization

Despite the interesting ability of the 43S pre-initiation complex to bind mRNA alone and promote translation in a 5' end-dependent manner, the 5'-UTR of eukaryotic mRNAs usually contains secondary structures which need to be unwound and prepared for the attachment of the 43S pre-initiation complex. This action requires other translation initiation factors as: (i) eIF4F holoenzyme binding the 5' terminal cap-structure and unwinding local structures assisted by (ii) eIF4B or (iii) eIF4H creating a fixation platform for the 43S pre-initiation complex (Pestova & Kolupaeva, 2002). The eIF4F is a complex comprising the cap-binding protein eIF4E, the DEAD-box RNA helicase eIF4A and eIF4G. In its turn eIF4G functions as a scaffold binding eIF4E, eIF4A, eIF3 and the **P**oly(**A**)-**B**inding **P**rotein (PABP).

Through its physical interaction with PABP, eIF4G allows the 5'-3' cross-talk of the mRNA (Imataka et al, 1998; Tarun & Sachs, 1996b; Wells et al, 1998), and performs mRNA circularization. In this manner the cap and the poly(A) tail cooperatively lead to an enhancement of translation (Preiss & Hentze, 1998; Tarun & Sachs, 1995; Tarun & Sachs, 1996b).It is still not clear whether the mRNA circularization enhances translation directly or only increases the affinity of certain initiation factors for the translated mRNA (Kahvejian et al, 2005). PABP's binding is regulated by the PABP - Interacting Proteins (PAIPs), PAIP1 and PAIP2. PAIP1 contains two binding domains for PABP that lie on either side of a region similar to the central portion of eIF4G (MIF4G domain (Roy et al, 2002)). PAIP1 stimulates translation by reinforcing the PABP-Poly(A) interaction whereas PAIP2 (A and B) strongly inhibits translation. PAIP2 shares homolog regions with PAIP1 (Khaleghpour et al, 2001; Roy et al, 2002). It has been shown that the inhibition of translation (in vitro and in vivo) mediated by PAIP2 is due to the decrease of PABP's affinity to the poly(A) tail and to eIF4G thus preventing the mRNA from the circularization, which is dependent on the 5′ and 3′ ends cross talk mediated via PABP-eIF4G and PABP-PAIP1-eIF4A (Gingras et al, 1999; Sachs & Varani, 2000; Tarun & Sachs, 1996a). Thus both PAIP1 and PAIP2 compete for PABP

binding. Further investigations have led to a more complex model PAIP1-PABP-eIF4G and PAIP1-eIF3-eIF4G form two ternary complexes. Taken together, these data demonstrate that the eIF3-PAIP1 and PAIP1-eIF4G interactions can promote translation initiation where eIF3-PAIP1 stabilize the interaction between PABP and eIF4G, making the RNA-looping possible (Martineau et al, 2008).

4.4.6.1.1.3. Ribosomal scanning of the 5'-UTR

Right after mRNA circularization follows the stalling of PIC on the mRNA and the formation of a 48S pre-initiation complex made up by 43S PIC, eIF3 and eIF4G (Imataka & Sonenberg, 1997; Korneeva et al, 2000; Lamphear et al, 1995; Ohlmann et al, 1996; Rau et al, 1996; Safer et al, 1978). Then the 48S-PIC scans in a 5' to 3' direction from the cap structure until it reaches an initiation codon. Commonly the initiation starts at the first AUG codon but initiation can occur at other codons such as: CUG, GUG, UUG, AUA or ACG (Kozak, 1995; Kozak, 1997; Wegrzyn et al, 2008). Scanning of long and structured 5'-UTR needs ATP-assisted unwinding. The initiation factors: eIF4A, eIF4G and eIF4B, eIF4A are also needed for proper scanning but the exact mechanism was not discovered until now. Some recent studies highlight the involvement of other RNA helicases such as DHX29 and DDX3/Ded1p in the scanning process (Jackson et al, 2010; Parsyan et al, 2009; Pisareva et al, 2008).

4.4.6.1.1.3. Ribosomal scanning of the 5'-UTR

The recognition of the initiation codon depends as well on its sequence as on the sequences upstream of it. The optimal sequence context seems to be the following: GCC(A/G)CCAUGG for AUG initiation codon (Chen et al, 2009; Chen et al, 2008; Kozak, 1995; Kozak, 1997). Once the ribosome encounters the right initiation codon the ribosome 40S small subunit becomes anchored on the RNA to be joined by the 60S subunit thereby forming an elongation-functional 80S ribosome. The ribosome association as well as the simultaneous dissociation of the eIF41, eIF41A, eIF3 and residual eIF2 are eIF5B-mediated.

4.4.6.1.1.5. Recycling

After the ribosome encounters a STOP codon, the synthesized polypeptide is released by a process that depends on eukaryotic release factors eRFs1 and eRF3 (Alkalaeva et al, 2006).

At the very end ribosomes are evicted from the RNA and the 40S ribosomal subunits are recycled for new rounds of translation by the action of eIF3j, eIF1A, eIF1 and ABCE1. These factors dissociate post-termination complexes by displacing elongation factors, 60S subunits, deacetylated tRNA and the mRNA allowing the formation of new pre-initiation complexes.

4.4.6.1.2. Cap-independent translation initiation

Studies of picornavirus translation have permitted to define a new mechanism which is mediated by *cis* acting RNA elements located in the 5'-UTR of the viral mRNA. These RNA structures present in the 5'-UTR allow internal binding of the 40S ribosomal subunit with the associated initiation factors and are defined as **I**nternal **R**ibosome **E**ntry **S**ites (IRES) (Balvay et al, 2009; Stoneley & Willis, 2004).

4.4.6.1.2.1. IRES

IRES are RNA sequences found in the 5'-UTR of the mRNA. Their function is to recruit ribosomes for translation initiation in a cap-independent manner. IRES elements were first discovered in viral RNA genomes more than 20 years ago and have been also found in many cellular mRNAs. Since then a huge diversity in size, sequence and structure has been reported. In total 56 viral and 73 cellular (the first being the immunoglobulin heavy chain binding protein) (Macejak & Sarnow, 1991) IRES elements have been characterized and so far no evidence for any conserved sequence was found, making a polyphyletic origin plausible (Balvay et al, 2007; Balvay et al, 2009). Despite their differences, all IRES elements can be defined as an RNA domain that has the ability to recruit ribosomes in a cap-independent manner. Although it is known that IRES elements require diverse cellular accessory proteins (collectively denominated **I**RES **T**rans-**A**cting **F**actors (ITAFs)) for proper function, the mechanism of cellular IRES function remains unclear. IRES-mediated translation occurs during many cellular stress conditions that are known to inhibit cap-dependent translation, including hypoxia, irradiation, apoptosis, angiogenesis, amino-acid starvation, continuous heat shock and mitosis. As a result, internal initiation allows the translation process to circumvent the cap-dependent control mechanisms, especially the steps involving.(Balvay et al, 2007; Balvay et al, 2009)

The advantage of IRES ribosome positioning for cellular RNA is obvious during shutdown of cap-dependent translation. The mRNAs involved for example in cell-cycle control, growth

and apoptosis, rely alternatively on internal ribosome entry sequences (IRES). If for any reason the cap-dependent translation is inhibited these RNAs remain bound to the polysomes and maintain active expression. Interestingly, it has been reported that mRNAs synthesizing proteins requested during mitosis undergo an IRES-driven translation initiation (Cornelis et al, 2000). Pyronnet et al. 2000; Sachs 2000). The precise mechanism of translation via cellular IRES and its regulation as well as how cellular mRNAs can switch from cap- to IRES-dependent translation initiation remains unclear.

4.4.6.1.3. Histone mRNA specific translation – towards a new translation model.

The expression of these cell-cycle regulated, multi-copy replication-dependent genes is a unique occurrence in living organisms. As mentioned before histone mRNAs are the only mRNAs ending with a conserved RNA hairpin element (Stem Loop) and lacking a poly(A) tail. Therefore it comes as no surprise that their translation has been shown to differ a great way from the classical model in both *Vertebrates* and *Drosophila*.

During the S phase a great number of histones is demanded. The histone genes are principally expressed in the mid-S phase (van der Meijden et al, 2002), meaning that during around one hour 60 million copies of each core histone have to be synthesized. Such a challenging task suggests the existence of an extremely efficient and fine controlled translation mechanism. As previously reported translation during the S phase is cap-dependent (Pyronnet et al, 2001; Pyronnet & Sonenberg, 2001).

The discovery of the importance of both the RNA hairpin element and its binding protein SLBP (or HBP) for histone mRNA translation revolutionized the view on cap-dependent translation.

4.4.6.1.3.2. Implication of the 5'UTR in the initiation of translation.

Until recently most of the studies of histone mRNA translation focused on the unique 3'UTR of the replication dependent histone genes, not taking into account the characteristics of the 5' terminus since it was known to present a usual cap-structure. As described before mRNA scanning is one of the crucial steps of translation initiation. Until recentely this step was believed to be an attribute of canonical cap-dependent translation initiation. Histone mRNAs are known to present particularly short 5'UTRs, especially those coding for histone H4. This feature means that these mRNAs are not fit substrates for the classical mechanism of

ribosomal scanning. Recent work by Martin and co-workers demonstrated that within H4 mRNA two structural elements are essential for translation initiation. The first recruits eIF4E while the second conceals the cap. Binding of eIF4E to the first element allows ribosomal tethering trough eIF4E even though the cap is not accessible. Thus the 43S PICis is directly loaded close to the start codon. The second structure is located 19 nt downstream of the initiation codon and forms a characteristic "three-way helix junction". Both of these elements are thus believed to assist in the correct placement of the ribosome on a relevant start codon.
The authors propose a combined-translation model where the cap being hidden in the three-way helix is not available so the ribosome has to be tethered directly on an AUG instead of scanning for the start codon. One notable advantage of such an initiation mechanism is the absence of a pioneer translation round to promote the CBC-eIF4E exchange (Ishigaki et al, 2001). This confers a greater efficiency of translation initiation in that the H4 mRNA-eIF4E complex is immediately "ready to go" for translation (Martin et al, 2011).
Interestingly, this model of histone translation model combines in an elegant way features of both the canonical and IRES-dependent models of translation initiation.

4.4.6.1.3.1 Implication of the 3'UTR in the initiation of translation.

Interestingly some similarities between Poly(A)-mediated and SL-driven translational model can be found. The analysis of SLBP interacting partners revealed the need of eIF4E, eIF4G, and eIF3 for correct translation initiation. Moreover SLBP co-purified with cap- and poly(A)-associated initiation complexes. It was shown that the interaction of SLBP with eIF4F was mediated by eIF4G and eIF3 indicating that SLBP is functionally similar to PABP. Thus the enhancement of translation initiation involves contacts of SLBP with the cap-interacting initiation factors (Gorgoni et al, 2005; Ling et al, 2002b).

4.4.6.1.3.1.1. SLIP-1 /MIF4GD

From the previously described models it can be concluded that SLBP occupies a central role in the histone translational model. Recent studies highlight a novel protein required for histone mRNA translation. It has been identified in a two hybrid screen performed with SLBP as bait which was accordingly named **S**LBP **I**nteracting **P**rotein **1** (SLIP-1) (Cakmakci et al,

2008b). This small 25 kD protein corresponds almost entirely to a middle domain of eukaryotic initiation factor 4G (MIF4G) and is therefore called as well **MIF4G D**omain Containing Protein (MIF4GD). The study by the Cakmakci group provides evidence for SLIP-1 strongly stimulating histone mRNA translation trough the interaction with SLBP. Surprisingly SLIP-1 interacts as well with eIF4G. This binding takes places on another site than the one known for PABP. Importantly this interaction seems to be direct unlike that between SLBP and eIF4G. This indicates that SLIP-1 acts as a bridge between SLBP and eIF4G and in this way helps the cross-talk between the 3' and 5' termini of histone mRNA.

In canonical RNAs the looping of the RNA is guaranteed by the formation of ternary complexes composed of PAIP1-PABP-eIF4G and PAIP1-eIF3-eIF4G (Martineau et al, 2008). Importantly Martineau et al. have shown that the interaction of PAIP1 with eIF3 takes place through the g subunit of eIF3. However they also revealed EIF3E (INT6) in a far-western experiment. Although the 3' end of the histone gene lacks a poly(A) tail, the general mRNP organization allowing efficient translation initiation might be very similar and thus the following histone translation model emerges: 5'RNA cap- eIF4E-eIF4G-eIF3(?)-SLIPl-SLBP-3' RNA SL structure. The couple SLBP-SLIP1 might though act in a similar way as PABP-PAIP1 by allowing looping of the RNA along with contacts with eIF4G and eIF3. This model is still incomplete. One can imagine other initiation factors, for example some of the IF3 subunits stabilizing the protein-protein interactions necessary for RNA circularization and/or initiating the translation machinery.

4.4.7. RNA degradation.

The initial step in degradation of most polyadenylated mRNAs in eukaryotes is shortening of the poly(A) tail by a deadenylase complex (Parker & Song, 2004). Deadenylated mRNAs can then either return to a translationally active state on polysomes, likely by the enzymatic action of cytoplasmic poly(A) polymerases (Coller & Parker, 2005), or can alternately be degraded from either terminus by a distinct set of nucleases.

Process	Canonical histone mRNA specific	Shared with polyadenylated mRNAs
Transcription	NPAT, HIRA(repression factor)	TBP, HINFd, YY-1, OCT1, OCA-S
Processing	SLBP, U7 snRNP (snRNA, Lsm-10, Lsm-11), ZFP100, FLASH	Symplekin, CPSF-100, CPSF73, FIP1, Sm
Export	SLBP	TAP, Rev
Translation	SLBP,SLIP,INT6	EIF3, EIF4GI, EIF4GII
mRNA Decay	SLBP, TUTase	Lsm1, exosome, UPF1, DCP, XRN1

Table 8. An overview of factors involved in canonical histone gene expression.

4.4.7.1. Histone mRNA degradation.

Following the end of S phase and the associated stop to DNA synthesis activities, the level of histone mRNAs promptly decreases. The half-life of histone mRNAs drops from 45-60 minutes to 10 minutes, leading to near complete elimination of histone mRNAs in G2 (Marzluff & Duronio, 2002). These results suggest that histone expression relies mainly on the posttranscriptional regulation of histone mRNAs (Fig.20)

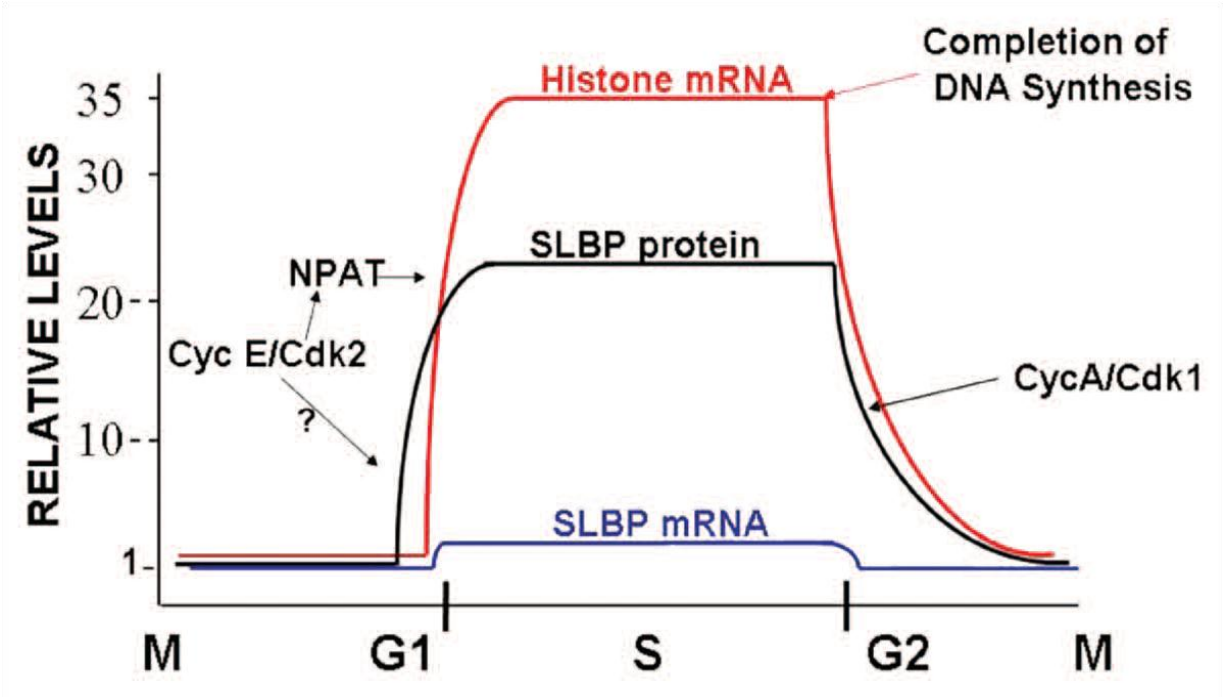

Figure 20. **Cell cycle regulation of canonical histone mRNA along with SLBP.** The presence of SLBP determines the maximal levels of replication-dependent histone mRNA that can accumulate in the cytoplasm. As cells reach the end of S phase, replication-dependent histone mRNA is rapidly degraded, along with SLBP

The degradation process is also dependent upon translation since cycloheximide treatment stabilizes histone mRNAs (Kaygun & Marzluff, 2005b). Histone mRNAs are degraded as a result of stopping DNA synthesis, and SLBP is degraded as a result of phosphorylation by cyclinA/Cdkl (Kaygun & Marzluff, 2005b). The expression of SLBP coincides with that of histones and is therefore cell-cycle dependent. SLBP accompanies the nascent histone mRNA from the processing in the nucleus until the end of the S phase where it undergoes itself rapid degradation. Regarding this process it has been shown that SLBP interacts with UPF1 (one of the main/core RNA decay factors) at the 3'end of histone mRNA immediately after inhibition of DNA replication and UPF1 plays therefore a direct role in histone mRNA decay (Kaygun & Marzluff, 2005b; Kaygun & Marzluff, 2005c). For proper decay a modification of the 3' end by oligourydilation (oligo(U)) (Mullen & Marzluff, 2008) is required.

The 8-12 nt oligo(U) tail added by the **T**erminal **U**rydylyl **T**ransferase (TUTase) at the 3' end provides a specific binding platform for Lsm 1-7, which together with SLBP, binds the histone mRNA. (Mullen & Marzluff, 2008) Lsm 1-7 complex probably recruits the enzymes executing the histone mRNA, as it does for the classical polyadenylated mRNAs (Parker & Song, 2004). Degradation involves the 5'-3' XRN1 exonuclease and the exosome in the 3'-5'

direction (Fig. 21). Together it results in a bidirectional decay mechanism for histone mRNAs in mammals.

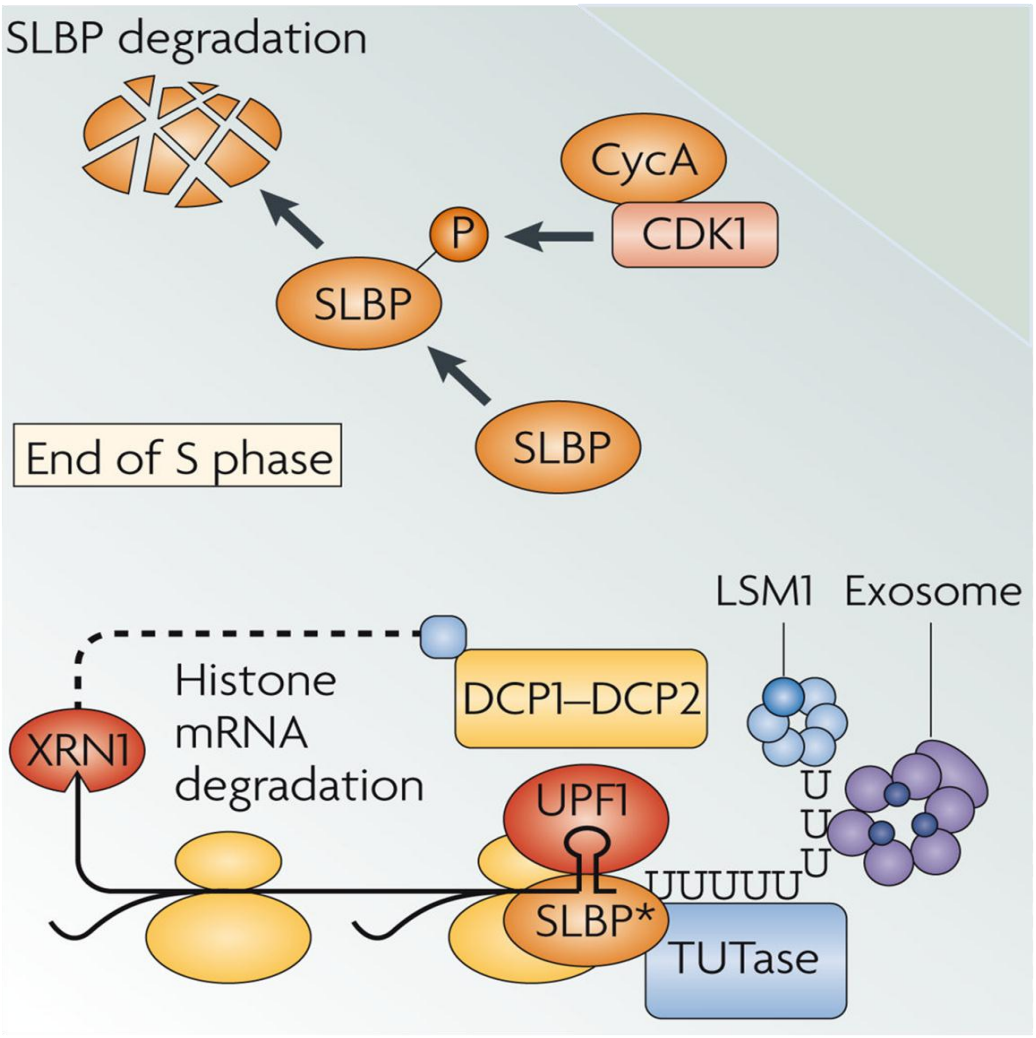

Figure 21 Model of histone mRNA and SLBP degradation. A proposed model of histone mRNA degradation showing the transition of histone mRNA from active translation as a circular mRNA, followed by the recruitment of Upf1 to the 3' end (Kaygun & Marzluff, 2005b) when DNA replication is inhibited, followed by oligouridylation and degradation. SLBP itself undergoes phosphorylation in the presence of kinases (CDK1, cyclin A).

4.4.8. Histone protein degradation.

Any excess of the histones in the cell is extremely hazardous because of their highly basic character resulting in non-specific binding to nucleic acids and non-nucleosomal aggregate

forming. Therefore it is absolutely critical for the cell to maintain a perfect balance between histone production and degradation.

4.4.8.1. Rad 53

Not many data are available concerning the degradation of histone proteins. Some reports coming from Yeast studies suggested a novel role of Rad53 in histone excess degradation. More specifically Rad53 is reported to prevent the accumulation of nonnucleosomal histone proteins in the cell. Cells which lack Rad53 are unable to deal with the consequences of histone overexpression. Although the precise mechanism remains unclear it is interesting to notice that Rad53 is able to directly co-purify with canonical histones. Since Rad53 is known to be the homolog of Chk2 present in higher eukaryotes it might be interesting to investigate the impact of Chk2 on histone degradation, in human cells (Gunjan & Verreault, 2003).

4.4.8.1. GrnA

In human cells the Cytotoxic T lymphocytes (CTL) protease Granzyme A (GrnA) induces caspase-independent cell death. Dong Zhang et al studies bring evidence for GrnA being involved in histone degradation. *In vitro* and GrnA completely degrades histone H1 and cleaves core histones into ~16-kDa fragments (Zhang et al, 2001).
Even if these two examples are interesting especially because they propose new functions for already known factors more research has to be done in order to elucidate a possible common model of histone protein degradation.

Objectives

"A perfection of means, and confusion of aims, seems to be our main problem."
Albert Einstein

Main objectives.

The main objective of this work was to gain further insights into the molecular mechanisms controlling gene expression on the posttranscriptional and translational level. In particular this work concentrated on the role of the protein INT6 in different RNA-related processes:

1. Study the role of the protein INT6 in the context of canonical histone mRNA translation. As seen previously, this process obeys to different mechanisms than that of classical mRNAs in line with the specific requirements associated with histone synthesis. INT6 had been previously shown to be part of the eIF3 translation complex but non-essential for global translation. Its interaction with SLIP-1 – one of the key players of the histone mRNA-specific translation mechanism – suggested that INT6 may participate in the still ill-understood process of histone mRNA translation.

2. Study the molecular interactions between INT6 and other components participating in the mRNA quality control quality Nonsense Mediated mRNA Decay (NMD), and try to understand the role played by INT6 in this process. Our laboratory has previously established an interaction between INT6 and two core NMD factors UPF1 and UPF2 and shown that INT6 itself participates in this surveillance mechanism, but its precise role in it is still incompletely understood.

3. Understand the role of the association of the HTLV-1 protein Tax with INT6 and in particular – since HTLV-1 mRNAs exhibits several key features common with the usual NMD targets – to understand whether HTLV-1 may not hamper the NMD pathway through this interaction.

Part II Results

Article I

Article I

The HTLV-1 Tax protein inhibits nonsense-mediated mRNA decay by interacting with INT6/EIF3E and UPF1.

Vincent Mocquet1,**6**, Julia Neusiedler1,**6**, Francesca Rende5, Jean–Michel Terme1*, Madeleine Duc Dodon2, Jürgen Wittman3, Christelle Morris1, Franck Mortreux1, Eric Wattel1, Vincenzo Ciminale5 and Pierre Jalinot1.

6 These authors equally contributed to this work.

Context of study and summary

Our previous results having shown an interaction between the protein INT6 and the HTLV-1 transcriptional activator Tax, we set out to characterize the effects and role of this association.

INT6 is a dispensable subunit of the translation factor EIF3, and previous work by our laboratory had shown that it participates in the Nonsense-Mediated mRNA Decay (NMD), a translation- and splicing-dependent mRNA quality control mechanism that recognizes premature termination codon (PTC)-containing mRNAs. Importantly, although NMD acts at the mRNA level, it is actually thought to be important for tumour suppression, as many potential mutations would result in the generation of abnormal mRNAs featuring a PTC that could be efficiently recognized and degraded by the NMD. We had observed that INT6 interacts with the core NMD factors UPF1 and UPF2 and that INT6 removal results in the stabilization of PTC-containing mRNAs, thus firmly establishing INT6 as a new NMD factor.

We thus decided to test whether Tax may affect NMD through its interaction with INT6. Because of the tendency of viruses to encode their genetic information in a minimal space, their RNAs are often atypical and many present features such as extensive alternative splicing, bicistronism, long 3'UTRs and leaky ribosome scanning, which could result in their targeting by the NMD.

We started by deciphering the interaction network between Tax, INT6 and the UPFs by immunoprecipitation experiments. Co-immunoprecipitation experiments demonstrated that

Tax interacts with UPF1, UPF2, UPF3b, and INT6, which are known to participate in NMD. More precisely the interaction with UPFs is mediated via UPF1 and especially its phosphorylated form. Our data suggest that the interaction of Tax and INT6 prevents the latter from interacting with the UPF1-UPF2 complex. Additional effects of Tax expression were an increase in size and abundance of UPF1-containing P-bodies. This could be explained by the stabilization of the interaction between the phospho-UPF1 and SMG5 observed in the presence of Tax. We further investigated the impact of Tax upon the mRNA. RNA-binding protein immunoprecipitation (RIP) experiments showed that Tax was able to associate with NMD-prone mRNA. The NMD tests demonstrated that Tax is able to increase the stability of transcripts normally subjected to NMD. This inhibition of NMD by Tax is INT6 dependent. We found these effects with different physiological RNAs as well as HTLV-1 transcripts. We concluded that HTLV-1 RNAs are sensitive to NMD and that Tax actively interferes with this mechanism.

Collectively, these data support the notion that Tax which trans-activates the expression of viral and cellular genes by binding to various enhancer-binding proteins, needed for transcription, has an impact on the NMD and interferes at the post-transcriptional level of gene regulation.

Our findings show that HTLV-1 is capable of manipulating the cell to make it more tolerant to external or internal messenger RNAs which are normally subjected to degradation. This is likely to favour the transformation of infected cells by allowing PTC-containing mRNAs derived from mutated genes to persist. This work will contribute to a better understanding of both the basics of NMD and some effects of HTLV-1 infection on cell transformation.

INT6 interacts with MIF4GD/SLIP1 and is necessary for efficient histone mRNA translation

Julia Neusiedler, Vincent Mocquet, Christelle Morris and Pierre Jalinot*

Laboratoire de Biologie Moléculaire de la Cellule, Unité Mixte de Recherche 5239, Centre National de la Recherche Scientifique, Ecole Normale Supérieure de Lyon, Institut Fédératif de Recherche 128 Biosciences Lyon Gerland, 46 Allée d'Italie, 69364 Lyon cedex 07, France.

Keywords : histone mRNA / translation / MIF4GD / INT6 / eIF3 / SLBP

Running title : INT6 role in histone mRNA translation

*Corresponding author: Tel: + 33 4 7272 8563. Fax: + 33 4 7272 8080.

E-mail: pjalinot@ens-lyon.fr

Abstract : The INT6/EIF3E protein has been implicated in mouse and human breast carcinogenesis. This subunit of the eIF3 translation initiation factor which includes a PCI domain exhibits specific features as presence in the nucleus and ability to interact with other important cellular protein complexes as the 26S proteasome and the COP9 signalosome. It has been previously shown that INT6 was not essential for bulk translation and this protein is considered to regulate expression of specific mRNAs. By performing a two-hybrid screen with INT6 as bait we characterize in this report the MIF4GD/SLIP1 protein as an interactor of this eIF3 subunit. MIF4GD was previously shown to associate with SLBP which binds the stem-loop located at the C-terminal end of the histone mRNAs and to be necessary for efficient translation of these cell-cycle-regulated mRNAs that lack a poly(A) tail. In line with the interaction of both proteins, we show using the RNA interference approach that INT6 is also essential to S-phase histone mRNA translation. This was observed by analyzing expression of endogenous histones and by testing heterologous constructs placing the luciferase reporter gene under the control of the stem-loop element of various histone genes. With such reporter plasmid, silencing and overexpression of INT6 exerted opposite effects. In agreement with these results INT6 and MIF4GD were observed to colocalise in cytoplasmic foci. We conclude from these data that INT6 by establishing interactions with MIF4GD and SLBP plays an important role in translation of poly(A) minus histone mRNAs.

Introduction :

The *int6* gene has been originally characterized as an integration site of the Mouse Mammary Tumour Virus (MMTV) in one preneoplastic mammary hyperplastic outgrowth line and two independent mammary tumours arising in unrelated mice (Marchetti et al., 1995). The human INT6 protein was further identified as a target of the Human T-cell Leukaemia Virus type 1 (HTLV-1) transforming protein TAX (Desbois et al., 1996) and also as the EIF3E subunit of the eIF3 translation initiation factor (Asano et al., 1997). EIF3 establishes multiple contacts with other translation initiation factors as EIF1, EIF1A and eIF4G (Hinnebusch, 2006; Hershey, 2010). It also binds the mRNA, the 40S ribosome subunit and favours association with the ternary complex thereby playing a key role in translation initiation. The general structure of eIF3 has been analyzed by electronic microscopy and revealed a five lobes organization (Siridechadilok et al., 2005). INT6 includes in its C-terminal part a Proteasome – COP 9 signalosme – Initiation of translation (PCI) domain which is also present in several subunits of the proteasome 19S regulatory particle, of the COP9 signalosome (CSN) and of eIF3. The PCI subunits of these complexes which are involved in protein degradation, SCF E3 ubiquitin ligase regulation and mRNA translation, respectively, are likely to play a scaffold role (Pick et al., 2009). Intriguingly INT6 has also been characterized to interact with several subunits of the proteasome and of the CSN and to associate in vivo with these complexes, although in lesser amounts as compared with eIF3 (Karniol et al., 1998; Yahalom et al., 2001; Hoareau Alves et al., 2002; Yen et al., 2003). In line with such interactions INT6 has been reported to control the stability of specific cellular proteins. Indeed we and others have previously shown that it acts positively on the stability of the MCM7 subunit of the DNA replication licensing factor MCM by interacting with its polyubiquitinylated forms (Buchsbaum et al., 2007; Grzmil et al., 2010). Conversely Chen et al. have shown that INT6 by binding to HIF-2α triggers its

proteolytic degradation (Chen et al., 2007; Chen et al., 2010). INT6 has also been shown to negatively control the stability of the steroid coreceptor 3 (SRC3) during mitosis (Suo et al., 2011). Besides these activities in protein stability, INT6 has also been shown to intervene in translation, however its effect seems restricted to specific proteins. Indeed both in fission yeast and in mammalian cells, knockdown of INT6 does not appear to modify significantly incorporation of 35S-labeled methionine in proteins or polysome profile (Bandyopadhyay et al., 2000; Zhou et al., 2005; Grzmil et al., 2010). This has been reported by different groups and correspond to our own observations. However current data does not exclude more specific activities of INT6 in this process. Indeed Zhou et al. have reported that two kinds of eIF3 complexes exist in fission yeast, one characterized by the presence of eIF3m and the other by that of eIF3e (Zhou et al., 2005). This latter type was found associated with a limited set of specific mRNAs. In a previous work we have also established that INT6 was required in human cells for the efficiency of the nonsense-mediated mRNA decay pathway (NMD) which prevents expression of truncated proteins which can exert deleterious effects (Morris et al., 2007). This activity is correlated with the ability of INT6 to interact with specific NMD factors like UPF1 and UPF2. It has also been recently reported that INT6 was able to act positively or negatively on the translation of specific mRNAs as evaluated by their presence in high molecular weight polysomes (Grzmil et al., 2010). From these various observations the picture emerges that INT6 selectively acts on translation in either a positive or negative way (Grzmil et al., 2010) but the exact underlying molecular mechanisms remain to be characterized.

A specific feature of the protein coding mRNAs is the poly(A) tail at their 3' end that binds several proteins including the poly(A) binding protein (PABP) which plays an important role in translation by creating interactions with initiation factors such as eIF4G and thereby causes circularization of the mRNA molecule (Amrani et al., 2008; Martineau et al., 2008). However

one family of cellular mRNAs lacks a polyA tail : that expressing the five classes of histone (H1, H2A, H2B, H3 and H4). Instead of the poly(A) signal these specific mRNAs include near their 3' end a 16 nucleotide stem-loop that interacts with the SLBP protein which plays a key role in the metabolism of these RNA (Zhao et al., 2004; Townley-Tilson et al., 2006). Indeed this protein forms a complex with other factors including symplekin and ZFP100 which in combination with the U7 mRNP and CSPF73 trigger cleavage of the histone mRNA a few nucleotide after the stem-loop (Dominski et al., 2005; Kolev & Steitz, 2005; Kolev et al., 2008; Sullivan et al., 2009b). This implies an hybridization between the 5' part of the U7 RNA and the HDE element which is located downstream the stem-loop (Dominski & Marzluff, 2007). Besides its role in formation of the 3' end, SLBP is also important for export of the histone mRNA (Ghule et al., 2008; Sullivan et al., 2009a), as well as their productive translation and this translational activity relies on interactions with eIF4G and eIF3 translation initiation factors (Ling et al., 2002; Gorgoni et al., 2005). More recently it has also been reported that this effect involves an interaction with a protein identified in a two hybrid screen performed with SLBP as bait which was accordingly named SLBP interacting protein 1 (SLIP-1) (Cakmakci et al., 2008). This small 25 kD protein corresponds almost entirely to a middle domain of eukaryotic initiation factor 4G (MIF4G) and has been designated accordingly as MIF4G domain containing protein (MIF4GD) by the HUGO Gene Nomenclature Committee, which name will be further used in this manuscript. After completion of DNA replication and ending of S-phase where histone genes are expressed, SLBP is itself degraded but is also important for degradation of the histone mRNAs (Kaygun & Marzluff, 2005). From the current data it clearly appears that SLBP plays a pivotal role in the metabolism of the histone mRNAs which are issued from the most cell-cycle regulated cellular genes, their S-phase specific expression relying on regulations at the transcriptional, maturation and translational steps.

In this report we identified an unanticipated interaction between INT6 and the MIF4GD protein. In line with this association we observed that INT6 is required for efficient translation of the histone mRNAs during S-phase. Our data support a novel specific role of this protooncoprotein in the translation of this specific type of cellular mRNAs.

Results

Interaction between INT6 and MIF4GD

A two-hybrid screen was performed with INT6 as bait using a cDNA library of immortalized human B lymphocytes. This screen led to identification of several INT6 interactors as RFP, MCM7 and several subunits of the eIF3 complex, of the CSN and of the 19S proteasome regulatory particle as previously reported (Morris-Desbois et al., 1999; Hoareau Alves et al., 2002; Buchsbaum et al., 2007). Among the clones obtained in this screen, three encoded MIF4GD. One clone, THI 22, included the complete MIF4GD coding sequence, while the other two lacked the first 16 N-terminal amino acids (Fig. 1A). After isolation from yeast and retesting against the INT6 bait all three clones were positive (Fig. 1A). To further test the interaction between both proteins in human cells, a vector expressing the complete MIF4GD coding sequence fused to the HA epitope at the C-terminal end was constructed. HeLa cells were transfected with this MIF4GDHA expression vector together with either a control plasmid or a construct expressing INT6 fused to the FLAG epitope at its C-terminal end. Immunoprecipitation using the antibody to HA showed a coprecipitation of INT6FLAG with SLIPHA (Fig. 1B). The same experiment was carried out with a vector expressing untagged INT6 and in the presence of RNAse. Under these conditions MIF4GDHA was also coprecipitated with the antibody to INT6 (Fig. S1A, lane 2). The experiment was also performed in the reverse way by transfecting cells with constructs

expressing various parts of INT6 fused to the FLAG epitope at their N-terminal end together with the vector expressing MIF4GDHA. Immunoprecipitation experiments using an antibody to FLAG followed by an immunoblot with the antibody to HA revealed binding of MIF4GD to all INT6 mutants (Fig. 1C), except that corresponding mainly to the C-terminal PCI domain (Fig. 1C, lane 6). All these INT6 mutants were precipitated in similar amounts (Fig. S1B). This indicated that the PCI domain of INT6 is not involved in the interaction and that MIF4GD interacts with different regions of the N-terminal part of INT6 as it binds with similar efficiencies the 9-195 and 246-445 INT6 mutants. These results established that INT6 binds MIF4GD in human cells through its N-terminal part.

INT6 is necessary for efficient translation of histone mRNAs in S-phase

It has been reported previously that MIF4GD interacts with SLBP and is required for efficient translation of histone mRNAs (Cakmakci et al., 2008). As INT6 is associated with the eIF3 translation initiation complex and binds MIF4GD, we further examined whether this protein is also important for histone mRNA translation. To this end, HeLa cells were synchronised in S phase by a double thymidine block (Fig. 2A) and transfected with siRNAs either control or directed against INT6 or MIF4GD. The efficiency of silencing was checked by RNA and protein analysis (Fig. S2 A and B). To control whether silencing of these proteins affects cell cycle distribution of the synchronized cells, a flow cytometry analysis was also carried out. Due to synchronization the majority of the cells were indeed in S phase and this was not significantly modified by the INT6 and MIF4GD siRNAs duplexes (Fig. S3), although that with the latter one a decrease in G1 cells correlated with a higher percentage of cells in G2/M (Fig. S3). Three hours after second block release, cells were incubated with 35S-labeled methionine for 10 min and the histones were purified by acidic extraction. After electrophoretic separation, the gel was first stained with Coomassie Blue (Fig. 2B, upper

panel) and radioactivity was further detected with a phosphorimager (Fig. 2B, lower panel). With three different INT6 siRNAs, a reduction of the radioactivity incorporated in the four canonical histones was observed (Fig. 2B, lower panel, compare lane 1 with lanes 2, 3 and 4). The experiment was repeated three times with the addition of a point corresponding to transfection of *MIF4GD* siRNAs and the radioactivity in the various histones was normalized with bands of the upper part of the gel (Cakmakci et al., 2008). As they were poorly separated the bands corresponding to histones H2B, H3 and H2A were grouped together for quantification. After this normalization it was observed that silencing of MIF4GD and INT6 led to similar reduction of radioactivity incorporation in histones (Fig. 2C). As previously shown this decrease was not due to a lower percentage of cells in S phase (Fig. S3). To verify further that this reduction was due to a translational effect, the amount of histone mRNAs was analyzed using the NanoString technology. As there are multiple copies of histone genes in the human genome, this method allowed a precise analysis with probes designed to be specific of the various genes. These were usually located in the region upstream of the stem-loop which is poorly conserved among the various histone genes. Such probes were designed for most of the histone genes, except those which are highly tissue-specific, such as genes expressed in the testis. In this list were also included seven normalization genes as well as a probe specific of INT6. Whereas *INT6* mRNA was strongly reduced by siRNA transfection (Fig. S2A) as expected, expression of the various histone genes were generally only slightly affected by INT6 silencing (Fig. 3). This was also the case for *MIF4GD* siRNA transfection (data not shown). Hence, it appears that INT6 silencing does significantly reduce translation of mRNAs coding for canonical histones during S-phase and that this effect does not result from a comparable decrease in mRNA amounts. In this analysis the effect of INT6 and MIF4GD were found to be similar.

To confirm this effect, another approach was undertaken. The firefly luciferase sequence was placed under the control of the promoter and stem-loop sequence of various genes coding for canonical histones. The sequence including the stem-loop extended in the 3' part to include the complete HDE. Such constructs were generated using these regulatory elements of the *HIST1H2AC*, *HIST1H2BG* and *HIST2H4A* genes. These vectors were transfected together with a control plasmid expressing the renilla luciferase and these experiments were performed in cells silenced for INT6 or MIF4GD by cotransfection of corresponding siRNAs. Transfection were repeated independently three times and the mean of the normalized firefly luciferase activity is represented. When the experiment was performed with a control vector including the firefly luciferase under the control of SV40 promoter and polyA elements, there was no effect of INT6 and MIF4GD silencing on luciferase expression (Fig. 4B, pGL3-P panel). For all three constructs including firefly luciferase under histone gene promoters and 3' end processing elements, a 60 to 80% reduction was observed when cells were silenced for INT6 or MIF4GD expression. The effect obtained with the two INT6 siRNA duplexes was similar and comparable to that resulting from MIF4GD silencing (Fig. 4B, panels PSL-H2A, PSL-H2B and PSL-H4). For this experiment also it was checked that the observed effect was not due to a decrease in the mRNA amounts. To this end, total RNA was prepared from the transfected cells and analyzed by RT-qPCR for firefly and renilla luciferase mRNAs. The INT6 silencing was not observed to reduce the firefly luciferase mRNA and even to slightly increase it in the case of the PSL-H2A and PSL-H2B constructs (Fig. 4C). This approach confirmed that, as for MIF4GD, INT6 silencing reduces translation of mRNA placed under the control of histone gene regulatory elements. As this effect was observed using various siRNA duplexes directed against *INT6* mRNA, the possibility of an off-target effect was unlikely. To further test this point a rescue analysis was done with the PSL-H4 construct. This plasmid was transfected in cells silenced either with a control or the I6.4 siRNA duplex. This

latter one is located in the 3' untranslated region of the INT6 mRNA. In such INT6-silenced cells, the protein was rexpressed from a vector lacking the 3' UTR. As observed above, silencing of INT6 reduced firefly luciferase expression, but reexpression of INT6 reverted this effect and even stimulated expression (Fig. 5A). Immunoblot analysis of INT6 and b-actin as control showed that these effects were correlated in lower and higher amounts of INT6 (Fig. 5B). These observations confirmed that the effect on translation was due to the absence of INT6 and also showed that overexpression of the protein can stimulate luciferase expression under the control of histone gene elements. These various observations establish INT6 as an important protein for translation of canonical histones.

Interaction of INT6 with SLBP

Considering the interaction of INT6 with MIF4GD and knowing that this latter protein binds SLBP, we examined the association of INT6 with SLBP. This was first analyzed by performing an immunoprecipitation experiment using the endogenous proteins present in a cellular extract. By analyzing proteins coprecipitated with endogenous INT6 a clear SLBP signal was detected (Fig. 6A, lane 3). This signal was absent when the antibody to INT6 was omitted in the reaction or when preimmune serum was used (Fig. 5A, lanes 1 and 2). We also analyzed if association between both proteins were dependent on RNA. For this HeLa cells were transfected with vectors expressing INT6 and SLBP tagged at its N-terminus with a FLAG epitope. Immunoprecipitation of INT6 in the presence of RNAse coprecipitated FLAG-SLBP (Fig. 6B, lower panel, lane 2). This experiment showed that RNA was not required to association of both factors. A possibility was that MIF4GD bridges SLBP and INT6 by interacting with both proteins. To test this, the INT6-SLBP coimmunoprecipitation was analyzed using extracts of MIF4GD-silenced cells. The removal of MIF4GD which was effective in the extract (Fig. 6C, upper panel, compare lanes 1 and 2) did not affect the

association of INT6 with SLBP (Fig. 6D, upper panel, compare lanes 2 and 3). From these data it appears that INT6 is able to interact with SLBP independently of MIF4GD. As it was previously shown that SLBP interacts with eIF3, we further tested if perturbation of this complex affects the INT6 SLBP interaction (Ling et al., 2002; Gorgoni et al., 2005). As for MIF4GD, silencing of EIF3B did not reduce coimmunoprecipitation of INT6 and SLBP (Fig. 6C, middle panel lane 3 and 6D, upper panel, compare lanes 2 and 4). Under these various conditions similar amounts of INT6 were immunoprecipitated (Figure 6D, lower panel). Thus these results indicate that there is a specific association of INT6 with SLBP which does not require MIF4GD or EIF3B, which is a core subunit of eIF3 and which is required for translational activity of the complex. This suggests that MIF4GD is likely recruited by both INT6 and SLBP and that its functional effect on translation might depends on association with other translational initiation factors than eIF3.

Colocalisation of INT6 and MIF4GD in the cytoplasm

To ascertain that INT6 and MIF4GD interact in the cellular context, we performed immunofluorescence studies. It was first checked that the MIF4GD subcellular localisation analyzed with the rabbit polyclonal antibody that we developed is similar to that detected by expressing MIF4GD fused to the HA epitope with the antibody to HA. Immunostaining with the antibody raised against the N-terminal first 20 amino acids of MIF4GD showed a weak nuclear punctate pattern with more intense cytoplasmic dots (Fig. S4, panel a). Analysis with the monoclonal antibody to HA showed a similar pattern with a clear colocalization of the cytoplasmic dots detected with both antibodies (Fig. S4). We concluded from these observations that MIF4GD is mainly localized in the cytoplasm in which it forms foci where the protein is concentrated. It was next tested how this localisation compares with that of

SLBP. Immunostaining of both endogenous proteins was analyzed using the antibody to MIF4GD that we developed, along with a mouse monoclonal antibody directed against SLBP. Both proteins were mainly detected in the cytoplasm where they form a punctate pattern (Fig. S4, panel B) and were clearly colocalized in some dots, but not in all (Fig. S4). Indeed some MIF4GD foci were not stained by the SLBP antibody. This indicates a partial cytoplasmic colocalisation of SLBP and MIF4GD. We then compared the subcellular localisation of MIF4GD and INT6. When cells were transfected with vectors expressing the INT6FLAG and MIF4GDHA fusion proteins and analyzed with the rabbit polyclonal antibody to INT6 along with the mouse monoclonal antibody to HA, INT6 was revealed both in the nucleus and the cytoplasm where it formed dots which showed a very good colocalization with those formed by MIF4GD (Fig. 7, upper panels). A very similar pattern was observed by transfecting the cells solely with the MIF4GDHA expression vector and staining the endogenous INT6 with the C-169 rabbit antibody to INT6 and MIF4GD with the monoclonal antibody to HA (Fig. 7, middle panels). Finally this pattern with nuclear and cytoplamic INT6 along with INT6-MIF4GD colocalisation in cytoplasmic dots was also observed by transfecting cells solely with the INT6FLAG expression vector and by revealing the proteins with the monoclonal antibody to FLAG and the rabbit polyclonal antibody to MIF4GD (Fig. 7, bottom panel). These observations are in agreement with the notion that MIF4GD binds INT6 and indicated that MIF4GD is mainly present in the cytoplasm, whereas INT6 can be detected in both nucleus and cytoplasm. They also showed that both proteins are colocalized in cytoplasmic foci in which SLBP can be observed, in agreement with all three factors participating to histone mRNA translation.

Discussion

INT6 as a eIF3 subunit necessary for histone mRNA translation

INT6 has been identified in numerous organisms from fission yeast to human and is highly conserved (Marchetti et al., 1995; Desbois et al., 1996; Morris-Desbois et al., 1999; Bandyopadhyay et al., 2000; Crane et al., 2000). It has also been characterized as one of the 13 subunits composing the 800 kD eIF3 complex (Asano et al., 1997) but its activity in this important translation initiation complex remains to be understood in details especially as some discrepancies can be considered by comparing results from different in vitro and in vivo studies. Mass spectrometry analyses have shown that each of the 13 eIF3 subunits are present at one copy and three different subcomplexes have been observed by favouring dissociation in response to increased ionic strength (Zhou et al., 2008). One includes the a, b, c, i and g subunits which correspond to the five core subunits of the s. cerevisiae eIF3 complex (Asano et al., 1998; Phan et al., 1998). Another subcomplex consists of the f, h and m subunits and the third subcomplex to the k, l, e, d and c subunits. Association of this latter with the other subunits is likely do depend on the c subunit with which INT6 interacts (Morris-Desbois et al., 1999). In the eIF3 complex some subunits are essential, probably mainly the a, b and c subunits, but others are likely to play a role in the translation of specific mRNAs or to allow specific regulations. Despite characterization of an interaction of INT6 with eIF4G (LeFebvre et al., 2006) and its requirement for reconstitution from baculovirus-expressed subunits of a minimal complex allowing binding of the 48S on the initiation codon (Masutani et al., 2007), several reports have ruled out a role of the protein for bulk translation (Bandyopadhyay et al., 2000; Zhou et al., 2005; Grzmil et al., 2010). Indeed it has been reported that deletion of the gene in fission yeast does not affect general translation or polysome profile (Zhou et al.,

2005). However in this organism it has been observed that eIF3e was necessary for stability of the complex when yeast were grown in minimal medium (Akiyoshi et al., 2001). Also in human cells, suppression of INT6 by RNA interference does not affect bulk translation and polysome profile (Grzmil et al., 2010). However it has been reported that INT6 was important for translation of specific genes. Grimzl et al. have observed that in MDA-MB-231 breast cancer cell line INT6 was able to act positively or negatively on the presence of several mRNAs in polysomes (Grzmil et al., 2010). In the same line, Zhou et al. observed in fission yeast the existence of two eIF3 complexes, one including eIF3e and the other eIF3m, each type of complex associating with different mRNA subsets. The data presented in this manuscript identify the genes encoding the canonical histones as a new class of genes of which translation depends on INT6/EIF3E.

Activity of INT6 in histone mRNA translation involves interaction with MIF4GD and SLBP

A specific feature of histone mRNAs is to lack a poly(A) tail and to include a particular stem-loop motif at their 3' end. This structure binds the SLBP protein which plays a key role in the 3' processing (Dominski & Marzluff, 2007), nuclear export (Ghule et al., 2008; Sullivan et al., 2009a) and translation (Ling et al., 2002; Gorgoni et al., 2005) of these mRNAs during the S phase. The effect of SLBP on translation has been shown to involve eIF3 and eIF4G. More recently Cakmakci et al. have also shown that this effect on translation involves an interaction of SLBP with MIF4GD/SLIP1 which was characterized in a two-hybrid screen with SLBP as bait (Cakmakci et al., 2008). Similarly we obtained a clone encoding the entire MIF4GD coding sequence in a two-hybrid screen using INT6 as bait. In agreement with this interaction we observed that INT6 is also an essential protein for efficient translation of the histones genes. This was observed by the RNA interference approach on

endogenous histone genes, but also with heterologous constructs including the luciferase reporter gene under the control of histone promoter and stem-loop regulatory elements. The effect observed by silencing INT6 was of similar magnitude as compared with suppression of MIF4GD or SLBP (Cakmakci et al., 2008) (J. Neusiedler, data not shown). Thus if INT6 is dispensable for the translation of the majority of cellular genes it plays an essential role for histone mRNAs. The interaction with MIF4GD is likely part of this effect. However the possibility of a direct binding of INT6 to SLBP exists although this aspect will require further investigation. Indeed immunoprecipitation experiments showed a clear SLBP signal when INT6 was precipitated and the association was not dependent on MIF4GD or EIF3B. Previous biochemical studies have shown the essential role of this core subunit in the assembly of the eIF3 complex (Asano et al., 1998; Phan et al., 1998; Masutani et al., 2007; Zhou et al., 2008). However it remains possible that the association between SLBP and EIF3E involves other eIF3 subunits, possibly those that form a subcomplex with EIF3E as C, K, L and D. It is likely that the SLBP-eIF3 association involves several contacts including those with MIF4GD which probably has a stabilizing effect.

Interestingly INT6 appears to interact with several proteins including a MIF4G domain. Indeed we have previously shown that it binds UPF2 which includes three MIF4G domains, as well as CBP80 which also includes one (Morris et al., 2007). Finally we show here that the small MIF4GD protein that mainly corresponds to a MIF4G domain binds INT6. Hence INT6 is likely to interact specifically with the MIF4G domain and as shown here this ability relies on the N-terminal two-third of the protein, but does not necessitate the C-terminal PCI domain which probably has a scaffold role permitting association with the other eIF3 subunits. LeFebvre et al. have described an in vitro interaction between the 1015-1118 region of eIF4G and the eIF3 complex which would involve a contact with INT6 as observed by performing partial proteolysis and mass spectrometry analyses (LeFebvre et al., 2006). Thus it

is possible that INT6 interacts simultaneously with both MIF4GD and eIF4G thereby playing a pivotal role. Both interactions would involve the N-terminal two-third of INT6 (LeFebvre et al., 2006). For poly(A)-tailed mRNA the looping of the molecule through contacts of poly(A)-bound proteins with eIF3 and eIF4G has been shown to be important for efficient translation initiation (Amrani et al., 2008; Martineau et al., 2008). In mammalian cells PABP does not contact directly eIF3 and this involves PAIP1 (Martineau et al., 2008). Martineau et al. have shown an interaction of PAIP1 mainly with EIF3G. However in a far-western experiment they also revealed bands migrating at the position of EIF3E and they show in this report that the stimulatory effect of PAIP1 overexpression is lost when INT6 is silenced. Interestingly PAIP1 also includes a MIF4G domain. Hence although the 3' end of the histone gene lacks a poly(A) tail, the general mRNP organization allowing efficient translation initiation might be very similar with SLBP-MIF4GD acting in a similar way as PABP-PAIP1 by allowing looping of the RNA along with contacts with eIF4G and eIF3. For the majority of mRNAs the absence of INT6 might be made up for by other contacts, possibly with EIF3G in particular (Martineau et al., 2008), whereas for histone mRNAs the absence of EIF3E appears detrimental.

Relationship between histone mRNA translation and other roles of INT6

Previous studies of INT6 function have led to several interesting observations which are likely related to its role in histone mRNA translation. In particular it has been reported that INT6 is partly located in the nucleus (Desbois et al., 1996; Morris-Desbois et al., 1999; Buchsbaum et al., 2007; Grzmil et al., 2010) and Watkins et al. have shown that in non-transformed cells this localisation was reduced in S phase (Watkins & Norbury, 2004). In agreement with a dual location in the nucleus and the cytoplasm, the protein has a N-terminal nuclear export sequence and an internal nuclear localisation signal, indicating that it is likely

to shuttle between both compartments (Guo & Sen, 2000). Mutation of the NES renders the protein exclusively nuclear. Also interestingly it has been observed that INT6 can be associated with the RNA polymerase II holoenzyme (unpublished results). Considering the interaction with SLBP, these observations raise the possibility that INT6 might be loaded on histone mRNAs cotranscriptionnally and thereby might participate to their export towards the cytoplasm. SLBP has been shown previously to play an important role in this process (Ghule et al., 2008; Sullivan et al., 2009a). This nuclear presence of INT6 is specific as other subunits of eIF3 are exclusively cytoplasmic (Watkins & Norbury, 2004). In the cytoplasm it is possible that INT6 by interacting with other subunits complexes favour assembly of the complete eIF3 on histone mRNAs as well as establishment of the various interactions with other general translation initiation factors. Alternatively, in the cytoplasm INT6 associated to SLBP might be replaced by a complete eIF3 complex. To test these notions it will be interesting to analyze in details the dynamics of the recruitment of the various eIF3 subunits to this particular type of mRNA and to verify that the nuclear presence of INT6 is indeed important to their translation.

In addition it is possible that INT6 intervenes at further steps of histone mRNAs metabolism. Indeed once genome replication is completed, histone expression stops and their mRNAs are degraded in a process which involves UPF1, a core NMD factor (Kaygun & Marzluff, 2005). Intriguingly we have previously shown that INT6 was important for the NMD process and was able to interact with factors like UPF2 and UPF1 (Morris et al., 2007). Hence it might be possible that after playing a positive role in S phase INT6 also participates to histone mRNAs degradation in G2. To better understand this point it will be important to determine the exact role played by UPF1 and RNA degradation factors in the process and also to identify what could make INT6 switch from a positive to a negative role.

Another activity of INT6 which might be related to its role in histone mRNA translation is the control it exerts on the stability of the MCM7 subunit of the MCM complex which is a key factor of DNA replication initiation and progression (Buchsbaum et al., 2007). Indeed we have previously shown that in S phase INT6 binds polyubiquitinylated MCM7 and protects it from degradation by the proteasome. In this work silencing of both INT6 and MCM7 were observed to impede normal DNA replication (Buchsbaum et al., 2007). By considering these two sets of data it is possible that INT6 acts as a sensor of DNA replication progression and allows coupling of this process with histone expression. One possibility would be that the INT6 chromatin-bound fraction by interacting with polyubiquitinylated MCM7 can not favour histone mRNA maturation and translation, thereby establishing a negative feedback loop allowing adjustment of histone production and DNA replication completion. Future studies should help to establish the validity of such a model and to determine precisely if both activities are independent or related.

In conclusion our observations have established an important role of INT6 for translation of the mRNA encoding canonical histone and thereby open new perspectives to understand how several activities of this protein might be related. They also might bring some interesting new ideas on the mechanisms allowing coupling of DNA replication and histone synthesis. Finally, this activity by influencing genomic stability is certainly important to consider for gaining a better understanding of the oncogenic and genomic instability effects resulting from INT6 alteration (Marchetti et al., 1995; Yen & Chang, 2000; Rasmussen et al., 2001; Morris & Jalinot, 2005; Mack et al., 2007).

Materials and methods

Two-hybrid assay

Two-hybrid screen of a cDNAs library of human lymphocytes immortalised by EBV (Durfee et al., 1993) with INT6 fused to the DNA binding domain of GAL4 was performed as previously described (Morris-Desbois et al., 1999). Three cDNA clones encoding MIF4GD were isolated and sequenced: THI 22 (full coding sequence amino acids 1 to 222), THI 2 and 21 (amino acids 17 to 222).

Constructs

Expression vectors for MIF4GD (NM-020679.2) alone or fused at its C-terminal end with the HA epitope were generated by PCR amplification from the SC113046 clone (OriGene Technologies, Rockville, MD, USA) with the 5' primer 5'-AGGAATTCTGGCTAGTCATGGGGGAGCCCAGTAGAGAG - 3' and as 3' primer either 5' - TCTGGAGATCTAGTCGGAGACTTCGCTGTAG - 3' or 5' - GTAGAAGATCTCGAGCTAGGCGTAGTCAGGCACGTCGTAGGGATACCCGTCGGA GACTTCGCTGTAG - 3' (HA fusion). The amplified fragments were digested by EcoRI and BglII restriction enzymes and inserted between between the EcoRI and BglII sites of the pTL1 expression vector (pSG5 derivative, Green et al., 1988). The constructs including the firefly luciferase under the control of the promoter and stem-loop elements of histone genes were constructed in two steps. First a sequence including the stem-loop and histone downstream element was amplified from total DNA prepared from Jurkat cells for the *HIST1H2AC*, *HISTH2BG* et *HIST2H4A* genes with the following primers: H2ACSL5': 5' - GTGATTCTAGAGGTATCTGAGCTCCCGGAAAC - 3'; H2ACSL3': 5' - TCCCCAGGATCCGAAAAGCAGTAATACGCTTTG - 3'; H2BGSL5': 5' - GTAAATCTAGACTTAGGTGCTTTAAAACTCAAAGG - 3'; H2BGSL3': 5' - CCACGGGATCCAAACTGGTCTCGATCCGCACGCC - 3'; H4ASL5': 5' - GGCCGCCTCTAGAGCTTTGCACGTTTCGATCCC - 3'; H4ASL3': 5' -

ACTTCGGATCCGATTGTCGCCCCACTGCCAAAG - 3'. The amplified fragments were digested with the XbaI and BamHI restriction enzymes and inserted between the XbaI and BamHI restriction sites of pGL3-promoter (Promega, Madison, USA). The promoter sequences of these histone genes were similarly amplified from Jurkat cells DNA using the following primers: H2ACP5': 5' - GTAAAGATCTGATTTCTGCTACTTATAGGG - 3'; H2ACP3': 5' - TCCAGCCATGGCAATCAGACAAAAATCACC - 3'; H2BGP5': 5' - ATCGGTAGATCTGTGAAAGGCGCAATTTGATTGG - 3'; H2BGP3': 5' - GGTTCAGCCATGGTGTCAGAAAACAATAACAGCAG - 3'; H4AP5': 5' - GCGTGTAGATCTCATCGTCGGAACGGCGCTTCC - 3'; H4AP3': 5' - TGCCGGCCATGGCCGCTGGAGCCCGATAGACAGC - 3' . The amplified fragments were digested by the XhoI and NcoI restriction enzymes and inserted between the XhoI and NcoI sites of the pGL3-promoter derivatives containing the stem loop sequences instead of the poly(A) signal giving expression vectors PSL-H2A, PSL-H2B and PSL-H4. The various constructs used in this study were controlled by DNA sequencing.

Cell culture and transfection

HeLa cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FCS (Invitrogen, Carlsbad, CA, USA), 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37°C in a 5% CO_2-humidified atmosphere. For plasmids and siRNA transfections, the amount of FCS used in the culture medium was reduced to 5% and antibiotics were omitted for siRNA transfection which was performed using the Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). siRNA duplexes I6.1, I6.3, I6.4 and anti-EIF3B have been described previously (Morris & Jalinot, 2005; Morris et al., 2007). For endogenous histone labelling experiments a *Luc* siRNA duplex was used. For experiments with luciferase constructs control siRNA was a scrambled

sequence of I6.1 (5'-GACGUGCCAGGAUGAUUGGdTdT-3'). For *MIF4GD* mRNA the sequence of the siRNA duplex on the sense strand was: 5'-CCAGUAGAGAGGAGUAUAAdTdT-3'. For DNA and siRNA transfection, HeLa cells were first transfected with the siRNAs using Lipofectamin 2000 and 24 h later the cell culture medium was changed and transfection of DNA vectors was carried out with the calcium phosphate procedure. After 16 h the culture medium was changed after a PBS wash and the cells were harvested 48 h later.

Metabolic pulse-labeling of cellular proteins

HeLa cells were transfected with the various siRNA duplexes as described above. For G1/S synchronization, 24 h after siRNA transfection 2.5 mM of thymidine (Sigma-Aldrich, StLouis, MO, USA) was added to the medium for 18 h, then removed for 10 h and added again at the same concentration for 14 h. Cells were then released from the double thymidine block and incubated in DMEM for 1.5 h. They were further cultured in DMEM lacking methionine for 30 min. Cells were then incubated in DMEM supplemented with ^{35}S-labeled methionine for 10 min. Cells were collected and histones were prepared from isolated nuclei by acidic extraction as previously described (Cakmakci et al., 2008). The histone fraction was analyzed by SDS-PAGE on 16% polyacrylamide gels which were first stained with Coomasie Blue and further analysed for radioactivity using a Fuji phosphorimager (Fujifilm, Tokyo, Japan). Radioactivity in the bands corresponding to histones was analysed using the Fuji ImageGauge software using the profile mode.

Immunoprecipitation and immunoblot

For immunoprecipitation, extracts of HeLa cells were prepared in RIPA buffer (Harlow & Lane, 1988) supplemented with TCEP (10 mM) and the Complete and Pefabloc protease

inhibitors (Roche, Basel, Switzerland). Lysates were centrifuged for 15 min at 14,000 rpm and the protein concentration of supernatants was measured with the DC Protein Assay kit (Biorad, Hercules, CA, USA). After adjustment to equal protein concentrations and overnight incubation with antibodies diluted 1:250, protein A sepharose beads (80µl) equilibrated in RIPA buffer were added. After incubation for 1.5 h beads were collected by centrifugation and washed three times in RIPA buffer. Proteins were eluted in 4x SDS sample buffer at 92 °C for 10 min. After separation by SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare Life Sciences, Uppsala, Sweden). For immunoblot, primary antibodies were used diluted 1:1,000 or at concentration indicated by the manufacturer. Revelation was performed by chemiluminescence using the ECL or ECL plus reagent (GE Healthcare Life Sciences) using secondary antibodies diluted 1:6,000 or 1:10,000, respectively.

Immunofluorescence and confocal microscopy

Immunofluorescence were carried out as previously described using 5 10^4 HeLa cells (Morris-Desbois et al., 1999). Briefly, cells were fixed for 20 min with fresh 4% paraformaldehyde and further washed 3 times with PBS. For permeabilization cells were incubated for 5 min in 1 ml of PBS-Triton X-100 0.1% (ds PBS). Free aldehydic groups were blocked by incubation with 0.1 M glycine for 15 min and cells were further incubated with 1% BSA at RT (15-30 min). Cells were washed 3 times with PBS and the primary antibody was incubated for 1.5 h at RT and cells were further washed 3 times with PBS. The secondary antibody conjugated with Alexa Fluor 488 or Alexa Fluor 546 (Invitrogen) was then incubated for 1 h in the dark and the cells were further washed 3 times with PBS and 1 time with distilled water. Finally cells were mounted in 10µl of Vectashield DAPI (1.5µg/ml) (Vector Laboratories, Burlingame, CA, USA). Slides were observed with an Zeiss Axioplan 2

LSM 510 upright confocal microscope (Carl Zeiss AG, Jena, Germany). Colocalization was either evaluated by visual inspection of signal overlap on merged images (Fig. S3) or by using the Colocalization Highlighter plug-in of ImageJ software (National Institutes of Health) (Fig. 6). Threshold settings for each image were automatically set with the threshold tool and assigned to the input window of the Colocalization Highlighter plug-in. The ratio of intensity was set at 50%. Two points are considered as colocalized if their respective intensities are higher than the threshold of their channels, and if their ratio of intensity is higher than 50%.

Antibodies

The antibodies used in this study were as follows: INT6 C-169 rabbit antiserum (Morris-Desbois et al., 1999), mouse monoclonal antibodies to FLAG (clone M2, Sigma-Aldrich) and to HA (clone 7, Sigma-Aldrich), goat polyclonal antibody to EIF3B (A-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal antibody to SLBP (clone 2C4-1C8, Novus Biologicals, Littleton, CO, USA). The antibody directed against MIF4GD was obtained by immunizing rabbit with a peptide corresponding to the first N-terminal 20 amino acids of the protein coupled to ovalbumin.

nCounter RNA analysis

Total RNAs were purified using the Total RNA isolation kit (Macherey-Nagel). Two sequence-specific probes for each transcript were designed by the NanoString company. The capture probe was complementary to a ~50-base region of the mRNA plus a short common sequence coupled to biotin. The adjacent reporter probe was complementary to a second ~50-base region of the transcript and was coupled to a digital fluorescent reporter composed of a unique combination of four spectrally nonoverlapping dyes. These probes were designed for

all the main genes encoding canonical or variant histones with the exception of those exhibiting a very low or highly tissue specific expression as inferred from the data given by the EST profile viewer of the Unigene resource (Boguski & Schuler, 1995) of the National Center for Biotechnology Information. The probes, which were designed to not cross hybridize with other histone mRNAs, were generally located in the divergent sequences located at the 3' end upstream of the stem-loop. To the list of 30 histone genes were added *EIF3E* and *MIFG4GD*, as well as a group of six genes (*CSNK2B*; *ENO1*; *GAPDH*; *GNB2L1*; RPL35A; *TKT*; *YWHAQ*) which were selected on the basis of their weak expression variations in siRNA experiments performed in HeLa cells as evaluated by microarrays analyses (P. Descombes, personal communication). Direct measurement of the mRNA levels of these genes was performed using the nCounter apparatus (Geiss et al., 2008), according to the manufacturer's instructions. Counts for each transcript were analyzed using an Excel macro made at the Genomics Platform, Centre Médical Universitaire, University of Geneva, Switzerland, for correction of background and normalization, as previously described (Beaume et al., 2011).

Real-time quantitative RT-PCR

Total RNAs were extracted using the Nucleic Acid and Protein Purification Kit (Macherey-Nagel, Düren, Germany). One step RT-PCR reactions were performed using the QuantiTect™ SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) and the LightCycler apparatus (Roche) according to the cycling conditions specified in the handbook of the kit. Gene specific primers were designed using the Primer3 software. The sequence of the sense and antisense primers used for quantitative PCR was as follows: firefly luciferase: 5'-

TCAAAGAGGCGAACTGTGTG -3', 5'- GGTGTTGGAGCAAGATGGAT-3'; Renilla luciferase: 5'- TCGTCCATGCTGAGAGTGTC -3', 5'- CTAACCTCGCCCTTCTCCTT -3'.

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Figure legends

Figure 1. The MIF4GD protein interacts with INT6. (***A***) Schematic representation of the three clones encoding MIF4GD identified by two-hybrid screen with INT6 as bait. The table represents the results of testing these clones by two-hybrid with vectors expressing the GAL4 DNA binding domain alone (pGBT9) or in fusion with INT6 (pGBInt6). As control the assay was also performed with a vector expressing the GAL4 activation domain alone (pAS2). The letters w (white) and b (blue) indicate the color of the colonies after 24 h. (***B***) HeLa cells were transfected with the pTL1-INT6FLAG construct, alone (lane 1) or together with the MIF4GDHA expression vector (lane 2). Cell lysates were immunoprecipitated with the antibody to HA and precipitated proteins were analyzed by immunoblot using the antibody to FLAG (upper panel). To monitor expression cell lysates were also analyzed by immunoblot with antibodies to FLAG (middle panel) and to HA (bottom panel). (***C***) Schematic representation of several INT6 deletion mutants fused to the FLAG epitope. COS7 cells were transfected with the MIF4GDHA expression vector together with the constructs expressing

the various parts of INT6 fused to FLAG as indicated. Lysates of these cells were used to perform immunoprecipitations with the antibody to FLAG and immunoblot analysis with the antibody to HA.

Figure 2. Effect of INT6 knockdown on histone protein synthesis. (***A***) Schematic representation of the protocol used to synchronize cells and to label histones. HeLa cells were synchronized by a double- thymidine block and released into the S-phase after the second block. Cells were then pulse-labeled with ^{35}S methionine. (***B***) Hela cells were transfected with either control (lane 1) or three different siRNA duplexes targeting INT6 (lanes 2 to 4). The proteins obtained after acidic extraction were separated on a 15% SDS-polyacrylamide gel which was stained by Coomassie Blue (top panel) or analyzed using a phosphorimager (bottom panel). The bands corresponding to histones H3, H2B, H2A and H4 are indicated. (***C***) The experiment was repeated several times and the amount of radioactivity in the H3/H2B/H2A bands as well as in the H4 band was quantified using the MultiGauge software with normalization with respect to bands of the upper part of the gels (Cakmakci et al., 2008). The mean of the ratios with respect with cells transfected with control siRNAs is represented with error bar corresponding to standard deviation. Data were analyzed with Student's t-test (two-tailed, unpaired) and the stars indicate a P-value of less than 0.05 (*), 0.01 (**) or 0.001 (***) with respect to control siRNA condition.

Figure 3. Gene-specific quantification of histone mRNA levels using the Nanostring technology. Hela cells were tranfected with control, I6.1 or I6.3 siRNA. Total RNAs were prepared and analyzed using probes specific of the histone genes indicated, as well as of *EIF3E* and *MIF4GD* mRNA, with the nCounter apparatus. The graph represents the mean of

either two, I6.1 (dark grey), or three, I6.3 (light gray), independent experiments as ratio with respect to control siRNA and error bars indicate standard deviation.

Figure 4. INT6 and MIF4GD requirement for efficient translation of mRNA ending with the histone stem-loop regulatory element. (***A***) Schematic representation of the four reporter constructs including the firefly luciferase coding sequence under control of SV40 promoter and polyA signal (pGL3-P) or under control of the promoter and stem loop elements of the following human histone genes: *HIST1H2AC* (PSL H2A), *HIST1H2BG* (PSL-H2B) and *HIST2H4A* (PSL-H4). (***B***) After treatment with control, I6.1, I6.3 or *MIF4GD* siRNA duplexes, HeLa cells were transfected with these different constructs together with a renilla luciferase expression vector to normalize transfection efficiency. Firefly and renilla activities were measured in protein extracts of these cells using the dual luciferase assay. The graphs represent the means of the ratio with respect to the control siRNA condition of three independent experiments for the various siRNAs and reporter constructs as indicated. Error bar corresponds to standard variation and stars are indicative of results of Student's T-test as described in legend to Figure 2. (***C***) In these experiments part of the transfected cells was kept aside to quantify by RT-QPCR the firefly and renilla luciferase mRNA. The graph represent the ratio of firefly luciferase mRNA amounts with respect to the control siRNA condition after normalization with renilla luciferase mRNA for the various reporter constructs and RNA.

Figure 5. ***(A)*** Rescue of INT6 siRNA effect by expression of a resistant form of INT6. Experiment was performed as described in legend to figure 3 with the PSL-H4 reporter construct and the control or I6.4 siRNA duplexes. The latter matches with a sequence of the 3' UTR of the INT6 mRNA. Together with the luciferase constructs cells were also

cotransfected with a control or a INT6 expression vector lacking the 3' UTR. Luciferase activity was measured and is represented as described in legend to Figure 3. ***(B)*** Extracts of cells used for the experiment shown in panel A were analyzed by immunoblot using an antibody to INT6 (upper panel) or to β-actin (lower panel). The position of the signal corresponding to these proteins is indicated

Figure 6. INT6 also interacts with SLBP. (***A***) Extracts of HeLa cells were used for immunoprecipitation experiments performed with pre-immune serum (p.i., lane 1), with proteinA-sepharose beads only (lane 2) and with the C-20 antibody to INT6 (lane 3). Immunoprecipitates were analyzed by immunoblot using a monoclonal antibody directed against SLBP. Position of the SLBP signal is indicated on the right of the gel together with the position of the 30 kD band of a molecular weight marker run in parallel. (***B***) HeLa cells were transfected with the pTL-INT6 (lanes 1 and 2) and pSG-FLAGSLBP (lane 2) expression vectors. Lysates from these transfected cells were analyzed by immunoblot using an antibody to INT6 (upper panel) and to FLAG (middle panel). With these cell extracts, immunoprecipitation were carried out using the antibody to INT6 (C20) and the immunoprecipitates were analyzed by immunoblot with the monoclonal antibody to FLAG (lower panel). The position of FLAGSLBP is indicated. ***(C)*** HeLa cells were transfected with control (lane 1), MIF4GD (lane 2) or EIF3B (lane 3) siRNAs. Extracts of these cells were analyzed with antibodies to MIF4GD (top panel), to EIF3B (middle panel) and to β-actin (bottom panel). ***(D)*** These extracts were used for immunoprecipitation experiments performed using the C-20 antibody to INT6 and immunoprecipitates were analyzed with the antibody directed to SLBP (top panel) as in A and with the antibody to INT6 (bottom panel).

Figure 7. Colocalisation in the cytoplasm of INT6 and MIF4GD. HeLa cells were transfected with the MIF4GDHA and INT6FLAG expression vectors in combination (panels a to e), with only with the former (panels f to j) or with only the latter (panels k to o). The cells were stained with the following combination of antibodies: C-169 antibody to INT6 and monoclonal antibody to HA (panels a to j), monoclonal antibody to FLAG and rabbit antiserum to MIF4GD (panels k to o). Representative confocal images are shown, as well as superimpositions of both stainings (panel c, h, m) and of the stainings with the corresponding transmission image (panels d, i, n). In panels c, h and m colocalization was evaluated by using the Colocalization Highlighter plug-in of ImageJ software and colocalized pixels appear in blank. Examples of cytoplasmic foci with colocalisation of INT6 and MIF4GD are indicated by white arrows. Scale bars 5 μ.

Supplemental Figure 1: ***(A)*** COS7 cells were transfected with the pTL-INT6 (lanes 1 and 2) and pSG-MIF4GDHA (lane 2) expression vectors. Whole cell lysates were analyzed by immunoblot using a mix of antibodies to INT6 and HA (upper panel). These lysates were used to carry out immunoprecipitaton using the antibody to INT6 (C20) in the presence of RNAse A. Immunoprecipitates were analyzed by immunoblot using the monoclonal antibody to HA (bottom panel). The positions of the INT6 and MIF4GDHA signals are indicated. ***(B)*** The FLAG immunoprecipitates of COS7 cells which were transfected with the MIF4GDHA expression vector along with the constructs expressing the various parts of INT6 fused to FLAG as described in Fig. 1C were analyzed by immunoblot using a monoclonal antibody to FLAG. The star and the cross indicated non specific bands corresponding to the immunoglobulin heavy and light chains. The position of the bands of a molecular weight marker run in parallel are indicated on the right.

Supplemental Figure 2: (***A***) The *EIF3E* mRNA level of the extracts used in Figure 3 were quantified under the same conditions. The graph represents the results obtained in these experiments for the EIF3E mRNA indicating its efficient silencing by the I6.1 and I6.3 siRNA duplexes. ***(B)*** Extracts of cells used for experiments shown in Figure 2 were analyzed by immunoblot using antibodies to INT6 (upper panel), to MIF4GD (middle panel) and to β-actin (lower panel).

Supplemental Figure 3: Flow cytometry analysis of synchronized cells silenced for INT6 and MIF4GD. ***(A, B, C, D)*** Cell cycle profiles of HeLa cells either non-synchronized (A) or synchronized in S phase by a double thymidine block which were transfected with the control (A and B), I6.1 (C) and MIG4GD (D) siRNA duplexes. (D) Graph representing the number of cells in the G1, S and G2/M phases of the cell cycle for the four experimental conditions.

Supplemental Figure 4: Colocalisation of MIF4GD and SLBP. (***A***) HeLa cells were transfected with the MIF4GDHA expression vector and immunostained with both the rabbit polyclonal antibody to MIF4GD (panel a) and the monoclonal antibody to HA and (panel b). Representative confocal images are shown, as well as superimpositions of both stainings (panel c and e) and of the stainings with the corresponding transmission image (panel d). Examples of colocalisation of MIF4GD and MIF4GDHA are indicated by white arrows. Scale bars 5 μ. (***B***) Non-transfected HeLa cells were similarly analyzed by immunostaining with both the antibody to MIF4GD and a monoclonal antibody to SLBP.

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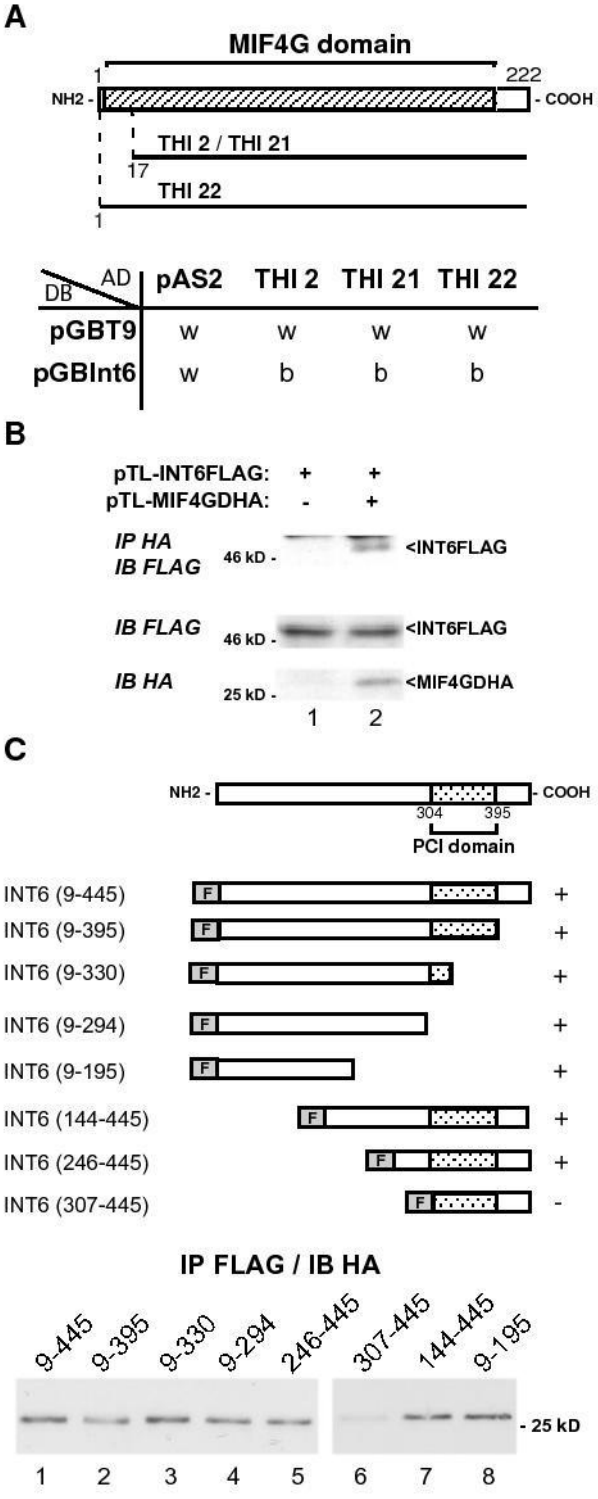

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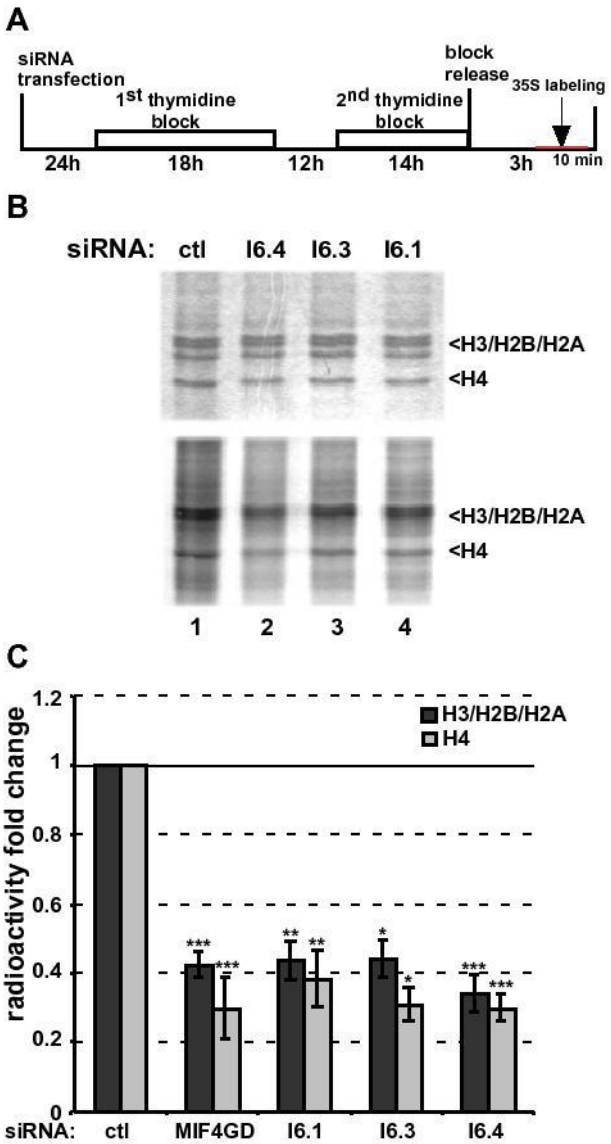

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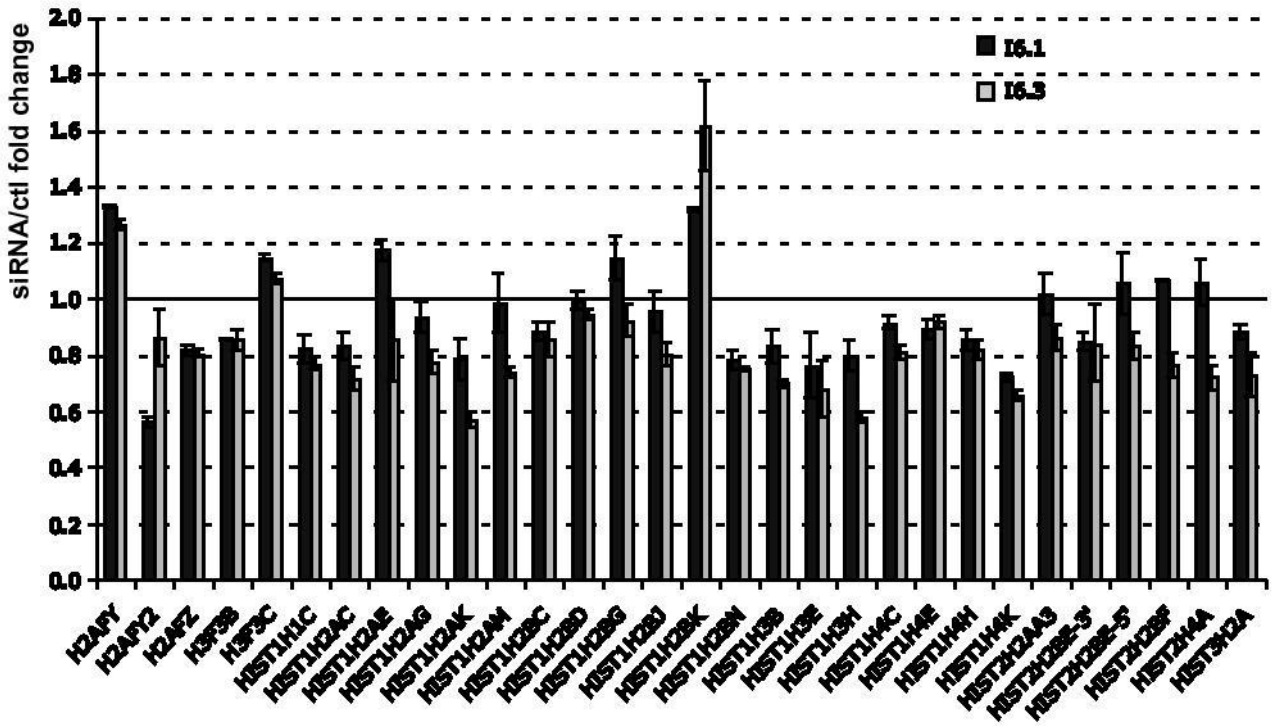

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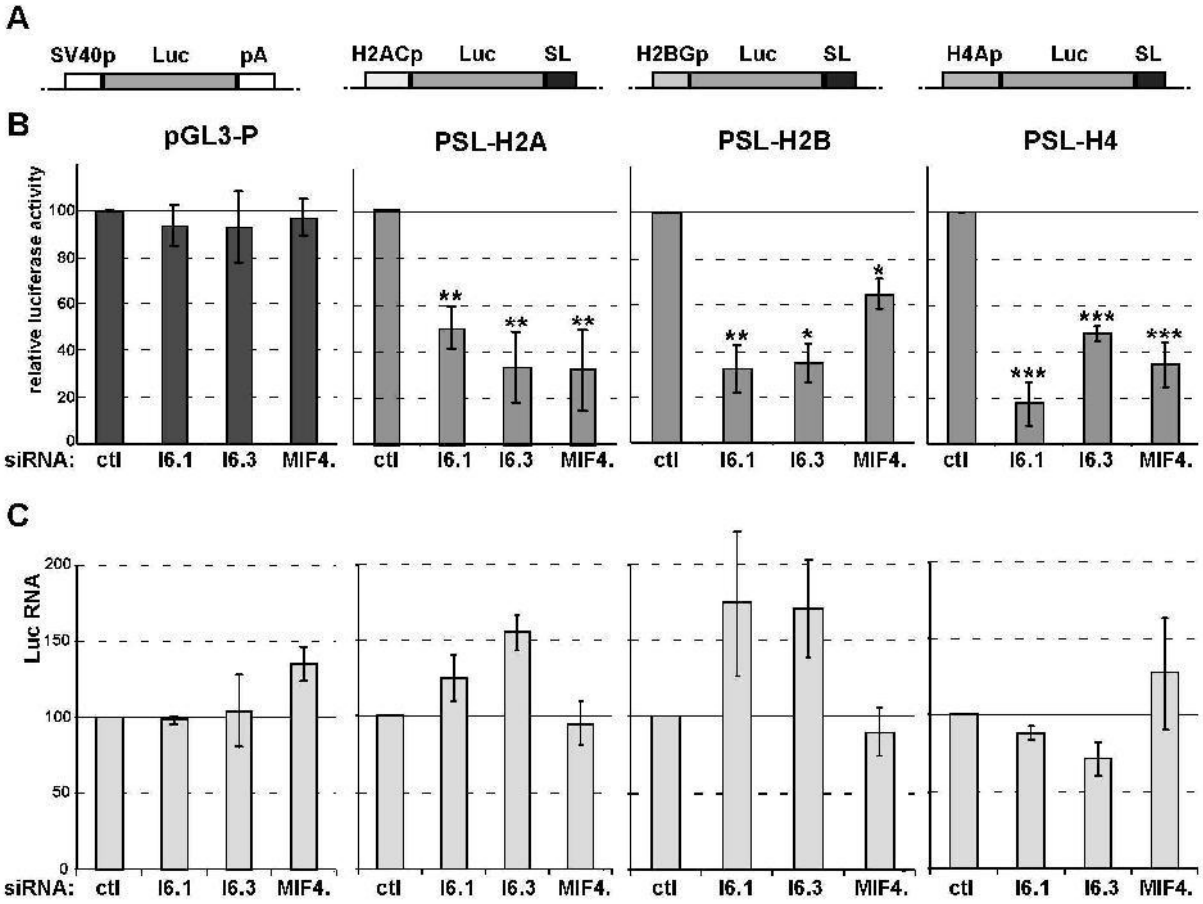

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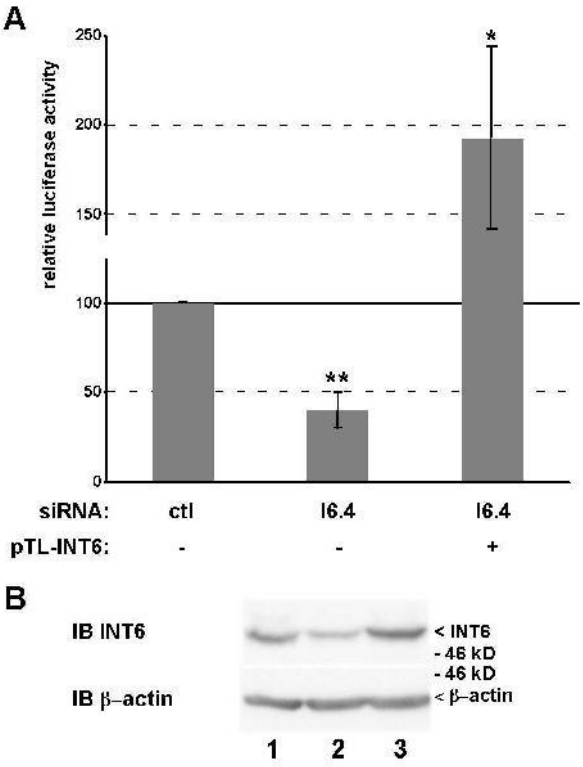

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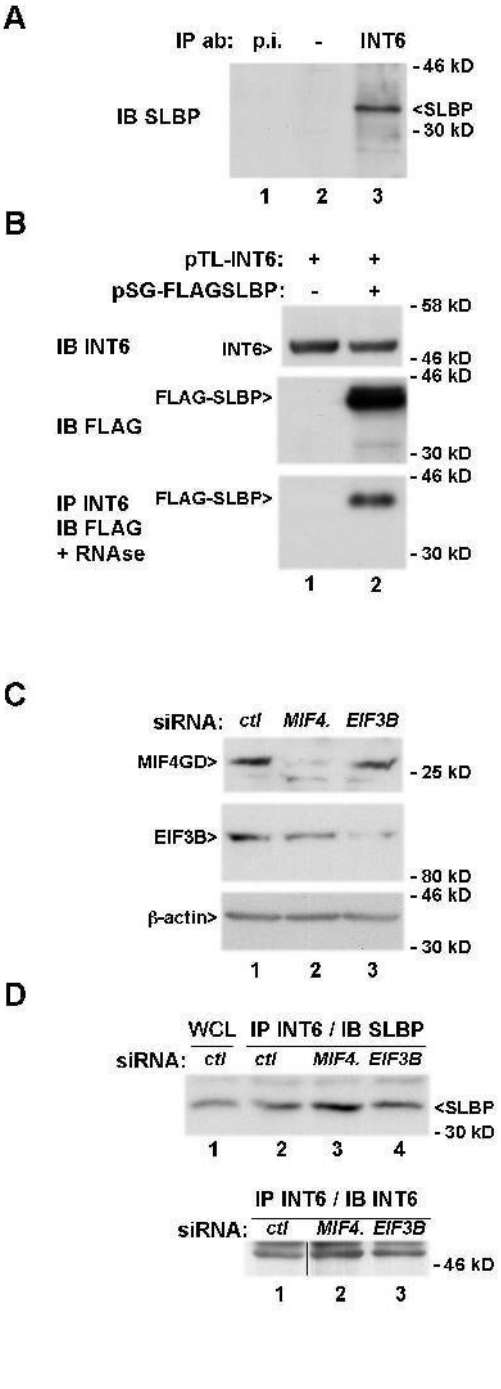

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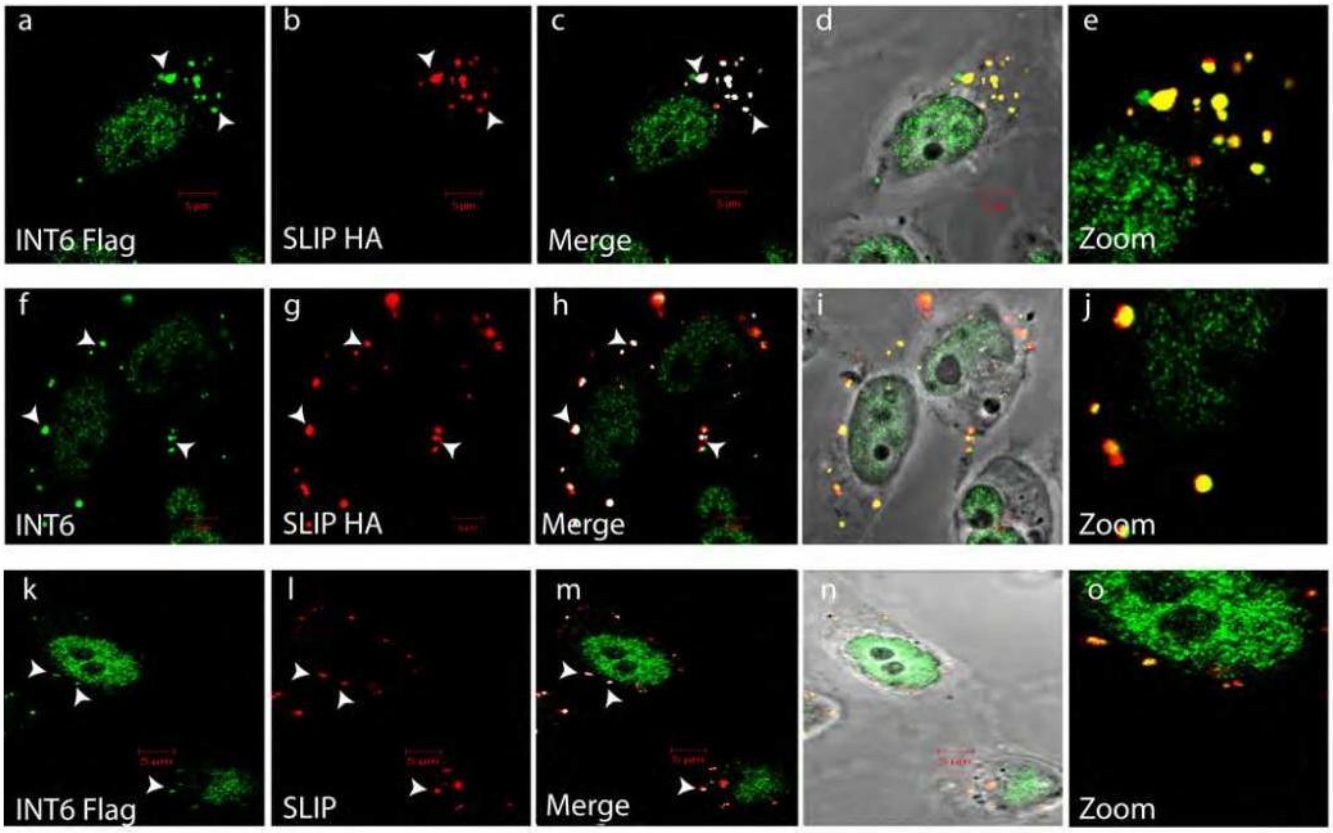

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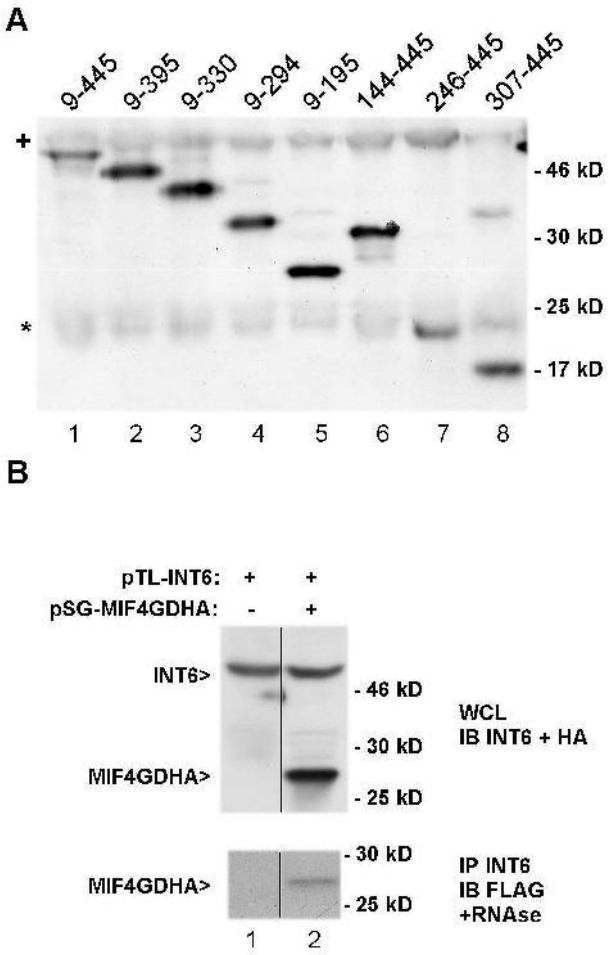
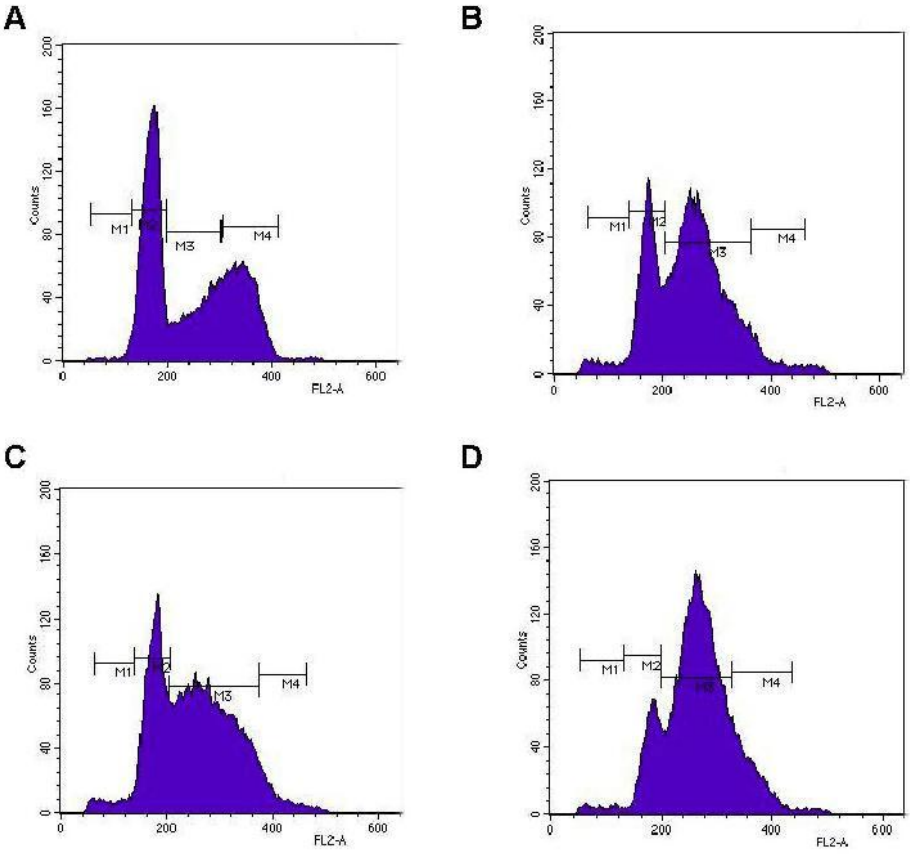
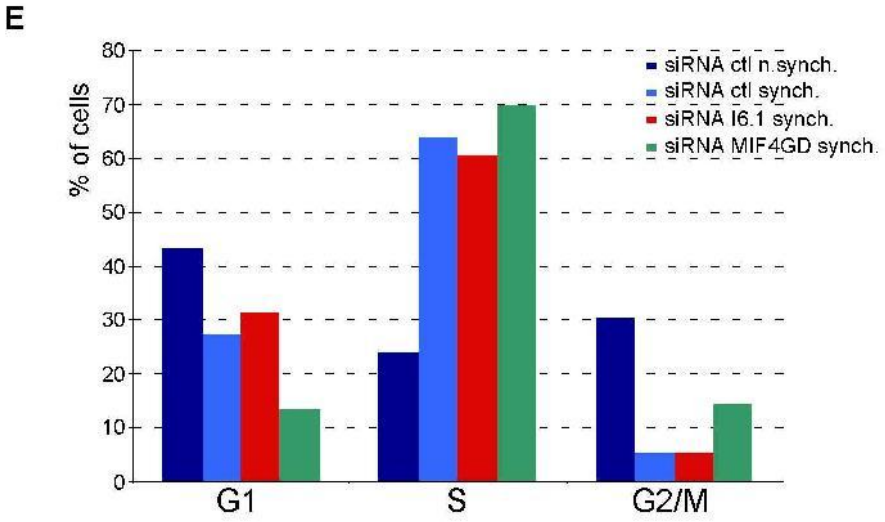


Supplemental Figure 1





Supplemental Figure 2

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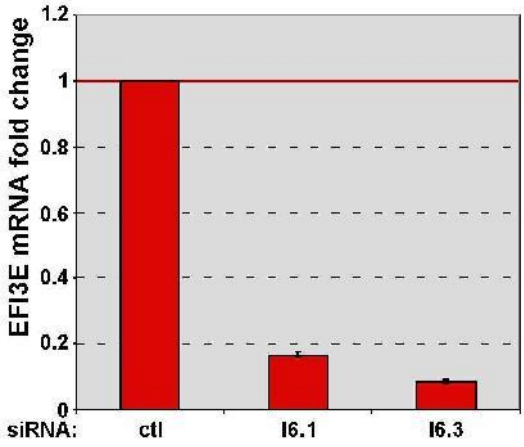

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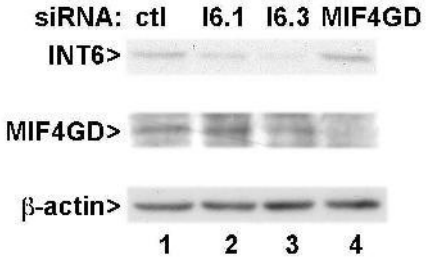

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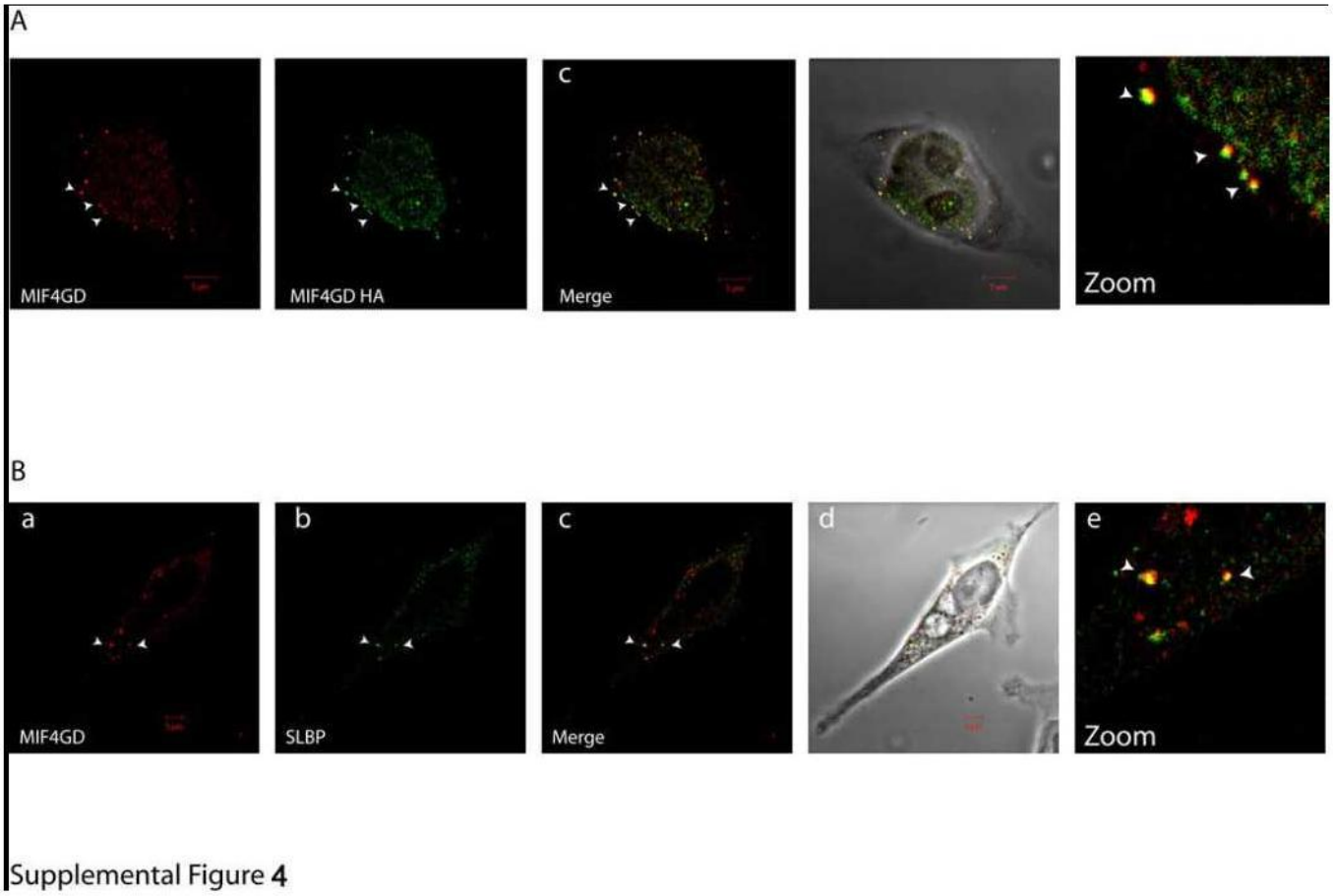

Supplemental Figure **4**

Article II

Article II

INT6 interacts with MIF4GD/SLIP1 and is necessary for efficient histone mRNA translation.

Julia Neusiedler#, Vincent Mocquet#, Christelle Morris and Pierre Jalinot*

# **Both authors contributed equally to this work**

Context of study and summary

This study focuses on the investigation of the translation of histone mRNAs which are derived from cell-cycle regulated, multicopy replication-dependent histone genes. Histone mRNAs are unique mRNAs ending with a conserved RNA hairpin element (steam-loop structure) and lacking a 3' poly(A) tail. In Metazoans, their translation and stability has been shown to be dependent on a 3' stem-loop that binds to SLBP and its interacting partner SLIP1. However the details of this process are only partially understood. In the following document we show that the INT6 protein binds to SLIP1 and SLBP. Moreover we demonstrate that INT6 itself is required for efficient translation of histone mRNA.

The INT6 protein (which is one of the 13 subunits of eIF3) has been shown previously to contact other translation factors including EIF1, 1A, and eIF4G. In our laboratory INT6 was shown to play an important role in cell cycle regulation. INT6 silencing by RNA interference in HeLa cells causes an increased number of cells in the G2/M phases of the cell cycle, along with mitotic defects and chromosome segregation defects. In accordance with our previous findings and given the interaction between INT6 and SLIP1 and SLBP we decided to investigate their functional with the aim of understanding the conserved mechanism of histone mRNA translation.

Indeed the data presented in this manuscript demonstrate that a knockdown of INT6 results in a 2 fold decrease in histone mRNA translation during S phase while the mRNA levels are not or only slightly affected. The down regulation of histone mRNA translation rate was validated by a luciferase reporter system and by measuring the level of newly synthesized endogenous histones. Similarly both luciferase-derived RNAs and endogenous RNA levels have been tested and no significant changes have been observed supporting the conclusion that the action of INT6 on the histone biogenesis takes place at the translational level.

This work brings new facts important for the understanding of the similarities and the differences between the classical translation mechanism and the histone mRNA specific one.

We conclude from these data that INT6 by establishing interactions with SLIP1 and SLBP plays an important role in translation of canonical histone mRNAs and thereby opens new perspectives to understand its role in oncogenesis.

The HTLV-1 Tax protein inhibits nonsense-mediated mRNA decay by interacting with INT6/EIF3E and UPF1.

Short title: HTLV-1 Tax protein inhibits NMD

Vincent Mocquet[1], Julia Neusiedler[1], Francesca Rende[4], Jean–Michel Terme[1*], Madeleine Duc Dodon[2], Jürgen Wittman[3], Vincenzo Ciminale[4], Christelle Morris[1] and Pierre Jalinot[1].

[1]Laboratoire de Biologie Moléculaire de la Cellule, Unité Mixte de Recherche 5239, Centre National de la Recherche Scientifique, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, Lyon, France

[2]Virologie Humaine, Unité 758, Institut National de la Santé et de la Recherche Médicale Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, Lyon, France

[3]Division of Molecular Immunology, Department of Internal Medicine III, Nikolaus-Fiebiger-Center, University of Erlangen-Nürnberg, D-91054 Erlangen, Germany

[4]Department of Oncology and Surgical Sciences, Institute of Neuroscience at the Department of Biomedical Sciences, Università di Padova, Via Gattamelata 64, Padua, Italy

Corresponding author: Pierre Jalinot: E-mail: pjalinot@ens-lyon.fr; Phone: + 33 4 7272 8563; Fax: + 33 4 7272 8080

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* Present address: Molecular Biology Institute of Barcelona (IBMB), CSIC, Barcelona Science Park, c/ Baldiri Reixac, 4-8, Torre R, 3era Planta, 08028 Barcelona, Spain

Abstract: The Tax protein expressed by the human T lymphotropic virus type 1 (HTLV-1) is a potent activator of provirus transcription and plays an important role in the initial steps of CD4+ T lymphocyte transformation by deregulating various cellular pathways. Tax binds INT6/EIF3E (hereafter INT6) that is a subunit of the EIF3 translation initiation factor required for efficient degradation of mRNAs by nonsense-mediated mRNA decay (NMD). In line with this association, we show here that Tax inhibits this important cellular pathway and also interacts with the NMD core factor UPF1. Through specific protein-protein interactions Tax alters the normal recycling of UPF1 and the morphology of processing bodies (P-bodies), the cytoplasmic structures which concentrate RNA degradation factors. Our data indicate that the effect of Tax on cellular genes expression is not restricted to transcriptional control and that this viral protein can favour production of viral RNAs by impeding their degradation.

Introduction

The human T lymphotropic virus type I (HTLV-I) infection is associated in 2-5% of patients with onset of severe diseases, mainly adult T-cell leukemia (ATL) and tropical spastic parapesis, also named HTLV-I associated myelopathy (for a review see[1]). These conditions are characterized by a long latency, infection often occurring in childhood and disease development at adult age. In line with the long latency period, it is estimated that ATL development involves several phases ending by acute proliferation of monoclonal ATL cells. At initial stage, lymphocytes are infected by viral particles leading to provirus integration and expression of various viral proteins. Among them, Tax plays an important role both by inducing transcription of the provirus and by stimulating proliferation of the host cell. To exert these functions, Tax binds and modulates the expression and activity of several key cellular proteins involved in transcriptional control, cell cycle progression, genomic stability, cell adherence and migration, protein degradation and RNA metabolism[2].

Among these various cellular proteins bound by Tax, we have previously characterized INT6, also known as EIF3E, one the 13 subunits of the translation initiation factor EIF3[3]. The complex between both proteins was found to be cytoplasmic, whereas in normal cells INT6 is present both in the cytoplasm and the nucleus[3,4]. In mammalian cells, silencing of INT6 seems to marginally affect general translation, but evidence has been recently obtained for a role of INT6 in the translation of specific genes[5,6]. Regarding its role in association with EIF3, we have previously shown that INT6 was important for the degradation of cellular mRNAs by nonsense-mediated mRNA decay (NMD)[7]. This process leads to degradation of mRNAs including a premature stop codon (PTC), which can arise from mutation or aberrant alternative splicing. NMD also regulates expression of mRNA with upstream ORF (uORF) or long 3'UTR sequences. After a first round of translation[8,9], the presence of a PTC more than 50 nucleotides upstream of a exon-exon junction leads to the association of the SMG1-eRF1-

eRF3-UPF1 complex (SURF) with the mRNA. UPF1 then interacts with the UPF2 and UPF3 proteins present at the nearby exon junction complex (EJC)[10,11]. These interactions lead to the phosphorylation of UPF1 by SMG1 and routing of the mRNA towards degradation[12] that can occur in cytoplamic compartments known as processing bodies (P-bodies)[13,14]. These structures concentrate various proteins such as the decapping enzyme DCP1 and the XRN1 exonuclease[15,16]. Concomitantly with degradation, UPF1 which is also present in P-bodies is dephosphorylated by SMG5 and PP2A and can then be recycled and binds novel mRNAs[17].

By considering the role of INT6 in the NMD pathway along with the effect of Tax on INT6, we asked whether Tax was able to modulate NMD. By using functional assays, we observed that Tax is indeed able to inhibit this mRNA surveillance pathway by establishing contacts with both INT6 and UPF1 and that Tax binds NMD-sensitive mRNA. In agreement with these capacities Tax expression can alter morphology of P-bodies and stabilizes specific cellular and viral RNAs by acting at this post-transcriptional level.

Methods

Constructs, cell culture and transfection

Plasmids used in NMD assays have been previously described: globin NS39 (GlNS39) and globin WT (GlWT)[7]; wild type and mutated β-globin fused to renilla luciferase[18]. The HBZ expression vector was generated from the *HBZ* ORF including the 3'LTR of the pCSHTLV-I plasmid[19] religated with the pUC 19 backbone from pCSHTLV-I .

JPX9 cells were transfected using Fugene6 (Promega) 24h before Tax expression induction with 150 μM ZnCl2. 293T cells were transfected with the calcium phosphate procedure as previously described[20]. In the case of DNA and siRNA cotransfection, 293T cells were first transfected with the siRNAs using the lipofectamin 2000 (Invitrogen) reagent according to the manufacturer's instructions. After 24h, the medium was changed and the tranfection of DNA

vectors was done with the calcium phosphate procedure. For viral mRNAs analysis, 48h after siRNAs transfection with lipofectamin 2000, cells were transfected with 2 μg of the HTLV-1 molecular clone pACH using the GeneJuice reagent (Novagen) for 20-24 h.

NMD assays, qRT-PCR/RTPCR

NMD assays in HeLa cells with the GlNS39 and GlWT constructs were performed as described previously[7]. In JPX9 cells, 2μg of WT or NS39 globin-renilla constructs with 1 μg of firefly reporter plasmid were transfected. The NMD assay was carried out following the dual luciferase assay procedure[18]. mRNA were measured with by qRT-PCR using the QuantiTect SYBR Green qRT-PCR kit (Qiagen) and appropriate primers as previously described[7]. Normalisation was carried out with respect to *GAPDH* mRNA. The values represented in the graphs correspond to the mean of at least three independent measures with error bars corresponding to standard deviation. The stars correspond to the results of Student's t-test (two-tailed, unpaired).

For analysis of HTLV-1 RNAs primer/probe sequences, cycling profiles and absolute quantitation of HTLV-1 transcripts were carried out as previously described[21]. The ΔΔCt method was applied to verify the silencing of *UPF1*. The data represented are the mean of five independent measures. For *HBZ* mRNA analysis, 8μg of pCS-HBZ and 1μg of a renilla luciferase expression vector plasmid were transfected in HeLa cells with lipofectamin 2000 together with or without pSG-Tax (1μg). The *HBZ* mRNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Roche applied science) using the primer 5'-AACTGTCTAGTATAGCCATCA-3' and then PCR amplified.

Immunofluorescence

Immunofluorescence was conducted as described in[22]. See supplementary methods for more details.

Immunoprecipitation and RIP assay

Immunoprecipitations were conducted as described[20] using antibodies listed in supplementary methods. The RIP assays were conducted as described[23]. More details are available in the supplementary methods.

Results

HTLV-1 Tax inhibits mRNA degradation via the NMD pathway.

In order to address the question of Tax effect on the NMD, we carried out assays using plasmids expressing β-globin mRNA either wild type (GlWT) or including a PTC at position 39 (GlNS39). HeLa cells were transfected with these constructs along with either a control or a Tax expression vector and the β-globin mRNA was quantified by qRT-PCR. The mRNAs quantification was performed with or whithout a 3h treatment with the RNA Polymerase II elongation inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). According to previous observations[24], in the absence of Tax the amount of GlNS39 before DRB addition was approximately 5% of that of GlWT (Figure 1A, lane 1). In agreement with its active degradation, this ratio dropped to 2% 3h after addition of DRB (Figure 1A, lane 3). In the presence of Tax the ratio GlNS39/GlWT was 10% (Figure 1A, lane 2), rising to 18% after 3h of DRB treatment (Figure 1A, lane 4 and Figure S1A). This indicated a specific effect of Tax on GlNS39 mRNA stability. Additionally, this effect was checked in JPX9 cells, which are Jurkat cells derivatives expressing Tax under the control of a metallothionein promoter which was induced by addition in the culture medium of Zn^{2+} cations[25]. After induction, we observed an increasing Tax expression (Figure 1D) which correlated with a significant stabilisation of the GlNS39 reporter compared to GlWT, their ratio going from ~7% in the absence of Tax to 35% at 6h post induction (Figure 1C). We also carried out a reversal of the Tax induction by washing the JPX9 cells after 6h of induction and reculturing them for an additional 48h. Under these conditions, the intracellular concentration of Tax decreased

(Figure 1D, compare lanes 3 and 4) and this correlated with a significant drop from 34% to 21% of the GlNS39/GlWT ratio (Figure 1C). Finally, we analysed the decay of these mRNA after transcription blockade by DRB, showing a specific stabilisation of GlNS39 in the presence of Tax (Figure S1B). Collectively these observations indicated that Tax is able to stabilise some NMD-sensitive transcripts.

By testing a series of NMD-prone mRNAs, we previously observed that the INT6 effect was restricted to a subgroup of them, which includes in particular *GADD45α*, *SLIT2*, *BAG1* and *ATF4,* but not *MAP3K14*[7]. To further evaluate an effect of Tax on NMD, we analysed the half-lives of these five endogenous mRNAs in JPX9 cells with or without Tax induction. Cells were harvested at 0h, 1.5h and 4h after addition of DRB and these mRNAs were quantified by qRT-PCR. Interestingly, the *GADD45α*, *SLIT2*, *BAG1* and *ATF4* mRNA half-lives were significantly increased in the presence of Tax ~3 to 4 times (Figure 1E-H). This was not the case for the *MAP3K14* mRNA (Figure 1I). These observations showed that the stability of these cellular mRNAs reacts similarly to the presence of Tax and to the silencing of INT6 by RNA interference[7].

Tax association with NMD-prone mRNA.

To understand how Tax can inhibit NMD, we first tested if Tax binds mRNA subjected to this process. RNA immunoprecipitation (RIP) experiments using the antibody to Tax were performed with extracts of the non Tax-expressing Jurkat cell line and of the Tax-stably-expressing cell line E12, in which the *GADD45α* and *MAP3K14* mRNA stability is affected by the presence of Tax (Figure S2 A and B). The presence of *GADD45α* and *MAP3K14* mRNA in Tax immunoprecipitates was analysed by RTPCR in these 2 cell lines as well as in C8166 cells. No amplification of *MAP3K14* mRNA was obtained (Figure 2A, bottom panel, lanes 1 to 3). For *GADD45α* a clear signal was obtained using extracts of E12 and C8166 cells, whereas Jurkat extracts only gave a background band (Figure 2A, bottom panel, lanes 4

to 6). When the reverse transcription step was omitted, no signal was obtained (Figure 2B). A similar result was obtained for *GADD45α* and *MAP3K14* mRNAs in HeLa cells transfected with a Tax expression vector (Figure 2C, lanes 1 and 5). RIP was also performed on the *GlNS39* mRNA in HeLa after transfection of the GlNS39 plasmid. We observed a specific association of Tax with this mRNA (Figure 2C, compare lanes 1 to 2 and Figure S2C-E). As the primers used for the PCR hybridize on the 5' side in exon 2 and on the 3' side in exon 3, the size of the amplified DNA fragment showed that Tax binds to spliced mRNA. Collectively these observations establish that Tax is part of the mRNP forming on mRNA targeted to NMD degradation.

Tax binding to INT6 and to the UPF1-UPF2 complex.

To assess further how Tax affects this process, we analysed its association with NMD core factors. Immunoprecipitations were done with antibodies to UPF1, UPF2, UPF3B or INT6, using RNAseA treated extracts from C8166 cells, a cell line expressing Tax from integrated provirus. The presence of Tax in these various immunoprecipitates was analysed by immunoblot. As control these experiments were also performed with extracts of Jurkat cells. The intracellular concentration of INT6 and of UPF2, UPF1 and UPF3b was not affected by the presence of Tax (Figure 3A, compare lanes 1 and 2). Interestingly, after immunoprecipitation using C8166 extracts a signal corresponding to Tax was detected specifically with all four antibodies except the pre-immune serum (Figure 3B, lane 1). As control, the Tax signal was absent when the same experiment was performed with a Jurkat cells extract (Figure 3B, lane 2). By showing that Tax expressed from an integrated provirus was able to associate with various NMD core factors, this analysis strengthened the notion that Tax hinders NMD via interaction with INT6 and other NMD factors such as the UPFs.

To decipher the molecular mechanisms leading to this inhibition, we further analysed the network of interactions between Tax, INT6, UPF1 and UPF2. 293T cells were transfected

with different combinations of vectors expressing Tax, INT6-FLAG, HA-UPF1 and HA-UPF2 (Figure 3C). Immunoprecipitations were targeting the HA epitope and immunoprecipitated proteins were analysed by immunoblot with antibodies to the HA epitope (Figure 3D, top panel), to UPF1 (Figure 3D, upper middle panel), to the FLAG epitope (Figure 3D, lower middle panel) and to Tax (Figure 3D, bottom panel). In agreement with the previous experiments, immunoprecipitation of both UPF1 and UPF2 led to a clear Tax signal (Figure 3D, bottom panel, lane 3). Similar results were obtained when HA-UPF1 and HA-UPF2 were immunoprecipitated separately (Figure 3D, bottom panel, lane 4 and 7 respectively), although in this latter case, the HA-UPF2 and Tax signals were markedly weaker. By precipitating Tax we confirmed its association with HA-UPF1, HA-UPF2 and INT6-FLAG (Figure 3E). In these experiments, the association of Tax with HA-UPF2 was possibly mediated by endogenous UPF1. In this regard, a weak endogenous UPF1 signal was detected after an immunoprecipitation of HA-UPF2 alone (Figure 3D, upper middle panel, lanes 6 and 7). In order to clarify this point, we silenced UPF1 by RNA interference while transiently expressing Tax and HA-UPF2. The level of Tax associated to HA-UPF2 was then reduced to a background level (Figure S3 A and B), indicating that UPF1 is likely to bridge Tax and UPF2. Equally, by using a UPF1 mutant that does not bind UPF2[10], it was observed that the binding of Tax to UPF1 was independent of UPF2 (Figure S3 C and D). Collectively these experiments confirmed that Tax associates with INT6 and demonstrated the binding of Tax with the core NMD complex through an interaction with UPF1.

Displacement of INT6 from UPF1 by Tax binding.

We further analysed the integrity of the NMD complex involving INT6 in the presence of Tax (Figure 3 panels C-E). Interestingly, we found that Tax expression strongly decreased the amount of INT6 immunoprecipitated by UPF1 and UPF2, suggesting that Tax prevents the association of INT6 with these NMD factors (Figure 3D, lower middle panel, compare lanes 2

and 3). Equally, while a clear association of Tax with HA-UPF1 alone was observed after immunoprecipitation of Tax or HA-UPF1, this binding was decreased when INT6 was co-expressed in both cases (Figure 3D lower panel and Figure 3E top panel, compare lanes 4 and 5). These data suggest that Tax associates with INT6, forming a complex that cannot associate with the UPF. We confirmed this with endogenous NMD proteins in HeLa cells where INT6 coimmunoprecipitaed much less endogenous UPF1 in the presence of Tax than in its absence (Figure S3F, compare lane1 and 2). This observation supports the notion that Tax competes for the binding of INT6 to UPF1. This point was also addressed by performing confocal microscopy analyses to look at the subcellular localization of these proteins. We observed that in the absence of Tax, ~80% of the rare HA-UPF1 cytoplasmic foci were also stained by the antibody to INT6 (Figure S4A panels a-d and B) while in the presence of Tax, no more than 20% of UPF1 spots colocalized with INT6 (Figure S4A panels e-h and B). These combined results led us to conclude that Tax, by interacting with both UPF1 and INT6 impairs their association.

Tax leads to the accumulation of UPF1 in P-bodies.

In order to better understand the effect of this association on the normal functioning of UPF1, we further analysed how Tax modifies the cellular UPF1 localization. It was reported that UPF1 is partially localized in P-bodies and that inhibition of mRNA degradation increases this localization[13]. HeLa cells were then transfected with vectors expressing HA-UPF1, DCP1-RFP (a component of the P-bodies), and either a control or a Tax expression vector (Figure 4A). In the absence of Tax and in agreement with previous reports, DCP1 fluorescence was mainly observed in a limited number (3 to 9) of small cytoplamic foci corresponding to the P-bodies[26] (Figure 4A, panel b and Figure S5). UPF1 was homogeneously distributed in the cytoplasm with rare small foci which were mainly colocalizing with DCP1 (Figure 4A, panels a-d). In the presence of Tax, the diffuse UPF1

staining concentrated in larger cytoplasmic foci (Figure 4A, panel e). Similarly, the DCP1 foci were much larger than in the absence of Tax (Figure 4A compare panels b and f, Figure S5). Since UPF1 and DCP1 still colocalized in the presence of Tax (Figure 4A panel g), these results suggested that Tax increases storage of UPF1 in P-bodies and causes their enlargement. In the absence of Tax, INT6 showed discrete cytoplasmic foci which colocalized with those corresponding to DCP1 fluorescence. This is in agreement with a role of INT6 in NMD. In the presence of Tax, the size of the INT6 foci did not increase as for UPF1 and they only marginally colocalized with the enlarged P-bodies (Figure S6). These observations show a clearly different behaviour for INT6 and UPF1 in the presence of Tax, supporting the notion that the viral protein causes disruption of the association of UPF1 and INT6. In order to confirm that the accumulation of UPF1 in P-bodies is related to its interaction with Tax, we first looked at the localization of the viral protein in cells expressing DCP1-RFP and p54-GFP, another component of the P-bodies. In the absence of Tax, DCP1 and p54 display a usual pattern for P-bodies (Figure 4B, panels b-c). An increasing amount of Tax led to the formation of DCP1 and p54 foci with an unusual size and number (Figure 4B panels f-o). In addition, to identify whether Tax colocalizes with UPF1 in these DCP1 foci, cells were transfected with an HA-UPF1 expression vector. In these cells several foci exhibiting a Tax, UPF1 and DCP1 costaining were observed (Figure 4C). These observations are in agreement with the interaction of Tax with UPF1 during RNA processing and suggest that Tax might play a direct role in the alteration of the P-bodies.

UPF1 is dephosphorylated by the phosphatase PP2A which is recruited via the SMG5-SMG7 complex[17] and then possibly re-enter a novel round of RNA processing[27]. Accordingly, we further tested if the accumulation of UPF1 in P-bodies was linked to alteration of this dephosphorylation step. To address this point, cells were transfected with the HA-UPF1 vector together with either a control of a Tax expression vector (Figure 5A). Extracts were

analysed with an antibody raised against phosphorylated UPF1[28] and another one which recognizes the S/TQ motifs phosphorylated by PI3KKs such as SMG1, the NMD UPF1 kinase. Expression of Tax raised the intensity of the signal detected with both antibodies (Figure 5A, middle and bottom panel, compare lanes 1 and 2) while the total amount of HA-UPF1 was unchanged (Figure 5A, top panel). Immunoprecipitation of Tax was also performed with these extracts and revealed that Tax associates with phosphorylated UPF1 (Figure 5B, middle and bottom panel). We further analysed the association of these phosphorylated forms of UPF1 with SMG5 which is part of the NMD dephosphorylation complex. Cells were transfected with different combinations of Tax, HA-SMG5 and UPF1 (without HA tag) expression plasmids. Immunoprecipitations were carried out against the HA tag of SMG5 and the presence of UPF1 in the immunoprecipitates was analyzed by immunoblot. In the absence of Tax, no significant level of UPF1 bound to HA-SMG5 was detectable (Figure 5D, compare lane 1 and 3). This point was previously described[17]: the SMG5-UPF1 interaction is so transient that only inhibition of UPF1 dephosphorylation allows its detection. Differently, in the presence of Tax and with similar levels of HA-SMG5, immunoprecipitated UPF1 was clearly detected (Figure 5D, lane 2). Collectively, these data indicate that Tax is able to inhibit NMD dependent UPF1 dephosphorylation, leading to an increase of SMG5-UPF1 association.

The NMD inhibition by Tax depends on its interaction with INT6.

In order to better understand the interactions involved in the NMD inhibition by Tax, we tested three different Tax mutants : Tax M22 which is inactive for NF-κB activation[29], Tax M47 which is inefficient in transactivating CREB responsive promoters[30] and Tax K1-10R in which all ubiquitinable lysines were mutated to arginine. This latter mutant is defective with respect to both NF-κB and CREB pathways[31,32]. The effects of these Tax mutants were assessed using the NMD assay. In agreement with the results of Figure 1A, the expression of

the wild type Tax in HeLa cells increased the ratio GlNS39/GlWT to ~9%. The expression of Tax M22 as well as Tax K1-10R led to a stronger increase with GlNS39 (~20% and 18% of GlWT, respectively). By contrast, the GlNS39/GlWT ratio with the Tax M47 mutant was similar to that observed in the absence of Tax (Figure 6A). Thus we further tested if the inactivity of the M47 mutant was due to a protein-protein interaction defect. Cells were transfected with vectors expressing HA-UPF1 and INT6-FLAG, together with a control plasmid or the different Tax expression vectors (Figure 6B-D). Tax immunoprecipitations were carried out and the presence of HA-UPF1 and INT6-FLAG in the immunoprecipitates was analyzed by immunoblot. Interestingly, Tax M22 binds more strongly to both INT6-FLAG (2 times) and HA-UPF1 (3 times) than Tax WT did. A similar result was obtained with Tax K1-10R. For Tax M47, binding to HA-UPF1 was slightly increased (1,5 times), but interaction with INT6-FLAG was markedly reduced (4 times less) (Figure 6D). In this experiment equal quantities of Tax protein were precipitated (Figure 6C, bottom panel). This observation indicates that the L319 and L320 Tax residues are necessary for association with INT6. By considering the ability of these Tax mutants to interact with UPF1 and INT6, it appears that the efficiency with which Tax inhibits the NMD correlates directly and positively with binding to INT6. The Tax M22 and K1-10R which binds INT6 more efficiently than wild type are more active in inhibiting the NMD, whereas Tax M47 which interacts poorly with it is inactive. The importance of the INT6/Tax interaction was also confirmed by an NMD assay in induced JPX9 cells since competing extra INT6 led to a significant decrease (~40%) in the inhibition of NMD by Tax (Figure S7).

Finally we also analysed the effect of the M22 and M47 Tax mutants on the P-bodies aspect. HeLa cells were transfected with DCP1-RFP and control or Tax expression vectors described in the Figure 4C and the Tax WT plasmid (Figure 6E, panel b,e) was replaced by Tax M22 or Tax M47 (Figure 6E, panel d, g). As expected, Tax M22 was able to induce many

DCP1-RFP foci (Figure 6E, panel c, f) which exhibited a size and number similar to that caused by Tax WT (in both case ~80% of the cells exhibited abnormal P-bodies, Figure 6E bottom graph), while only a limited number of abnormal DCP1-RFP foci were observed with Tax M47 (~11% of the cells). These data show a correlation between the ability of Tax to functionally inhibit NMD and to modify the aspect of P-bodies.

HTLV-1 mRNAs are sensitive to NMD.

Characterization of the inhibitory effect of Tax on RNA degradation through the NMD pathway raised the question of the sensitivity of the various viral mRNAs to this process. To address this question, we transfected the pACH plasmid which includes the entire HTLV-1 provirus in cells which were previously treated with either control or UPF1 siRNAs. 24h after provirus transfection, RNA were prepared from these cells and analysed by qRT-PCR[21]. Under these conditions the UPF1 mRNA was reduced to ~20% of its level in control cells, indicating an effective silencing (Figure 7A). Although an increase was seen for all viral mRNAs, their sensitivity to UPF1 silencing was variable. The effect was weak for mRNA encoding Gag, Env, Rex/Tax and p21rex, but more pronounced for those coding for p12, p13, p30tof and both forms of HBZ (Figure 7B). This analysis suggested that some viral RNA are prone to UPF1-mediated degradation. In order to determine if Tax can cause stabilization of such viral RNAs we analysed the *HBZ* transcript which was selected as its transcription is much less sensitive to Tax than that of the other sense transcripts (VM, unpublished observations). Cells were transfected with a plasmid including the 3' moiety of the provirus and allowing the expression of the *HBZ* mRNAs under the control of the 3' LTR. The decay of the *HBZ* sp1 mRNA after addition of DRB was analyzed by qRT-PCR with or without Tax. This experiment showed that Tax increases ~2.6 times the half-life of the *HBZ* sp1 mRNA (Figure 7C). Collectively, these data support the notion that Tax enhances viral expression at the post-transcriptional level.

Discussion

The observations presented in this report add evidence for a role of INT6 in mRNA degradation by the NMD pathway. While UPF1 inhibits translation by associating to EIF3[12], our data suggest that INT6 participates in this EIF3-UPF1 complex. An issue which will require further evaluation is the dynamics of the process. Indeed, some considerations indicate that INT6 might be present at early, as well as at late steps of the processing of a mRNA targeted for degradation[5,7,33]. Interestingly, it has been reported that a fraction of INT6 was associated with chromatin[34] and purification experiments have shown its presence in the RNA Pol II holoenzyme (PJ and J-M Egly, unpublished observations). The possibility exists that INT6 can be loaded via its interaction with the CBP80 subunit of the Cap Binding Complex[7]. When the pioneer round is completed on WT mRNA, the mRNP is reconfigured[35] and routed towards active translation with an EIF3 complex devoided of INT6 if unnecessary[5,6]. Alternatively, in the presence of a PTC, UPF1 is phosphorylated, inhibiting the translation and leading to the mRNA degradation [12,36]. This degradation can occur in the P-bodies, where we observed the partial localization of INT6, to the contrary of other EIF3 subunits[26]. Therefore, INT6 is likely to stick to the mRNP until its degradation, whereas the EIF3 complex might be previously detached.

The HTLV-1 Tax protein has been described as a potent transactivator of provirus expression but also as an immortalising protein with pleiotropic activities. In this report we show a novel effect of Tax in inhibiting the NMD pathway. In line with functional effects observed with reporter and endogenous mRNAs, Tax binds directly INT6 and UPF1, but inhibits the association between both proteins. Interestingly Tax was able to markedly enhance the presence of UPF1 in the P-bodies, in which Tax can also be detected, and also increase the number and the size of these P-bodies foci. This effect is likely due to inhibition of mRNA degradation as observed in other cases[27]. In line with this effect, we observed that

Tax expression causes an increase in the amount of phospho-UPF1, a form that interacts with degradative factors. It has been shown that SMG5 can act as an adaptor to link UPF1 to PP2A which dephosphorylates it and thereby allows its recycling. Interestingly it has been shown that Tax was able to maintain IKKγ in an active state by inhibiting its dephosphorylation by PP2A[37,38]. It is possible that in a similar manner the presence of Tax impedes the dephosphorylation of UPF1 by inhibiting PP2A activity. As a consequence, the Tax-UPF1 complexes accumulate in the P-bodies preventing the recycling of this core NMD factor. However, the analysis of Tax mutants indicated that the Tax/INT6 association is also important to impact the NMD. Then by interacting with both INT6 and UPF1, Tax would inhibit the processing of NMD-prone mRNAs at two different steps (FigureS8).

Moreover it is known that ~30% of the cancers would be due to premature stop codons in tumor suppressor gene escaping the NMD and leading to truncated dominant negative proteins. The Tax dependent NMD inhibition might extend such an effect to in frame PTC due to mutations or alternative splicing, what frequently occurs in T cells[39]. Here we limited our analysis to mRNAs already described to be stabilised after silencing of UPF1, UPF2 or INT6 [7,40,41]. Future systematic studies of NMD-sensitive transcripts increased by Tax should help to define how this activity of Tax contributes to cell transformation. Among the analysed genes, ATF4/CREB-2[42] has been involved in the HTLV-1 LTR transactivation. This effect of Tax might then participate to transcriptional induction of HTLV-1 provirus. The role of ATF4 during HTLV-1 infection has also been related to its heterodimerization with AP-1 family transcription factors and has been associated to T-cell transformation through AP-1 responsive genes[43].

Our data also show that some HTLV-1 mRNAs are sensitive to UPF1-mediated degradation. This suggests that viral mRNAs expression downstream of activated transcription is also regulated at the posttranscriptional level. Thus, it is likely that in addition

to its important effect on transcription Tax also intervenes by allowing escape of the RNAs from degradation. While NMD could be an effective protective mechanism against viral infection, Tax allows an escape from it, favouring expression and persistence of the virus. Among the viral genes, expression of the antisense gene *HBZ* is clearly detectable all along the infection at the RNA level[44,45]. The current hypothesis is that the *HBZ* gene has a dual functionality: *HBZ* mRNA promotes T-cell proliferation[46], while HBZ protein suppresses Tax-mediated viral transcription[47]. In line with its sensitivity to UPF1-mediated degradation, our data show that Tax significantly stabilizes *HBZ* mRNA. This observation illustrates the complexity of the cross talk between both viral proteins.

In conclusion, although NMD has already been associated to the host-pathogen biology[48,49], we report here for the first time that an HTLV-1 protein actively interferes with this pathway. Our observations also establish that Tax not only controls viral and cellular transcription but also alters the post-transcriptional outcome of mRNAs. Then it is important to consider this NMD effect for our understanding of the virus oncogenic effect.

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Author contribution

VM, JN and FR performed the experiments which were designed and analyzed by them plus VC and PJ. J-MT, MDD, JW and CM provided important materials and advice for realization of the experiments. VM and PJ wrote the manuscript which was critically corrected by all other authors.

Conflict of interest

None of the authors have a financial interest related to this work.

Figure Legends

Figure 1: Effect of Tax on stability of NMD-prone mRNA. A: NMD assays were performed in HeLa cells in the absence (lanes 1 and 3) or presence of Tax expression (lanes 2 and 4), without (lanes 1 and 2) or with addition of 100 µg/ml DRB for 3h (lanes 3 and 4). The amount of GlNS39 mRNA is represented as a percentage of GlWT mRNA in a bar graph with error bar corresponding to standard deviation (*: $P < 0.05$; **: $P < 0.01$). **B**: extracts used in A were analyzed by immunoblot with an antibody to Tax. **C**: NMD assays were performed in JPX9 cells. Renilla luciferase activity from NS39 plasmid was normalized and expressed as a percentage of that from WT plasmid, without (0h) or with Tax induction for 3h or 6h. The 6h+W48h bar corresponds to cells washed and further cultured for 48h after 6h of induction (Student's t-test results refer to the 0h condition, except for the 6h+W48 (6h induction). **D**:

Extracts used in C were analyzed by immunoblot with antibodies to Tax and to β–actin as indicated. **E**: The half-life of the *GADD45α* mRNA was measured in non-induced (squares and full line) and 6h-induced (crosses and dotted line) JPX9 cells. *GADD45α* mRNA amounts were measured by qRT-PCR at each time point after addition of DRB, normalized to *GAPDH* mRNA and the natural logarithm of the values expressed as fractions to time 0 were plotted against time. Each point correspond to the mean of three independent measures and error bar indicates standard deviation. Half-life of the mRNA in both conditions was calculated and is indicated in the graph. **F, G, H, I**: Same as E, but for *BAG1*, *ATF4*, *SLIT2* and *MAP3K14* mRNAs, respectively.

Figure 2: Tax associates with NMD-prone mRNAs. A: RIP were carried out with extracts from E12 (lanes 1, 4), Jurkat (lanes 2, 5) and C8166 (lanes 3, 6) cells using an antibody to Tax. The immunoprecipitates were analysed by RTPCR using primers specific for *MAP3K14* (lanes 1-3) and *GADD45α* (lane 4-6) mRNAs (lower panel). RTPCR were also carried out on cell lysates (upper panel). **B**: RIP assays were performed as described in A, except that the reverse transcription step was omitted. Lanes 4 and 5 are positive controls of PCR amplification for each mRNAs . **C**: RIP experiments were carried out as in A with HeLa cells transfected with a construct expressing GlNS39 as well as Tax (lanes 1, 3, 5) or a control vector (lanes 2, 4, 6). *GlNS39* (lanes 1, 2), *GADD45α* (lanes 3, 4) and *MAP3K14* (lanes 5, 6) mRNAs were analyzed by RTPCR from the same IP. Tax expression in the cell extracts was monitored by immunoblot (bottom panel).

Figure 3: Interaction network between Tax, INT6 and the NMD core factors UPF1 and UPF2. A, B: endogenous NMD factors were immunoprecipitated in C8166 (B, lane 1) and Jurkat (B, lane 2) extracts after RNAse A treatment. From the upper panel to the lower: UPF2, UPF1, UPF3b, INT6, Tax and pre-immune serum. The cell extracts (A, lanes 1 and 2) as well as the immunoprecipitates (B, lanes 1 and 2) were analysed by immunoblot for the

indicated proteins **C-E, C**: Tax, INT6-FLAG, HA-UPF1 and HA-UPF2 expression vectors were transfected in 293T cells as indicated. The expression of INT6-FLAG was analysed by immunoblot using antibodies to FLAG. **D**: Extracts of these transfected cells were used to carry out immunoprecipitations using the antibody to the HA epitope. Immunoprecipitates were analysed by immunoblot using antibodies to HA, to UPF1, to FLAG and to Tax. Ig marks the signal of the immunoglobulin heavy chain. **E**: extracts from D were immunoprecipitated with an antibody to Tax and immunoprecipitates were analysed by immunoblot with the antibodies to HA, to FLAG and to Tax.

Figure 4: Colocalization of Tax and NMD factors with P-bodies. A: Confocal microscopy analysis of HeLa cells transfected with HA-UPF1 and DCP1-RFP expression vectors together with a control (panels a-e) or Tax (panels e-h) expression vector. Immunostaining was done with an antibody to HA and appears in green (panels a, e). The red fluorescence from DCP1-RFP was also analyzed and is represented alone (panels b, f) or with the HA fluorescence (panels c and g). Panels d and h are an enlargement of P-bodies examples indicated by arrows. **B**: Confocal microscopy analysis of HeLa cells transfected with vectors coding for two components of the P-bodies DCP1-RFP and p54-GFP. 0, 0.1 and 0.5µg of Tax plasmid were cotransfected (panels f-j and k-o). Immunostaining of Tax appearing in blue (panels a, f, k,) along with green fluorescence from p54 (panels b, g, l) and red fluorescence from DCP1-RFP (panels c, h, m) are shown as in A. **C** : Cells were treated and depicted as before except that immunostaining was done using antibodies to Tax (blue, panel a) and UPF1 (green, panel b).

Figure 5: Tax stabilises the phosphorylated forms of UPF1. A: 293T cells were transfected with Tax and HA-UPF1 expression vectors as indicated. Cell extracts were analysed by immunoblot with an antibody to HA, phosphorylated UPF1 or phosphorylated S/TQ motifs. The signals of phosphorylated UPF1 were quantified and normalised with respect to total UPF1. **B**: extracts from A were immunoprecipitated with an antibody to Tax

and immunoprecipitates were analyzed by immunoblot with the indicated antibodies. **C**: 293T cells were transfected with HA-SMG5, UPF1 and Tax expression vectors as indicated. Cell extracts were analysed by immunoblot using antibodies to UPF1 and to Tax. **D**: Extracts from C were immunoprecipitated with the antibody to HA and immunoprecipitates were analyzed with antibodies to HA and to UPF1.

Figure 6: Tax mutants affect differently NMD efficiency. **A**: NMD assays were carried out as described in Figure 1A with cotransfection of control (-), Tax wild type (WT), Tax M22, Tax M47, and Tax K1-10R expression vectors. (p-values refer to the control point (-) * $P < 0.05$). **B**: cells were transfected with the HA-UPF1, INT6-FLAG and Tax expression vectors as indicated. Extracts from these cells were analyzed by immunoblot with antibodies to HA and to FLAG. **C**: Extracts from B were immunoprecipitated using an antibody to Tax and immunoprecipitates were analyzed with antibodies to HA, to FLAG and to Tax. Ig marks the immunoglobulin heavy chain. **D**: Signals corresponding to HA-UPF1 and to INT6-FLAG in the immunoprecipitates from C were quantified, normalized with respect to the signals detected in the extracts and plotted in a bar graph (white bars : INT6 ; black bars : UPF1). **E**: confocal microscopy analysis of HeLa cells transfected with constructs expressing DCP1-RFP together with a control (panel a) or vectors expressing either WT Tax (panels b,e), Tax M22 (panels c,f) or Tax M47 (panels d,g). The red fluorescence from DCP1-RFP and Tax immunostaining (green) are shown. The proportions of normal P-bodies ($n < 9$ and $\varnothing < 1.5\mu m$) and unusual P-bodies ($n > 9$ and/or $\varnothing > 1.5\mu m$) is represented in a bar graph below.

Figure 7: The HTLV-1 mRNA are sensitive to the NMD. A, B: HeLa cells were transfected first with a control or UPF1 siRNA and 48h later with the HTLV-1 molecular clone construct pACH. UPF1 mRNA from these cells was quantified to control silencing efficiency (A). The various HTLV-1 transcripts were also quantified by qRT-PCR and the ratio of the amounts determined in UPF1-silenced versus control cells is represented on the

bar graph (B). Error bar corresponds to standard deviation (* $P < 0.05$; ** $P < 0.01$). **C**: The half-life of the *HBZ sp1* mRNA was measured in HeLa cells transfected with or without Tax. *HBZ* mRNA amounts were measured by qRT-PCR at each time point after addition of DRB, normalized to renilla mRNA and the natural logarithm of the values expressed as fractions to time 0 were plotted against time.

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Figure 1

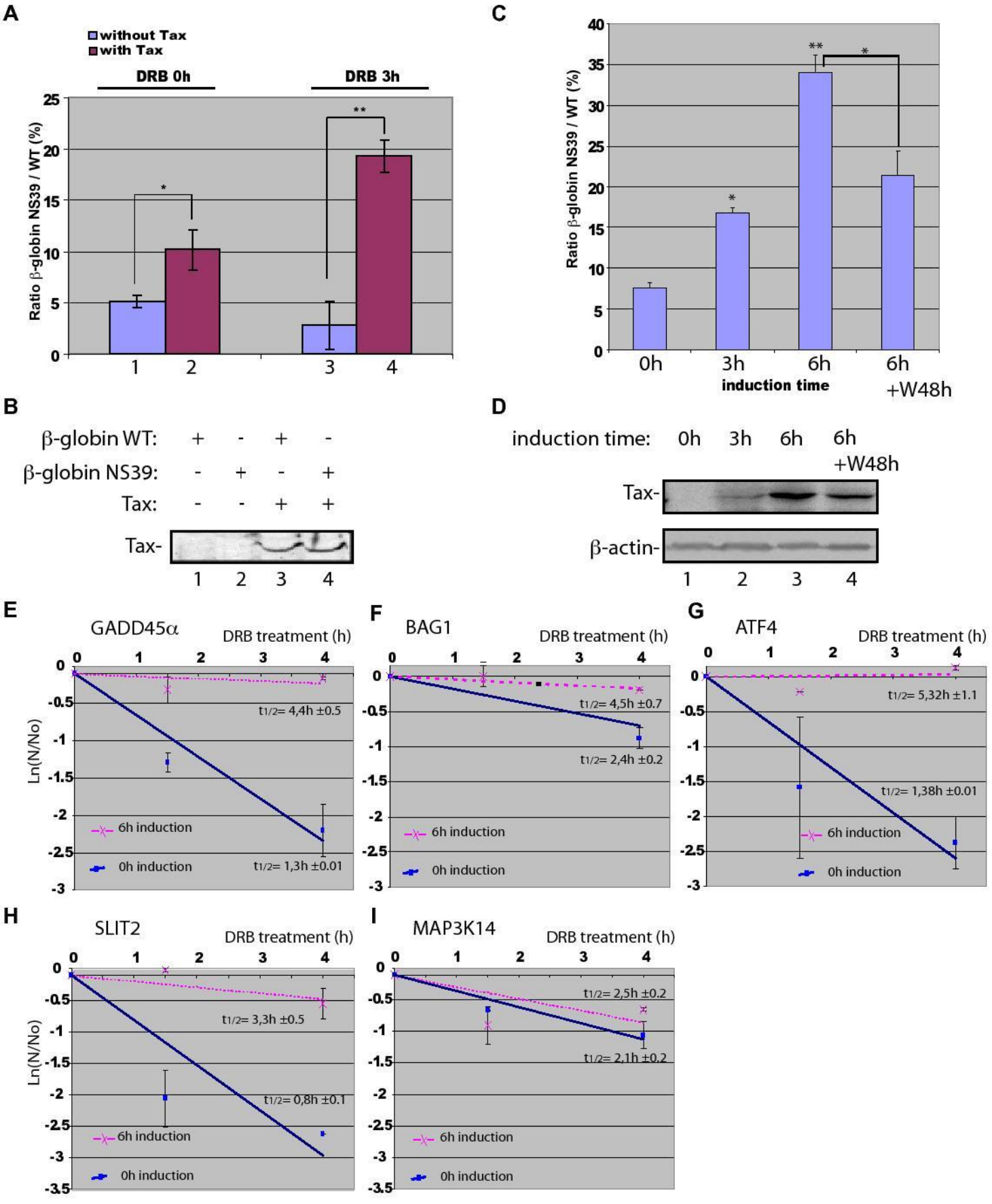

A
without Tax
with Tax
DRB 0h
DRB 3h
Ratio β-globin NS39 / WT (%)
1
2
3
4
B
β-globin WT: + - + -
β-globin NS39: - + - +
Tax: - - + +
Tax-
1 2 3 4
C
Ratio β-globin NS39 / WT (%)
0h
3h
6h
6h +W48h
induction time
D
induction time: 0h 3h 6h 6h +W48h
Tax-
β-actin-
1 2 3 4
E
GADD45α
DRB treatment (h)
Ln(N/No)
t1/2= 4,4h ±0.5
t1/2= 1,3h ±0.01
6h induction
0h induction
F
BAG1
DRB treatment (h)
t1/2= 4,5h ±0.7
t1/2= 2,4h ±0.2
6h induction
0h induction
G
ATF4
DRB treatment (h)
t1/2= 5,32h ±1.1
t1/2= 1,38h ±0.01
6h induction
0h induction
H
SLIT2
DRB treatment (h)
Ln(N/No)
t1/2= 3,3h ±0.5
t1/2= 0,8h ±0.1
6h induction
0h induction
I
MAP3K14
DRB treatment (h)
t1/2= 2,5h ±0.2
t1/2= 2,1h ±0.2
6h induction
0h induction

Figure 2

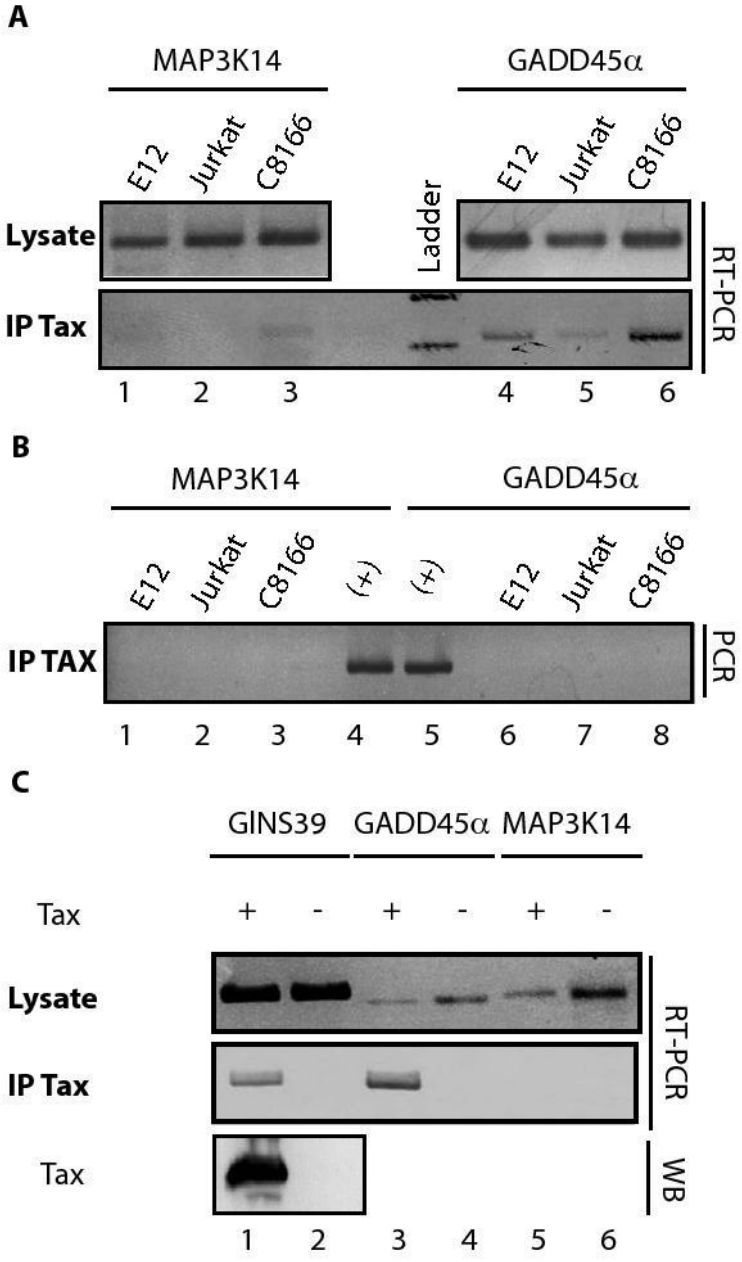

A
MAP3K14
GADD45α
E12
Jurkat
C8166
Ladder
E12
Jurkat
C8166
Lysate
IP Tax
RT-PCR
1 2 3 4 5 6
B
MAP3K14
GADD45α
E12
Jurkat
C8166
(+)
(+)
E12
Jurkat
C8166
IP TAX
PCR
1 2 3 4 5 6 7 8
C
GlNS39
GADD45α
MAP3K14
Tax
+ - + - + -
Lysate
IP Tax
Tax
RT-PCR
WB
1 2 3 4 5 6

Figure 3

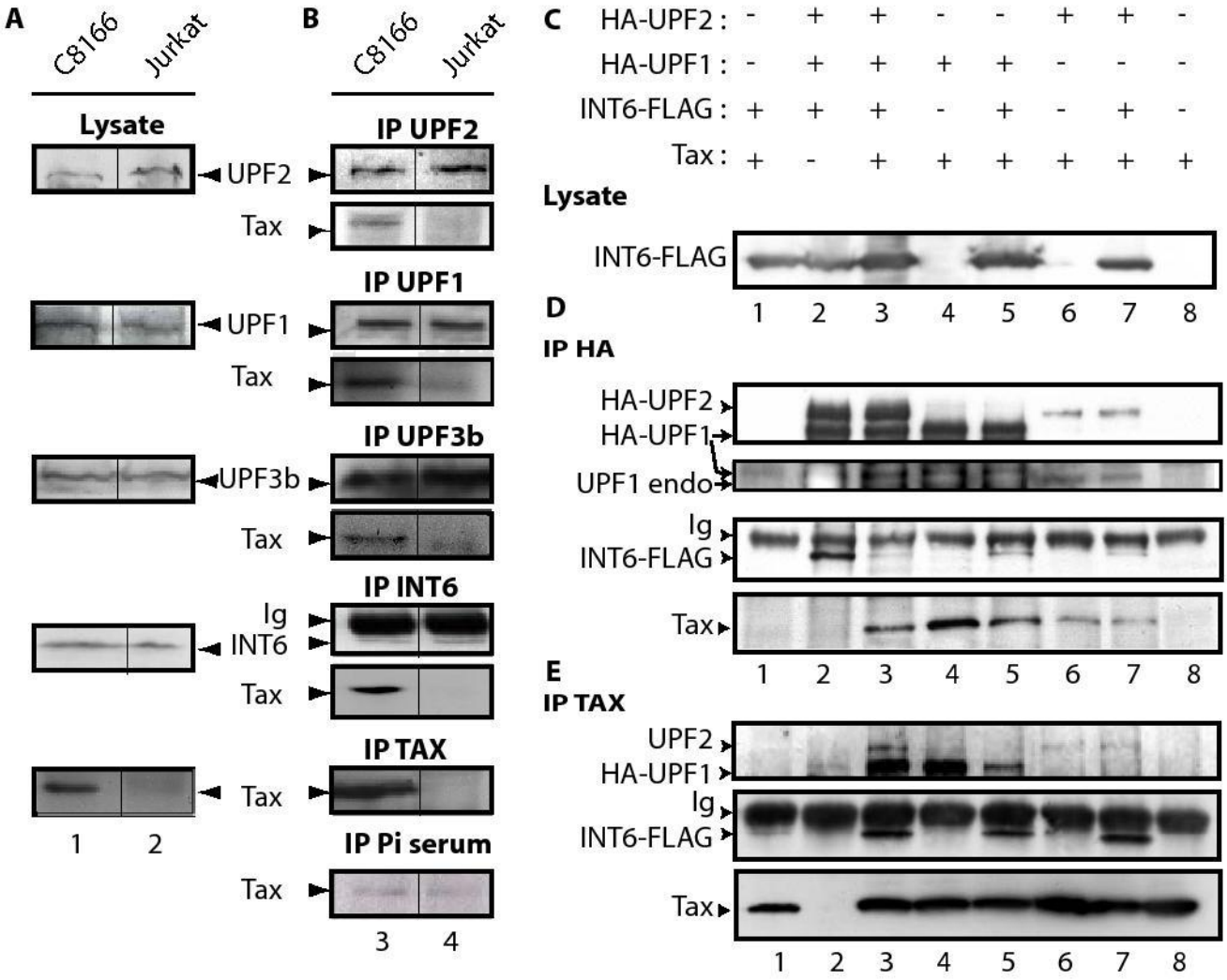

A
C8166
Jurkat
Lysate
UPF2
UPF1
UPF3b
Ig
INT6
Tax
1
2
B
C8166
Jurkat
IP UPF2
Tax
IP UPF1
Tax
IP UPF3b
Tax
IP INT6
Tax
IP TAX
Tax
IP Pi serum
Tax
3
4
C
HA-UPF2 : - + + - - + + -
HA-UPF1 : - + + + + - - -
INT6-FLAG : + + + - + - + -
Tax : + - + + + + + +
Lysate
INT6-FLAG
1 2 3 4 5 6 7 8
D
IP HA
HA-UPF2
HA-UPF1
UPF1 endo
Ig
INT6-FLAG
Tax
1 2 3 4 5 6 7 8
E
IP TAX
UPF2
HA-UPF1
Ig
INT6-FLAG
Tax
1 2 3 4 5 6 7 8

Figure 4

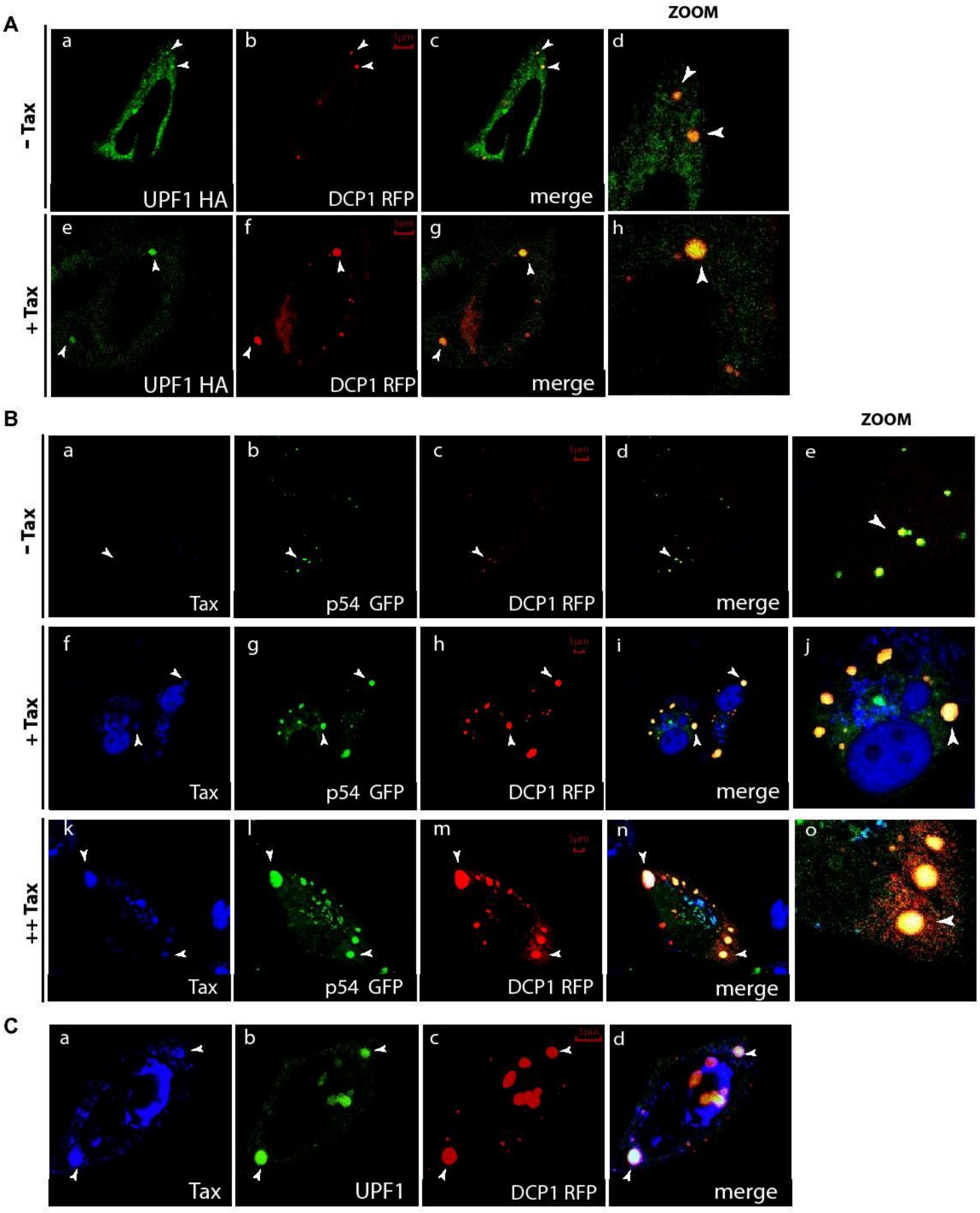

A
ZOOM
- Tax
+ Tax
a
b
c
d
e
f
g
h
UPF1 HA
DCP1 RFP
merge
5µm
B
ZOOM
- Tax
+ Tax
++ Tax
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
Tax
p54 GFP
DCP1 RFP
merge
5µm
C
a
b
c
d
Tax
UPF1
DCP1 RFP
merge
5µm

Figure 5

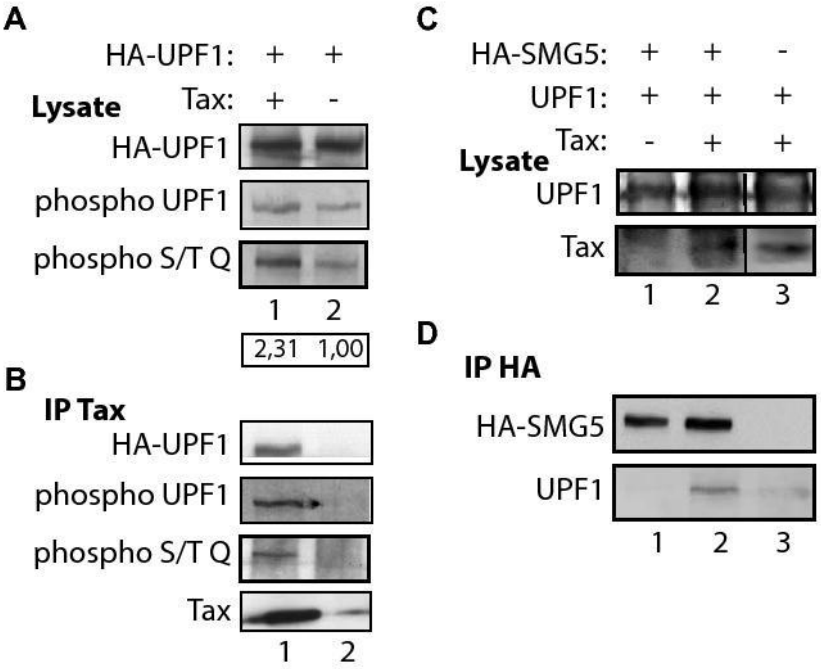

A
HA-UPF1: + +
Lysate Tax: + -
HA-UPF1
phospho UPF1
phospho S/T Q
1 2
2,31 1,00
B
IP Tax
HA-UPF1
phospho UPF1
phospho S/T Q
Tax
1 2
C
HA-SMG5: + + -
UPF1: + + +
Tax: - + +
Lysate
UPF1
Tax
1 2 3
D
IP HA
HA-SMG5
UPF1
1 2 3

Figure 6

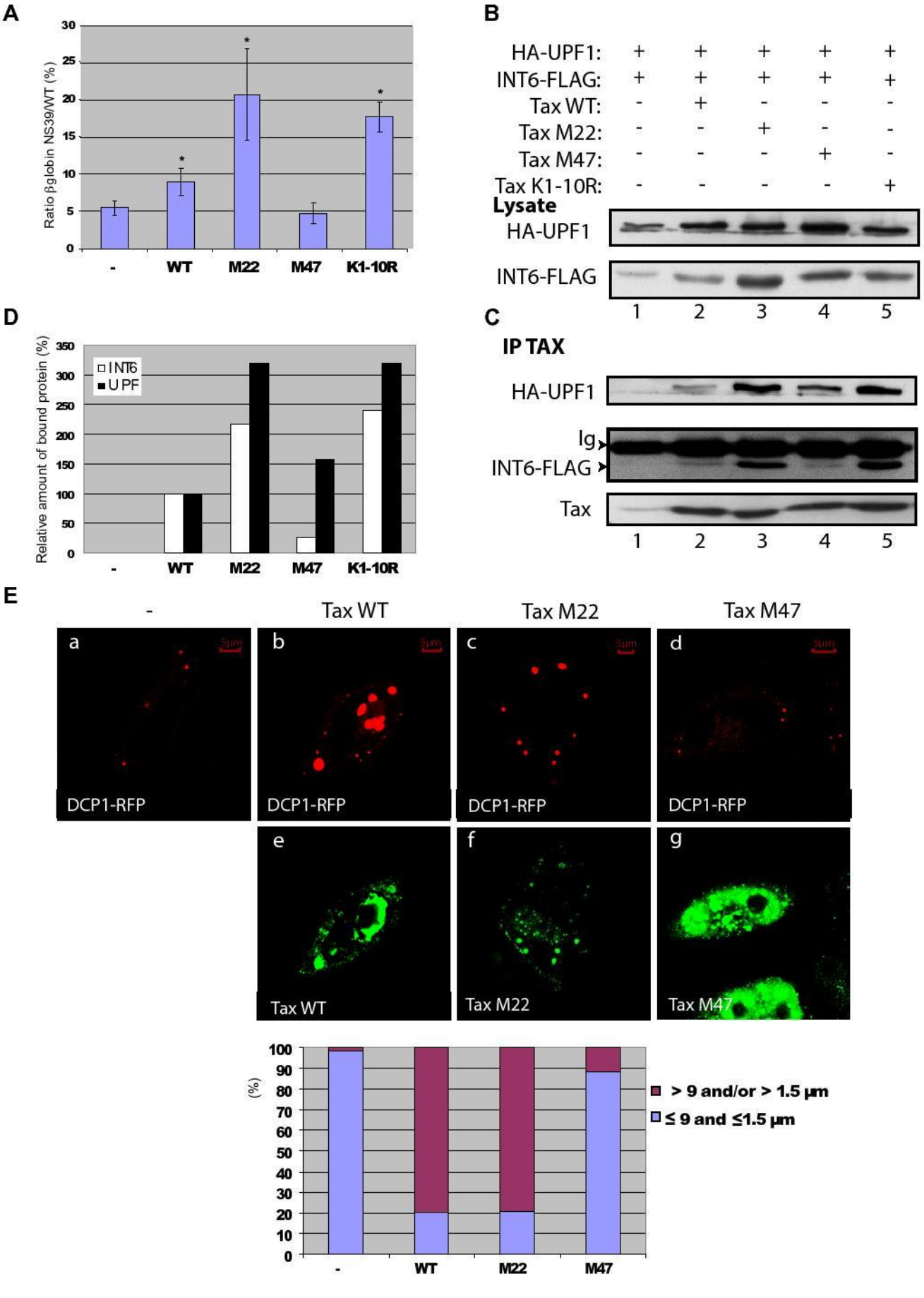
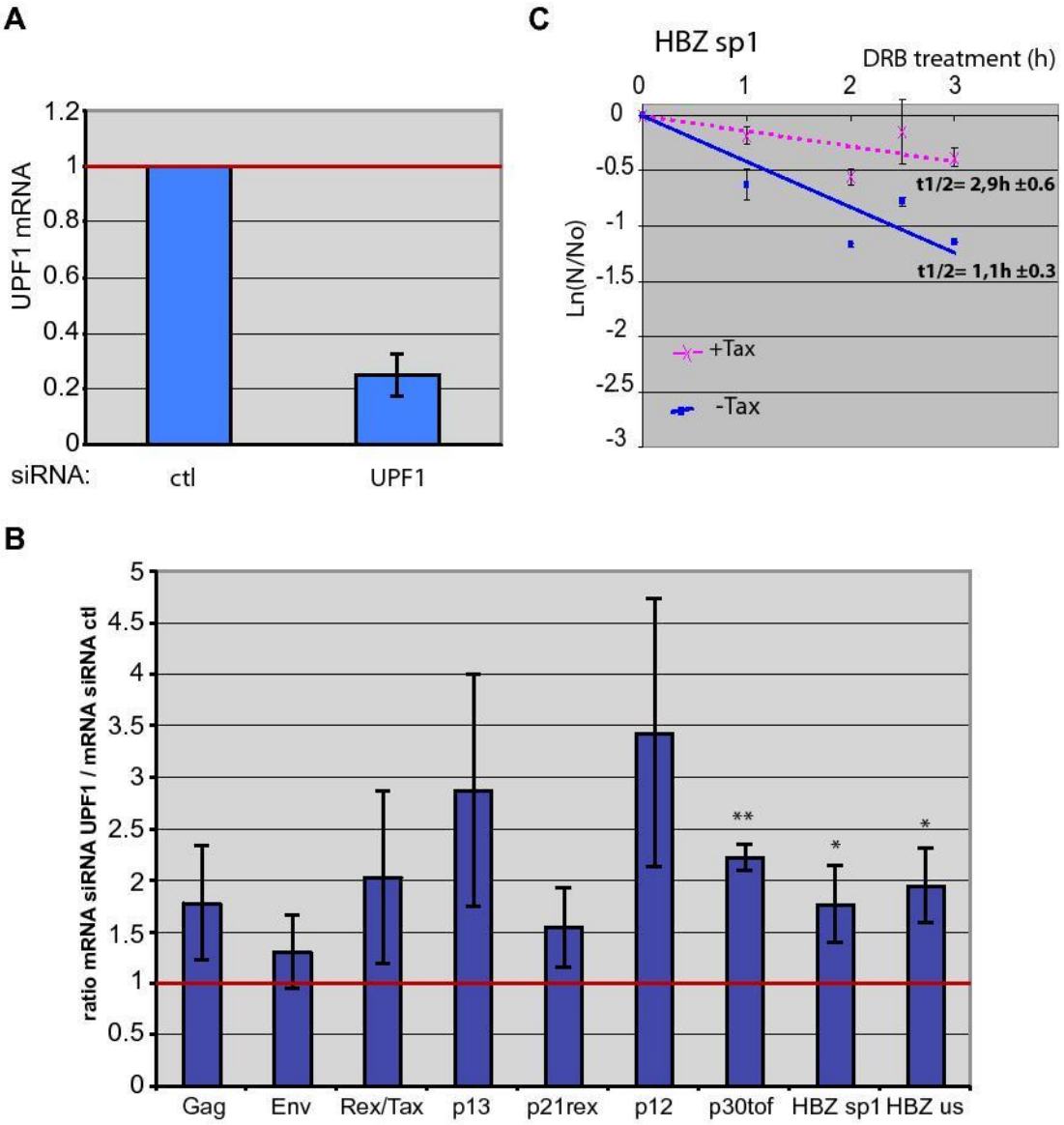
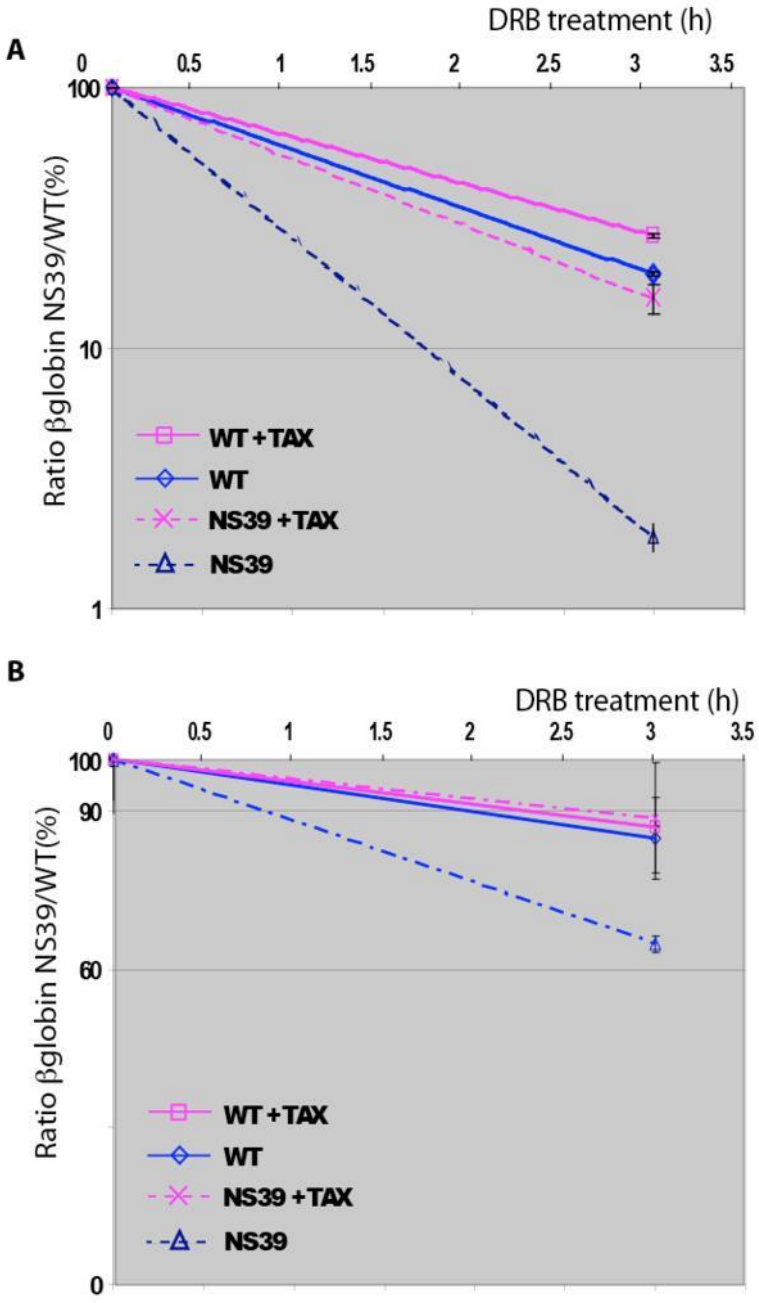
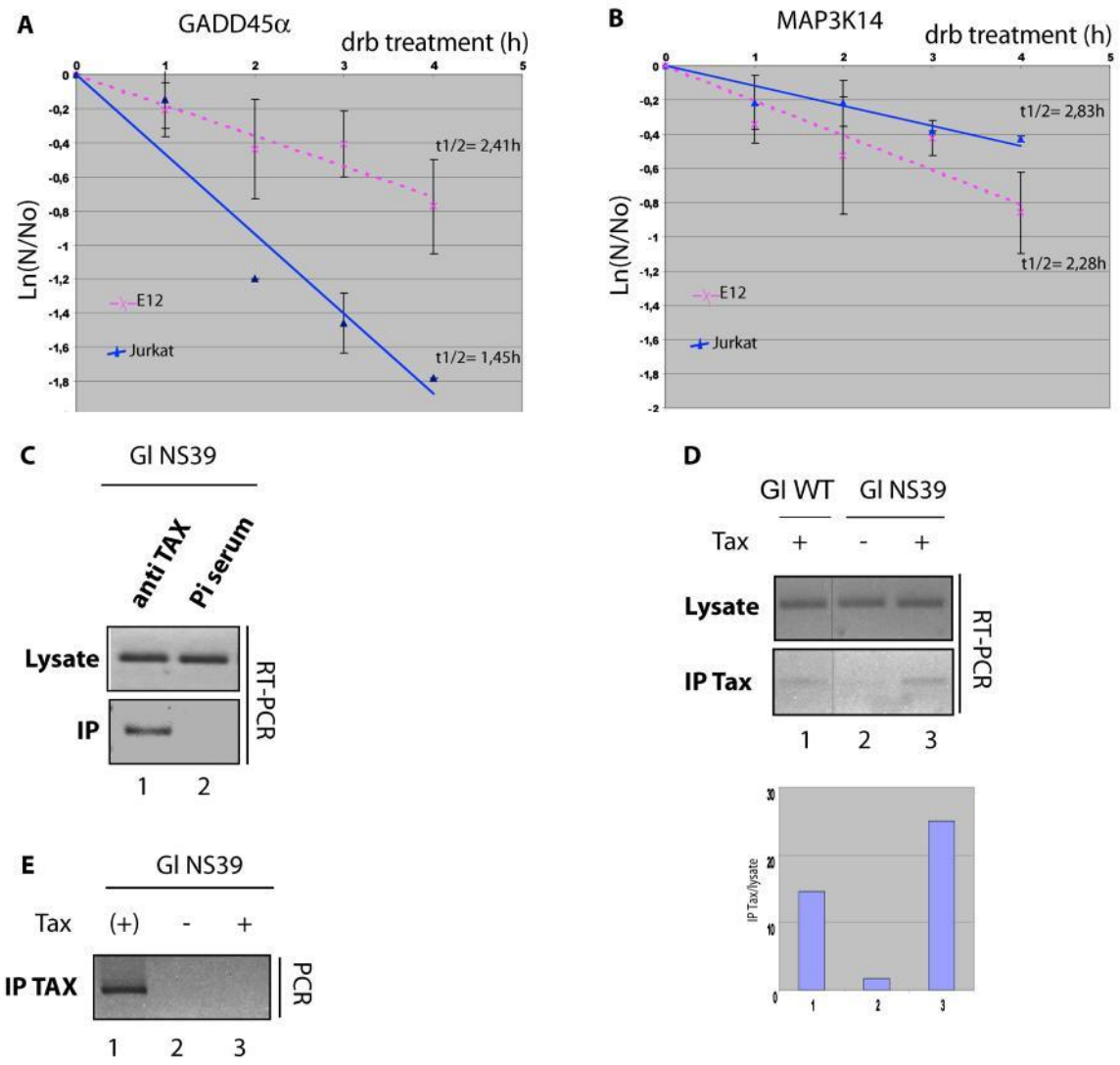

A
Ratio βglobin NS39/WT (%)
30
25
20
15
10
5
0
-
WT
M22
M47
K1-10R
B
HA-UPF1: + + + + +
INT6-FLAG: + + + + +
Tax WT: - + - - -
Tax M22: - - + - -
Tax M47: - - - + -
Tax K1-10R: - - - - +
Lysate
HA-UPF1
INT6-FLAG
1 2 3 4 5
C
IP TAX
HA-UPF1
Ig
INT6-FLAG
Tax
1 2 3 4 5
D
Relative amount of bound protein (%)
350
300
250
200
150
100
50
0
INT6
UPF
-
WT
M22
M47
K1-10R
E
-
Tax WT
Tax M22
Tax M47
a
b
c
d
5µm
DCP1-RFP
DCP1-RFP
DCP1-RFP
DCP1-RFP
e
f
g
Tax WT
Tax M22
Tax M47
100
90
80
70
60
50
40
30
20
10
0
(%)
-
WT
M22
M47
> 9 and/or > 1.5 µm
≤ 9 and ≤1.5 µm

Figure 7

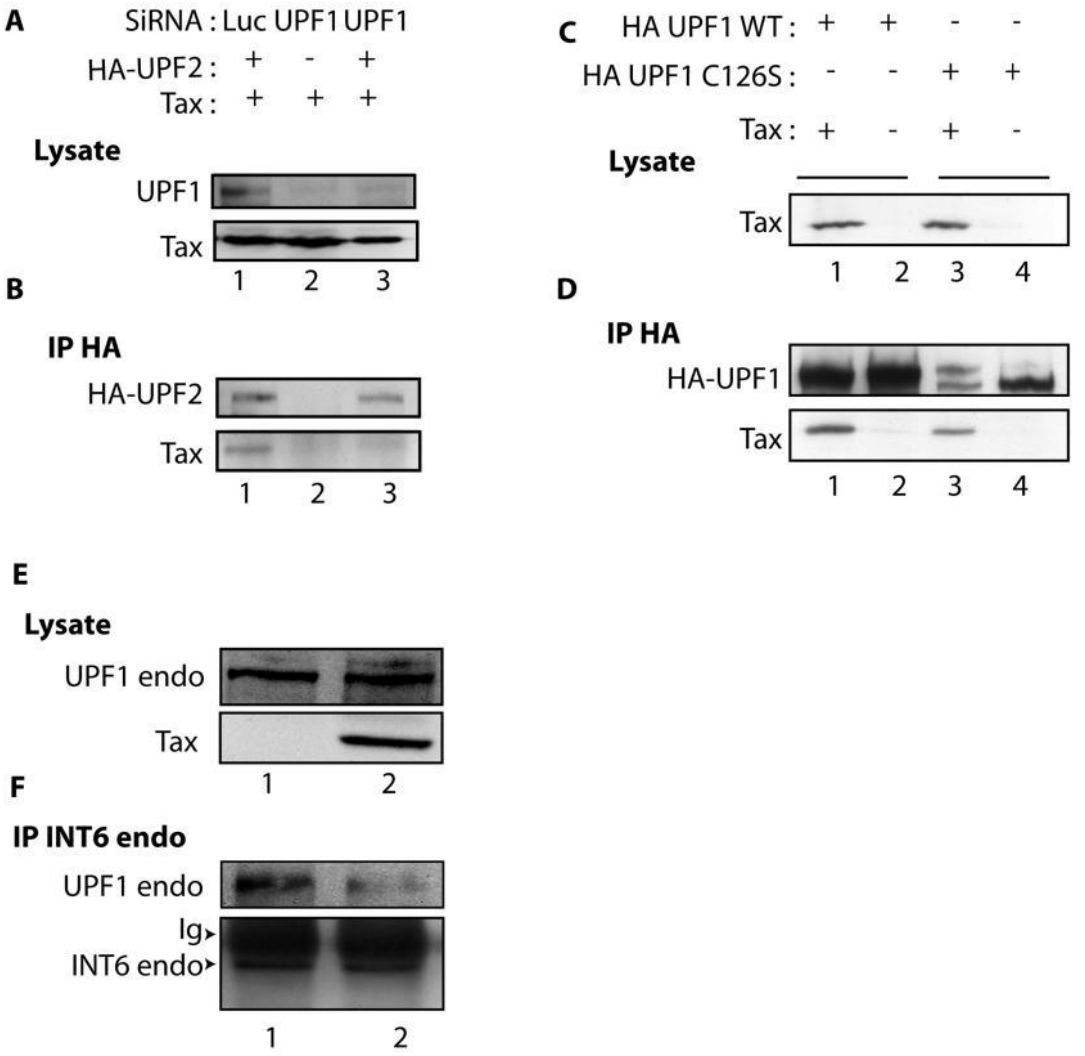

A
UPF1 mRNA
1.2
1
0.8
0.6
0.4
0.2
0
siRNA:
ctl
UPF1
C
HBZ sp1
DRB treatment (h)
0
1
2
3
0
-0.5
-1
-1.5
-2
-2.5
-3
Ln(N/No)
t1/2= 2,9h ±0.6
t1/2= 1,1h ±0.3
+Tax
-Tax
B
ratio mRNA siRNA UPF1 / mRNA siRNA ctl
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
*
*
Gag
Env
Rex/Tax
p13
p21rex
p12
p30tof
HBZ sp1
HBZ us



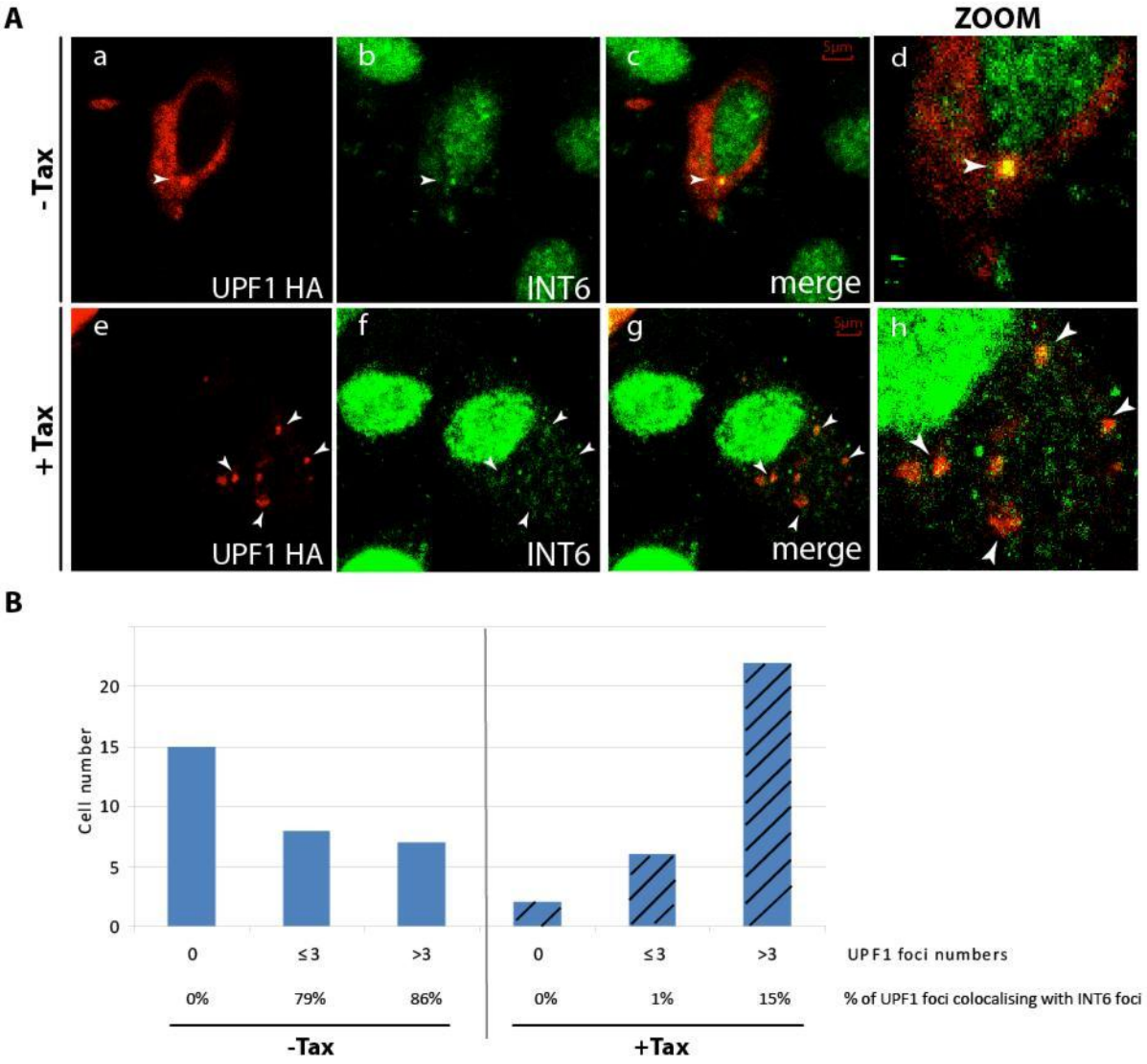
Supplemental Figure 1: Decay of the *GlWT* and *GlNS39* mRNA in the absence and presence of Tax. A : HeLa cells were transfected with β-globin reporter plasmids (Gl NS39 or Gl WT) and a control (triangle/dotted line or diamond/full line, respectively) or a Tax expression vector (cross/dotted line or square/full line, respectively). Cells were treated or not with 100 µg/ml DRB for 3h. Total mRNA was extracted and the globin mRNA was quantified by qRT-PCR and normalised to the *GAPDH* mRNA. The globin mRNA decay for each condition is represented in the graph and shows that Tax specifically stabilises the *Gl NS39* mRNA as compared to the *Gl WT*. **B** : NMD assay was carried out with JPX9 cells (see methods) with 6h of Tax induction (cross/dotted line or square/full line, respectively) or without (cross/dotted line or square/full line, respectively). Cells were treated or not with 100 µg/ml DRB for 3h. Renilla luciferase signal was measured and the luciferase mRNA decay was represented in a graph. Similarly as for A, it shows that Tax specifically stabilises the premature stop codon containing mRNA as compared to the WT mRNA.



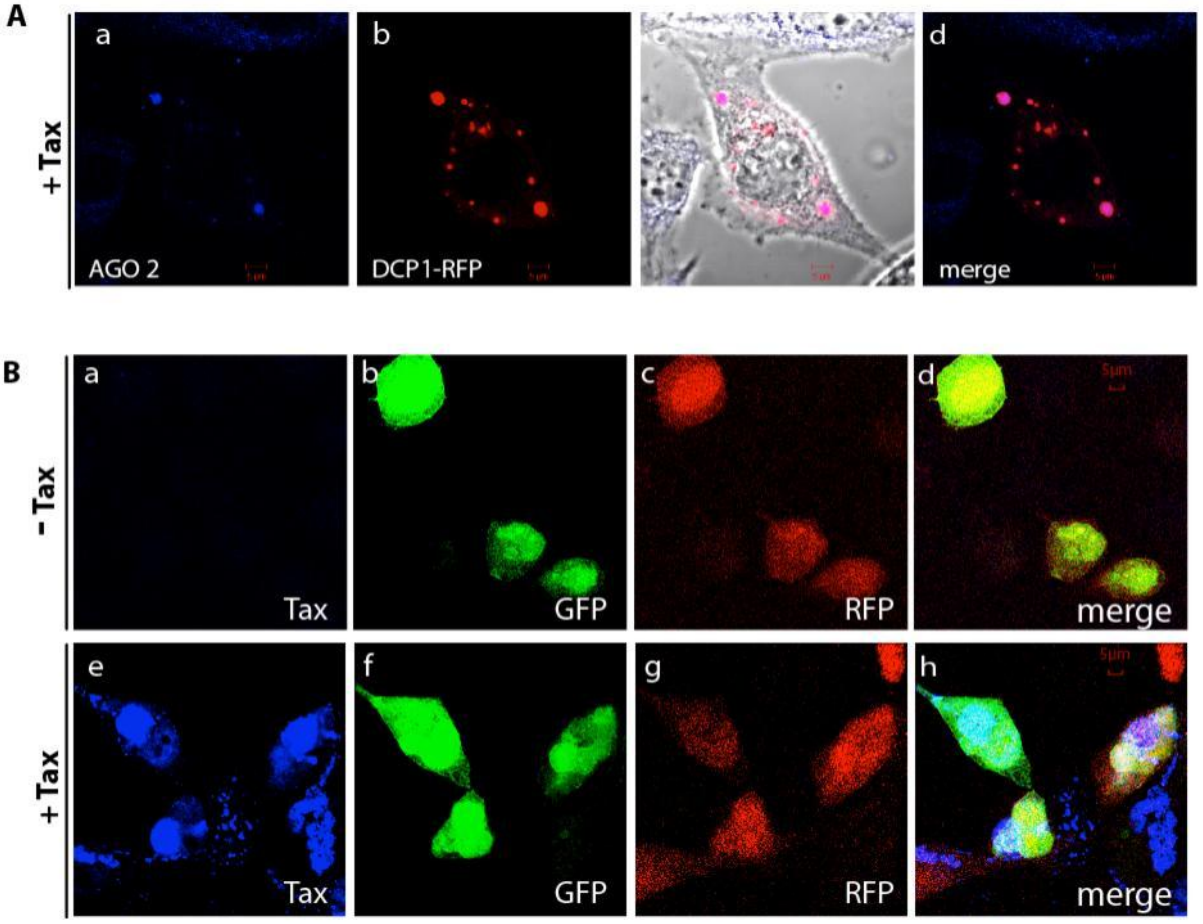
Supplemental Figure 2: Association of Tax with NMD-prone mRNA. A : Jurkat and E12 cells (Jurkat cells stably expressing Tax) were treated for 1h, 2h, 3h and 4h with 100 µg/ml DRB. *GADD45α* mRNA amounts were measured by qRT-PCR at each time points and the natural logarithm of the values expressed as fractions to time 0 were plotted against time. The half-life of the mRNA was calculated for each cell line. **B :** Same as A with the *MAP3K14* mRNA. These graphs show that similarly to JPX9 cells, the presence of Tax significantly stabilises the *GADD45α* mRNA, but not the *MAP3K14* mRNA. **C**: A RIP was carried out in HeLa cells expressing Tax and *GINS39* mRNA as in Figure 2C, except that the antibodies used were targeting Tax (lane 1) or preimmune serum (lane 2). This shows the specificity of the signal detected after Tax immunoprecipitation and strengthens the notion that Tax is stably associated to the mRNA targeted by the NMD machinery **D** : RIP experiments were carried out after transfection of HeLa cells with the GINS39 or GIWT constructs as indicated and vectors expressing wild type Tax (lane 1 and 3) or a control plasmid (lane 2). The signals were quantified, normalised to that of the lysate and plotted in a bar graph. While the level of *GINS39* prior to IP was 10 fold less as that of *GIWT* mRNA (data not shown), the level of *GINS39* mRNA after Tax IP was 1.3 fold more than *GIWT* showing that Tax binds *GINS39* mRNA more efficiently than *GIWT* mRNA, what is in agreement with published data concerning UPF1[5]. **E** RIP assays were performed as described in D, except that the reverse transcription step was omitted. Lanes 1 is a positive control of PCR amplification.



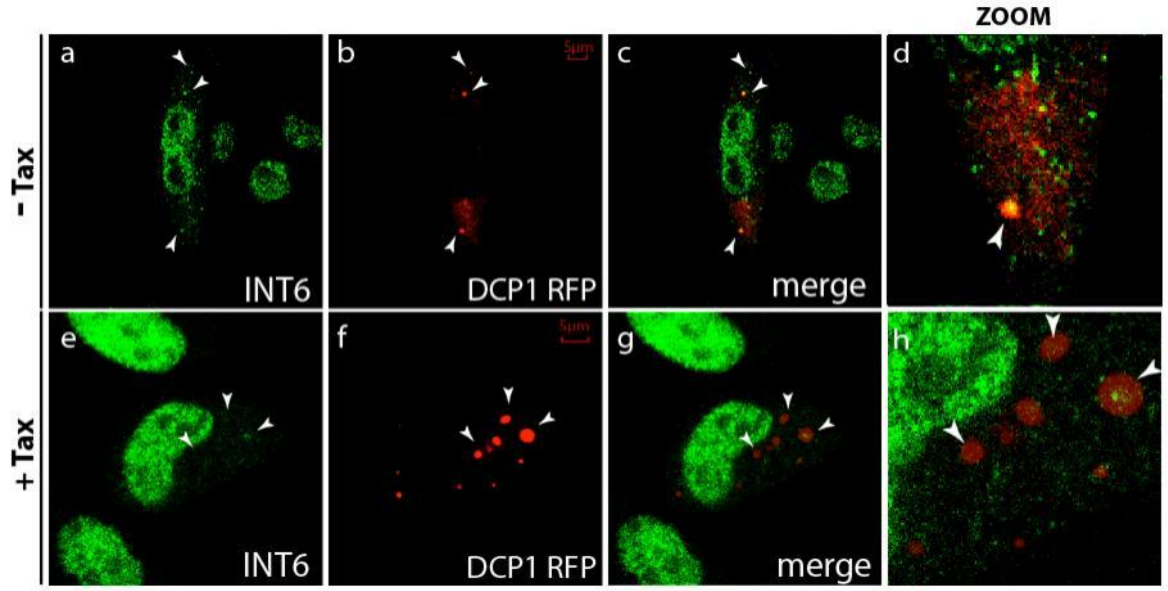
Supplementary Figure 3 : Tax needs UPF1, but not UPF2 to bind NMD factors and competes for INT6 interaction. **A**: 293T cells were transfected with either control (luciferase, lane 1) or anti-UPF1 (lanes 2 and 3) siRNA duplexes, as well as with vectors expressing Tax (lanes 1 to 3) and HA-UPF2 (lanes 1 and 3). Protein levels of endogenous UPF1 (upper panel) and Tax (lower panel) were monitored by immunoblot. **B**: Extracts of these transfected cells were used for immunoprecipitations carried out with the antibody to HA and immunoprecipitates were analysed by immunoblot with antibodies to HA (upper panel) and to Tax (lower panel). The absence of association between Tax and UPF2 when cells were cotransfected with a siRNA directed towards UPF1 (lane 3) confirmed that the interaction observed in lane 1 was mediated by UPF1. **C**: 293T cells were transfected with vectors expressing HA-UPF1, either wild type (lanes 1 and 2) or including the C126S mutation (lanes 3 and 4), and Tax (lanes 1 and 3). Tax expression in the cell extracts was monitored by immunoblot. **D**: Extracts of these transfected cells were used for immunoprecipitations using the antibody to HA and co-immunoprecipitated proteins were analysed by immunoblot with antibodies to HA (upper panel) and to Tax (lower panel).Tax was able to associate with the WT as well as the C126S UPF1 mutant (lane 1 and 3), although expression of the latter was weaker. This indicates that UPF2 is not needed for this association. **E**: HeLa cells were transfected with a control (lane 1) or a Tax (lane 2) expression vector. Levels of endogenous UPF1 and Tax were analysed by immunoblot. **F**: extracts from E were immunoprecipitated with an antibody to INT6 (N-19) and immunoprecipitates were analysed by immunoblot with antibodies to UPF1, INT6. The strong signal above INT6 due to the Ig heavy chain is indicated (Ig).



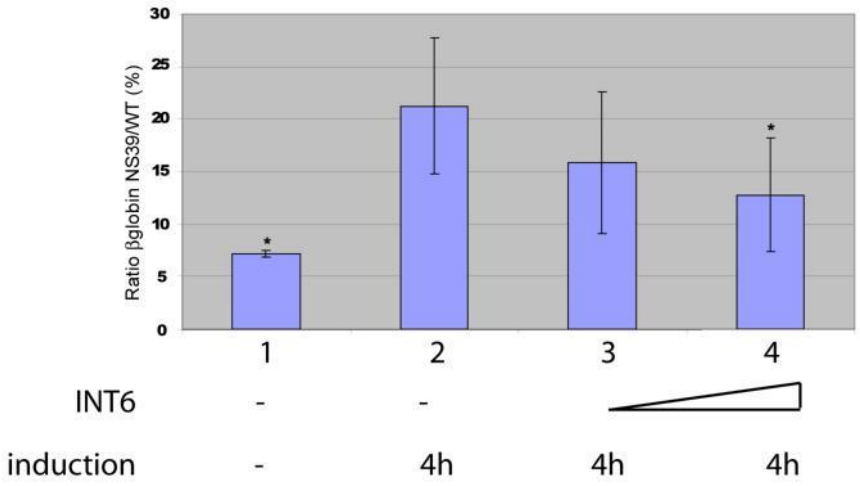
Supplemental Figure 4 : Tax inhibits colocalization of UPF1 and INT6. A: Confocal microscopy analysis of HeLa cells transfected with HA-UPF1 and with a control (panels a-d) or Tax (panels e-h) expression vector. Immunostaining was done with an antibody to HA (red, panels a,e) and to INT6 (green, panels b,f). Superposition of both stainings is shown in panels c and g. Panels d and h are an enlargement of cytoplasmic foci showing both HA-UPF1 and INT6 stainings. **B**: UPF1 foci number was quantified (0, ≤3, >3) in several cells in the absence or presence or Tax and results are indicated below bars corresponding to number of cells. Colocalization of HA-UPF1 and INT6 in these foci was also analysed. These data show that the presence of Tax increases the number of UPF1 foci, but decreases the presence of INT6 in these structures.



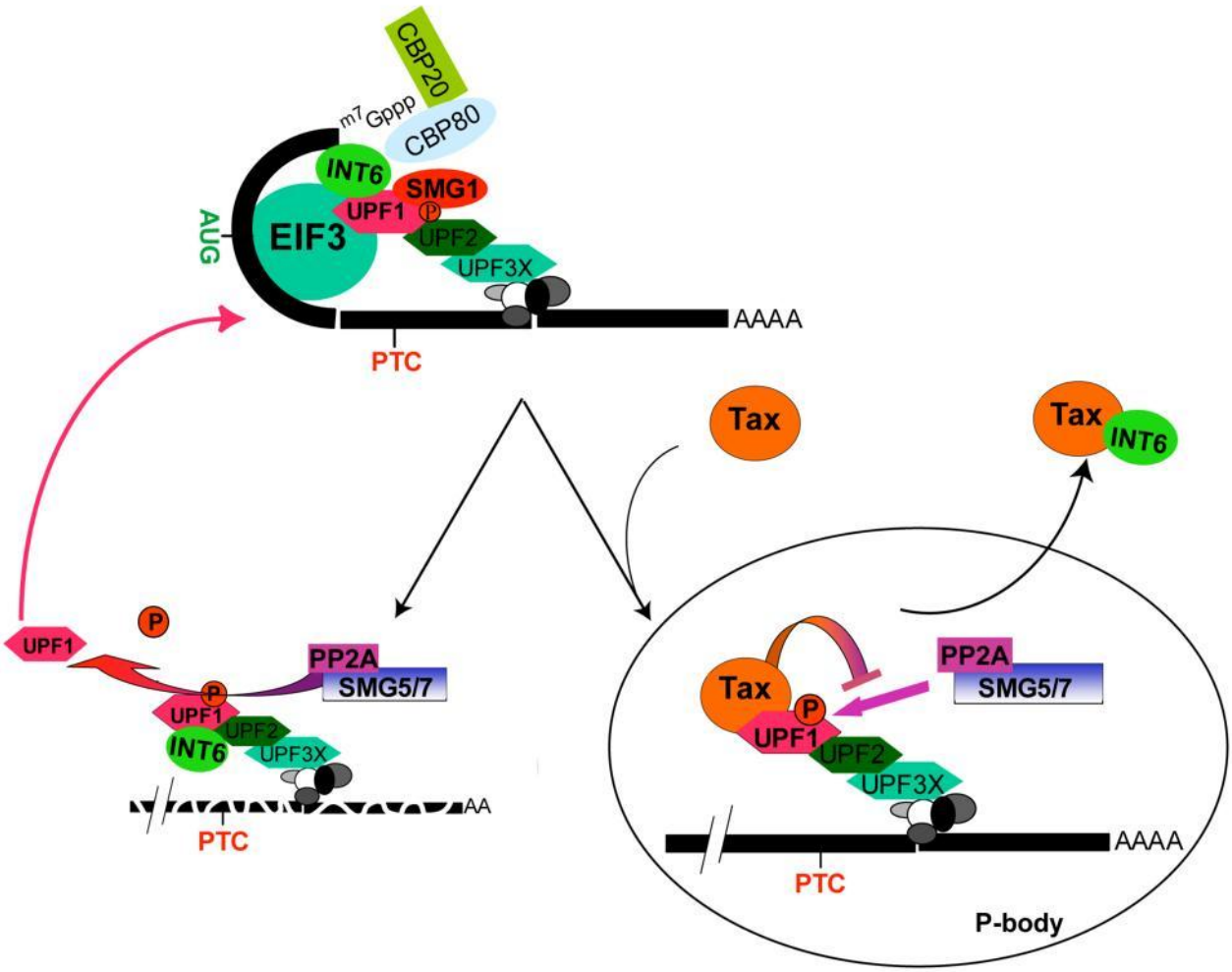
Supplemental Figure 5 : P-bodies are modified in the presence of Tax. **A** : HeLa cells were transfected with Tax and DCP1-RFP expression vectors. Immunostaining with an antibody directed against endogenous AGO2 (blue, panel a), as observed by confocal microscopy is shown for a representative cell, along with red fluorescence of DCP1-RFP (panel b). Superposition of both stainings with (panel c) or without transmission image (panel d) are shown. These pictures confirm the effect of Tax on P-bodies with an endogenous component of these structures. **B**: HeLa cells were transfected with expression vectors for GFP and RFP. Control (pSG5; panel a-d) or Tax (panels e-h) expressing plasmids were also transfected. Immunostaining with antibody to Tax (blue, panels a, e) along with GFP green fluorescence (panels b, f) and RFP red fluorescence (panels c, g) as observed by confocal microscopy are shown for representative cells. Superposition of all three stainings is also shown (panels d,h). This control shows that Tax by itself does not affect subcellular localization of the GFP and RFP proteins when they are not fused to another one.



Supplemental Figure 6 : INT6 is delocalized from the P-bodies in the presence of Tax. HeLa cells were transfected with the DCP1-RFP expression vector, together with control (pSG5, panels a- e) or Tax (panels e-h) expression vectors. Cells were treated and depicted as described for Figure 3A except that immunostaining was carried out using an antibody directed against INT6 (C-169) (panels a, e).



Supplemental Figure 7: INT6 overexpression reverses NMD inhibition by Tax. NMD assays were performed using induced JPX9 cells as described in Figure 1C, except that an increasing amount of INT6 expression vector (0.7 μg and 2.1 μg) was cotransfected with the renilla luciferase/globin plasmids. (Student's t-test results refer to the lane 2. **P*<0.05). These data show that overexpression of INT6 is able to decrease by ~40% NMD inhibition, demonstrating the competitive relationship between Tax and INT6 in the process.



Supplementary Figure 8: Summary scheme of Tax effect on the NMD pathway. In the case of a NMD-prone mRNA, UPF1 is phosphorylated by the SMG1 kinase and associates with UPF2 and UPF3. In these conditions, UPF1 represses translation initiation by interacting with EIF3. Phosphorylated UPF1 also triggers association with mRNA decay factors such as DCP1, XRN1 and the exosome component EXOSC2. UPF1 is further dephosphorylated by the SMG5/7/PP2A complex and recycled (left bottom part of the scheme). In a cell expressing the HTLV-1 provirus, from the data presented in this report Tax alters NMD efficiency by binding INT6, thereby impairing its association with UPF1 and functionally inhibiting mRNA degradation. By also binding phospho-UPF1 Tax associates with the NMD-prone mRNA and prevents normal UPF1 dephosphorylation by PP2A, thus causing accumulation of phospho-UPF1 in the P-bodies (right bottom part of the scheme) which then show an enlarged aspect.

Supplementary methods

Immunofluorescence and confocal microscopy : $0.05x10^6$ HeLa cells were transfected with the calcium phosphate procedure. Cells were fixed 20 min with fresh 4% paraformaldehyde, washed 3 times and incubated with PBS, 1% BSA. Cells were incubated for 1.5 h at RT with the primary antibody, washed 3 times, and further incubated 1h with the secondary antibody. Finally cells were mounted in 10 µl of Vectashield DAPI (1.5µg/ml) (Vector laboratories). Slides were observed with an LSM 510 confocal microscope (Zeiss).

Antibodies: Antibodies used in this study were the following: rabbit antisera to INT6 (C-20, N-19 or C-169 as indicated in the figures)[1,2]; rabbit polyclonal antibodies to UPF1, UPF2 and UPF3b[3]; mouse monoclonal antibody to phospho-UPF1 was kindly provided by A. Yamashita (clone 8E6[4]); rabbit polyclonal antibody to phospho-(Ser/Thr) ATM/ATR substrate (#2851; Cell Signaling Technology); mouse monoclonal antibody to Tax (clone 474); mouse monoclonal antibodies to FLAG (clone M2, Sigma) and to HA (clone 7, Sigma) epitopes.

RIP assay

~$7x10^6$ cells were fixed with 0.1% formaldehyde for 20 min at room temperature. Then 0.25 M glycine was added for 5 min before washing. The cell pellet was resuspended in 2 ml of buffer B (50 mM Tris–Cl, pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 0.05% SDS, 1 mM EDTA, 150 mM NaCl) containing protease and RNAse inhibitors. The cells were lysed by sonication. 2.5h of immunoprecipitation at 4°C were proceeded and protein A beads were added for 2h before extensive washing. The beads were resuspended in buffer D (50 mM

Tris–Cl, pH 7.0. 5 mM EDTA, 10 mM DTT and 1% SDS) and incubated at 70°C for 45 minutes.

Supplementary references

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Part III Discussion

„One never notices what has been done; one can only see what remains to be done."

Maria Skłodowska - Curie

General discussion and perspectives.

1.1 INT6/EIF3E is required for translation of specific mRNAs, notably for histone mRNA.

Previous studies of INT6 function seem to strongly support its role in histone mRNA translation. Although human EIF3E/INT6 was originally described as a subunit of a translation initiation factor (Asano et al, 1997a), its role in this process remains controversial. However a specific subset of mRNAs has been observed to be regulated by INT6 (Grzmil et al, 2010). Further evidence for INT6 being implicated in the translation of specific mRNAs is provided by very recent studies showing that the expression of one of the truncated forms of INT6 provokes a shift from cap-dependent to IRES-mediated translation initiation. The mutant used to study this phenomenon is called 3e5 and is a result of MMTV integration at intron 5 (one of the natural sites of integration of the MMTV).

This integration has been formerly shown to be sufficient to induce malignant transformation of mammalian cells (Mack et al, 2007; Mayeur & Hershey, 2002; Rencus-Lazar et al, 2008). It has been suggested that the expression of 3e5 was the cause of tumorigenesis, rather than the loss of an *eif3e* allele (Chiluiza et al, 2011). The authors propose a model in which the expression of the C-terminal truncated mutant would compete with the wild-type form for the binding to eIF4G. This would result in the inhibition of mRNA binding to the 43S PIC. The translation of mRNAs which is strictly cap-dependent would the most affected since its efficiency depends on the eIF4G-associated unwinding machinery of the secondary structures in the 5'UTR. These findings support the idea that INT6 is important for the cap-dependent translation of a subgroup of mRNAs (i.e. GAPDH, actine). This is interesting from the point of view that histone mRNAs are also translated in a cap-dependant manner; this has been at least demonstrated in the case of H4 coding mRNA (Martin et al, 2011).

Another interesting point to consider is the fact that most – but not all – mRNAs are translated at the strongest rate in the G_1 phase of the cell cycle and at the lowest rate during mitosis (Pyronnet & Sonenberg, 2001). These changes correlate with the activity of several canonical translation initiation factors, modulated during the cell cycle to regulate translation. However, some mRNAs, like histone mRNAs, have very different patterns of cell cycle-dependent regulation. INT6 is set apart from the other eIF3 subunits by the fact that it exists both in the nucleus and the cytoplasm and can shuttle between both compartments. Watkins and Norbury have reported that in primary human fibroblasts, but not their transformed counterparts, a subpopulation of cells has reduced nuclear EIF3E/INT6. After synchronization in S phase, the percentage of cells where nuclear INT6 is reduced reaches 40% (Watkins & Norbury, 2004a). These findings suggest that during S phase INT6 could be required in greater quantities in the cytoplasm, and it is tempting to suggest that its function could be in part to allow the assembly of the complete eIF3 on histone mRNAs as well as the establishment of various interactions with other general translation initiation factors. INT6 associated to SLIP1, SLBP and through them to the histone mRNAs in the nucleus, might be replaced by a complete eIF3 complex once it reaches the cytoplasm. To test this notion it would be interesting to analyse in details the dynamics of the recruitment of the various eIF3 subunits to this particular type of mRNA. Another, non-exclusive hypothesis is that INT6 could be required for histone mRNA export. Some studies indicate that histone mRNAs are exported via a TAP dependent mechanism (Huang & Steitz, 2001). Studies of the implication of SLBP itself in the export process remain controversial. Indeed, although SLBP has been shown to be required for this process (Ghule et al, 2008; Sullivan et al, 2009a), it has not yet been demonstrated to present an NES, while INT6 does have one. Thus, it would be of great interest to test the importance of the existence of a nuclear pool of INT6 and of correct INT6 shuttling for accurate histone mRNA export. Finally, since INT6 is also important for translation of many different classes of mRNA, such as mRNAs involved in apoptosis. For instance it has been shown that the translation of the mRNA coding for the antiapoptotic protein BCL-XL is stimulated by INT6 (Grzmil et al, 2010). It would also be interesting to test whether modifications of the subcellular distribution of INT6 correlate with the conditions in which these classes of mRNAs are produced and translated. Alternatively, S phase relocation of INT6 may be a way to regulate the function of other complexes it associates with, such as the COP9 signalosome.

1.2. The diversity of INT6 functions and its link with histone mRNA translation.

The discovery that histones are more than just a protection for the double-helix and a tool to organize it properly in the cell nucleus provoked an avalanche of studies. Histones are involved in the control of DNA replication, transcription, repair and recombination, which makes of them one of the chief proteins of DNA metabolism. Any alteration of histone synthesis has thus extreme consequences for the phenotype of the cell and can ultimately result in cell death or transformation.

A previous study performed in our laboratory revealed the presence of INT6 at chromosomes in the mid-zone of the mitotic spindle (Morris & Jalinot, 2005). The same study reported that the loss of INT6 causes significant mitosis defects such as spindle aberrations and ultimately results in faulty chromosome segregation. This effect was demonstrated to be due to the inhibition of cyclin B-Cdk1 kinase activity. The data presented in this report revealed that this inhibition correlates with a prolonged phosphorylated state of Cdk1 which in turn resulted from the absence of INT6.

Intriguingly the INT6/EIF3E protein has been also identified as an interacting partner of MCM7, and the interaction is thought to be direct (Buchsbaum et al, 2007; Grzmil et al, 2010) This DNA replication licensing factor is part of the **M**ini-**C**hromosome **M**aintenance complex (MCM2-7) which is essential for the initiation and progression of eukaryotic genome replication and is thought to be the replicative helicase. INT6 has been shown to stabilize polyubiquitinylated, chromatin-bound MCM7. Interestingly, both MCM7 and INT6 silencing resulted in a significant slowing down of the replication machinery (Buchsbaum et al, 2007).

The question arises if it is possible that INT6 acts as a sensor of DNA replication progression and by doing so allows the synchronisation of this process with histone expression. INT6 might favour histone synthesis in the cytoplasm, while its nuclear pool would be rather an enhancer of replication by stabilizing the MCM7. This way INT6 could accelerate both of these phenomena. The fine tuning of these two processes could be regulated via the control of the speed of nuclear import-export of INT6 and through it the relative size of the cytoplasmic and nuclear pools of the protein, authorizing both replication in the nucleus and translation of histone mRNAs in the cytoplasm. Such a control mechanism would aid to establish the delicate balance between the amount of histones and the newly doubled DNA. Future studies

should help to establish the validity of such a model and to determine precisely if both of these activities of INT6 are independent or related.

1.3. The activity of INT6 in histone mRNA translation involves interaction with SLIP-1 and SLBP

Our group has identified two new interaction partners of INT6: SLIP-1 (MIF4GD) and SLBP. These two proteins have been shown previously to be required for histone mRNA translation (Cakmakci et al, 2008a; Ling et al, 2002a). We propose that INT6 plays a role in this mechanism through these interactions. Previously LeFebvre et al have described an *in vitro* interaction between the 1015-1118 region of eIF4G1 and the eIF3 complex. This interaction would involve a contact with INT6 as observed by performing partial proteolysis and mass spectrometry analyses (LeFebvre et al, 2006). SLIP-1 in turn has been observed to interact with eIF4G through its N-terminal region (amino acids 27 to 170, (Cakmakci et al, 2008a)). Thus it is possible that INT6 interacts simultaneously with both MIF4GD and eIF4G1, thereby playing a pivotal role in histone mRNA translation. The question arises as to whether INT6 forms separate complexes with SLIP-1, SLBP and eIF4G1 or if all these proteins are present in a single complex (Fig. 22). Sub-complexes could be formed during the initiation of translation. This idea would be supported by the fact that SLIP-1 is a dimer protein and thus could easily bind to more than just one partner. Our preliminary data suggest the existence of a subcomplex made up of INT6, SLIP-1 and SLBP. Although we observed that SLIP-1 is not necessary for the interaction between INT6 and SLBP, it seems that this interaction is more stable when all three proteins are present.

Additionally INT6 binding near the carboxyl end of eIF4G and SLIP-1 at its amino end might result in possible conformation changes of the scaffold protein eIF4G. This conformational change could be the event that brings together the two termini of the mRNA.

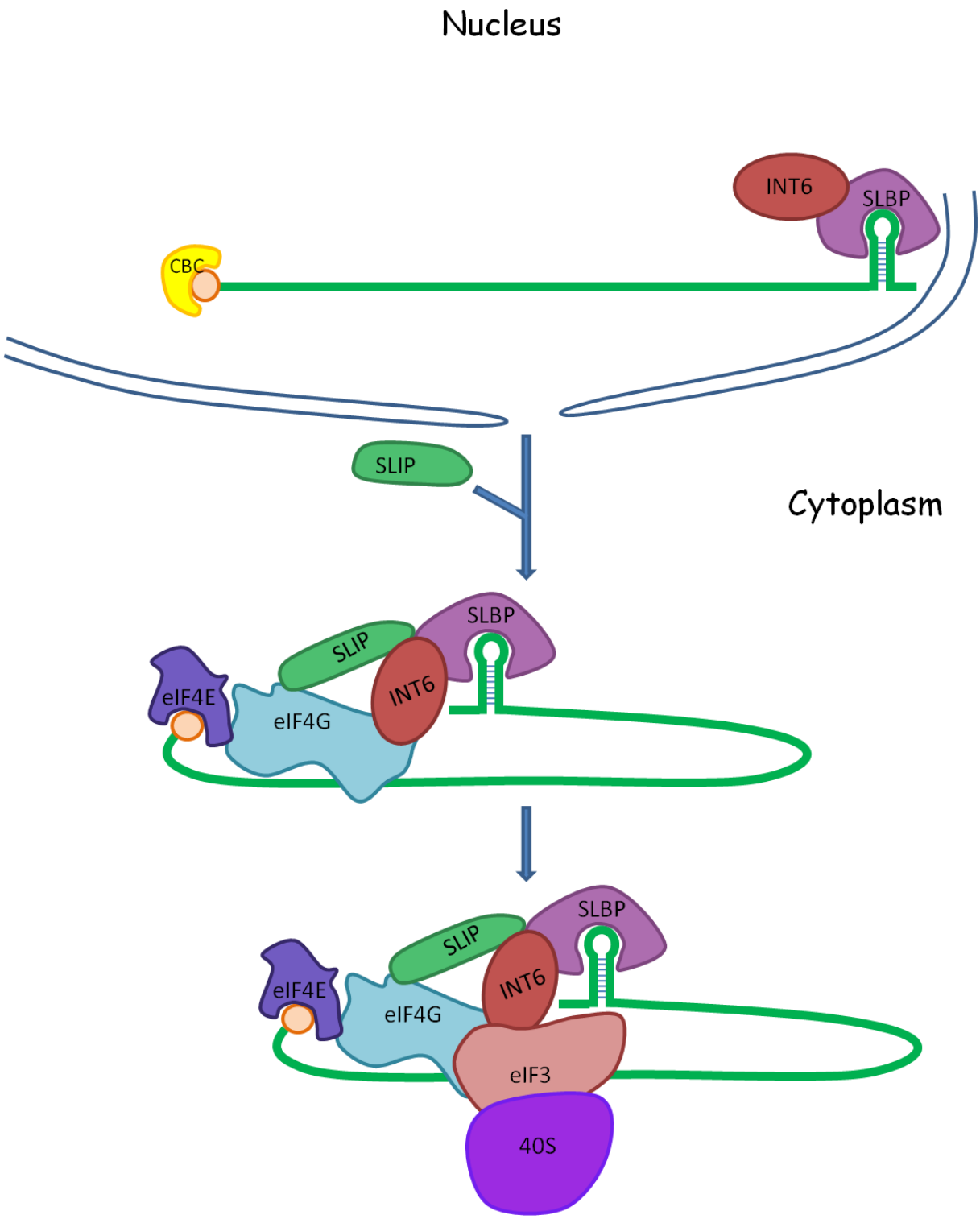

Figure 22 Model of Histone mRNA translation. Here we propose a hypothetic model of the association of known factors required for histone mRNA translation. In this model INT6 is loaded early on the RNA via the interaction with SLBP and accompanies it from the nucleus to the cytoplasm. A translationally active complex is formed in the cytoplasm once SLIP-1 joins the other initiation factors.

1.4. Histone mRNA and protein degradation.

Although the present work concentrated on the involvement of INT6 in histone mRNA translation, it is possible that INT6 also intervenes at (a) further step(s) of histone mRNAs metabolism. Indeed, once genome replication is completed histone expression stops and their mRNAs are degraded in a process which involves SLBP and UPF1, the latter being a core NMD factor (Kaygun & Marzluff, 2005a). Our previous studies demonstrated that INT6 interacts with UPF2 and UPF1 and is important for the NMD process (Morris et al, 2007). Given our results which show that INT6 interacts strongly with SLBP, it would be interesting to investigate the effect of INT6 on histone mRNA in late S-phase and G2/M. To better understand this point it will be important to determine the exact role played by UPF1 and RNA degradation factors in the process. Our preliminary data from G2/M-synchronized and INT6-depleted cells might aid to understand this process. To our surprise we observed in these experiments an increased histone level in INT6-deficient cells. However, this effect cannot be (uniquely) explained by changes in mRNA levels since those stayed stable or were only slightly increased. Hence it is possible that after playing a positive role on histone synthesis in S phase, INT6 also participates in histone degradation in G2. Since it is known that INT6 interacts with the 26S proteasome subunits (Hoareau Alves et al, 2002) and that in *Schizosaccharomyces Pombe* it positively regulates the proteasome 26S via an association with the regulatory subunit Rpn5 (Yen et al, 2003) it would be interesting to test if INT6 might participate in histone or SLBP degradation via one of these complexes. Little is known about the degradation of excess free histones or their direct degradation via the proteasome. From studies performed in budding yeast we know that histone protein levels are regulated by phosphorylation and the ubiquitin-proteasome pathway. The CHK-1/2 homolog Rad53 is implicated in this phosphorylation process. In fission yeast Ams2 activates histone transcription at the G1/S transition. As the cells exit the S phase, Ams2 is phosphorylated by DDK, leading to its degradation via the SCF^{Pof3}-ubiquitin proteasome pathway. It has been also proposed that SLBP is degraded via the ubiquitin-proteasome pathway (Takayama & Toda, 2010). Thus it seems that rather than histone degradation, another lead to follow might be the decay of SLBP at the S/G2 border. As previously established this protein is actively degraded at the end of the S-phase and is indispensible for histone synthesis. However, the molecular basis underlying this degradation is not known. Before degradation SLBP has to be phosphorylated and ejected from the SL (Zheng et al, 2003). The phosphorylation takes place on two threonines 60 and 61 and requires the cyclin A/cdk1 and CK2 (Kaygun & Marzluff, 2005b). Even if the molecular details of regulatory systems may vary between species, the

underlying principles of histone homeostasis, maintained via ubiquitin-mediated proteolysis could be conserved from yeast to human beings. Interestingly we observe a stabilization of histone protein levels in absence of INT6 in the G2/M phase. This phenomenon could be also due to the stabilization of SLBP on histone mRNAs. INT6 might be implicated in the degradation of SLBP via the proteasome since INT6 has been shown to associate with the lid of the 26S proteasome.

1.5. Implication of INT6 in multiple degradation pathways.

INT6 has been reported to control the stability of specific cellular proteins. As described above INT6 stabilizes chromatin-bound MCM7, which also happens to be the polyubiquitylated fraction of the protein. Different evidence has been brought for HIF-2α (**H**ypoxia **I**nducible **F**actor 2) to be stabilized in the absence of INT6. INT6 has also been shown to negatively control the stability of the steroid coreceptor 3 (SRC3) during mitosis (Suo et al, 2010). The fact that INT6 acts in a different manner on the stability of selective proteins might be related to its presence in two different protein degradation complexes, the Proteasome and the CSN.

Interesting data related to the subject of the role of INT6 in protein degradation have been obtained from studies of the INT6 fission yeast homolog, Yin6. It has been shown that in *yin6* null cells chromosomes are improperly segregated. This improper segregation has been proposed to be due to the inhibition of securin/Cut2 degradation via the proteasome (Yen et al, 2003). Another recent report coming from the same group suggests that Yin6 might be important for the regulation of the ERAD-degradation pathway (Otero et al, 2010). ERAD stands for **E**ndoplasmic **R**eticulum **A**ssociated Protein **D**egradation and is a pathway which targets misfolded proteins of the ER for polyubiquitylation and subsequent degradation by the proteasome. According to these data, Yin6 acts together with two interacting partners, Cdc48 and Moe1, to control the ERAD degradation pathway (Otero et al, 2010). It is interesting to note that the properties of these proteins seem to be evolutionarily conserved in humans. Human Cdc48 is notably able to rescue the lethality of the yeast cdc48Δ mutant, and Int6 and Moe1/eIF3d bind Cdc48 in human cells. This might open new possibilities to explain how these proteins are involved in specific protein degradation. It would be very interesting to link the potential role of INT6 in proteasome-mediated protein degradation to its function in the pioneer translation round. During this pre-scanning of mRNAs short peptides are synthesized which have to be degraded. One could imagine a role of INT6 in this degradation.

1.6. Tax, histones and INT6: is there any link?

Since Tax – the transcriptional activator of HTLV-1 – has been demonstrated to interact with INT6 (Desbois et al, 1996) and this later protein has been revealed in the current work as important for histone mRNA translation we were wondering if Tax may have an impact on the histone mRNA translation.

Our understanding of the multitude of ways in which viruses either commandeer or are controlled by cellular pathways is constantly evolving, and far from complete.

Tax has been shown to interfere with a broad range of cellular mechanisms, such as cellular proliferation, DNA repair and cell cycle control. All of these effects of Tax are thought to cooperate in the development of ATL. A recent study claimed that the expression of Tax in HTLV-1 infected T-cells decreases histone protein levels and that the expression of Tax in Jurkat cells diminishes the level of replication-dependent histone transcripts. The group of P.J. Leybourn demonstrates that both HTLV-1 infection and Tax expression result in reduction of the H1 linker histone protein level and the core H3 histone level in human Tcells (Bogenberger & Laybourn, 2008). In line with earlier studies showing that Tax, by blocking checkpoint controls, results in unregulated initiation of S phase, impairs histones synthesis and provokes octamer disassembly, the impact of Tax on histone biogenesis is not very surprising. Tax inhibition of histone gene expression could result in incomplete chromatin assembly during DNA replication in S phase, leading to increased incidence of DNA double strand breaks and greater chromosome instability. In normal somatic cells, histone gene expression is tightly coupled to DNA replication in S phase and is mainly regulated at the mRNA level. These results suggest a novel mean by which Tax may induce genomic instability. However, preliminary results obtained in our team do not seem to confirm the results of the Leyboum group. Indeed, in our experiments Tax expression in JPX9 inducible cells instead resulted in a stabilization of histone protein levels, suggesting a stimulation translation.

Importantly, the increased histone protein level observed in presence of Tax did not correlate with any changes at the transcript level. The same result was observed for two different experiments, one in which reporter mRNA derived from a transfected plasmid were monitored, another in which endogenous histone mRNA levels were analysed by RT qPCR. The drastic differences between our data and the ones already published may be due to (i) the use of different cell types by both groups, (ii) different core histones studied, (iii) the use of a

luciferase-reporter system (in our case), (iv) a non-specific effect – such as transcriptional squelching - due to non-physiological levels of expression of the transfected protein in one of the conflicting experiments. In our study we observed an increase of H2A and H4 in the presence of Tax whereas Leybourn's group concentrated on the linker histone H1 and the core histone H3. Furthermore we were able to detect a significant reduction of SLBP levels in presence of Tax. This could be due to an enhanced degradation of SLBP, which is induced by its phosphorylation by the CDK2 kinase (Koseoglu et al, 2008). CDK2 has been previously identified as being stimulated by Tax (Haller et al, 2002). This potential effect makes the correct interpretation of the phenotype of Tax expression very complicated and leaves many possibilities open. We would like to investigate an overall effect of Tax expression on the protein level of endogenous histones. Tax may act on one or several levels during histone biogenesis. Proteins like HIRA, NPAT, SLBP or SLIP-1 which function downstream of cyclin E-Cdk2 in histone gene regulation, might be affected.

The plausible implication of INT6 in the post-ERAD short peptide degradation and the hypothesis of INT6 controlling the stability of SLBP, bring us to the second part of my discussion which will be more focused on the implication of INT6 in the NMD pathway (Morris et al, 2007) and another role of the interaction between the viral protein Tax and INT6 (Desbois et al, 1996).

1.7. INT6 as an emerging, central player in the NMD pathway

The work described in this manuscript strengthens the prior findings of an important role of INT6 in the NMD. It has been shown that INT6 removal from the cell clearly results in the stabilization of PTC-containing mRNAs (Morris et al, 2007). In the present work we confirm also the interaction between INT6 and Tax (Desbois et al, 1996) and the one between INT6 and UPF1 (Morris et al, 2007).

An as yet unresolved issue, that is immediately relevant to the newly discovered role of INT6 in the NMD, is the precise timing of its association with RNA. Previous work indicates that INT6 might be present at early as well as at late steps of the processing of an mRNA targeted for degradation (Asano et al, 1998; Hoareau Alves et al, 2002; Morris et al, 2007; Zhou et al, 2005). From our own unpublished data we know that a fraction of INT6 is associated with

chromatin and with the RNA polymerase II holoenzyme (P Jalinot and J-M Egly, unpublished data). Although this remains to be firmly established, the possibility exists that INT6 could be loaded very early on the nascent mRNA, and its interaction with the CBP80 subunit of the Cap Binding Complex is in line with such a notion (Morris et al, 2007). This early loading of INT6 is compatible with INT6 being implicated in the pioneer round of translation and thus in the NMD.

1.8. The HTLV-1 Tax protein as a tool to study the role of INT6 in the NMD pathway

Since their discovery viruses have been shown to have evolved mechanisms not only to evade eradication by cellular pathways, but also to manipulate them for enhanced viral replication and gene expression. Our understanding of how many of these pathways are normally targeted is still incomplete, as new discoveries are made on a frequent basis. Ironically, because of their tight association with the complex cellular networks, viruses are powerful tools to help deconstruct those same networks, in our case those which govern eukaryotic RNA stability.

INT6 was first identified as a product of a gene which is frequently disrupted by Mouse Mammary Tumour Virus (MMTV) integration in MMTV-induced tumour cells; it was subsequently discovered independently a second time as an interactor of Tax. This example of two independent discoveries of a same protein through its targeting by viruses is only one of a plethora illustrating how viruses can help to discover cellular proteins and investigate their function.

Given our findings that INT6 is both involved in NMD and a target of Tax, we addressed the question of a putative Tax impact on the NMD. The features of the HTLV-1 genome make it a possible target of NMD. Indeed we observed that Tax was able to inhibit the NMD suggesting a possible protective mechanism the virus might have developed. This will be described further in this chapter.

We started deciphering the interaction network between the viral protein, INT6 and the UPFs. We were able to demonstrate that Tax binds directly both proteins and by doing so disrupts their association. Studies using Tax also led to the observation of a delocalization of NMD factors in the cells upon *tax* expression. This was observed by performing immunofluorescence analysis of the subcellular localisation of these proteins. In Tax transfected cells immunofluorescence experiments revealed that Tax disrupted the partial localization of INT6 within P-bodies. Interestingly we were able to detect Tax itself within the P-bodies. Moreover, whereas under normal conditions UPF1 is hardly detectable in P-bodies

– although expression of an HA-tagged form of the protein slightly facilitates observations of cytoplasmic foci colocalizing with those formed by the P-body marker DCP1-RFP – Tax was able to markedly enhance the presence of UPF1 in these P-bodies. Tax expression also modified the aspect of these subcellular structures. Indeed the number of P-bodies increased and they were also larger than in cells lacking Tax. This effect is likely to result from the inhibition of mRNA degradation, as a similar one has been observed in other cases (Durand et al, 2007a; Sheth & Parker, 2006). P-bodies have been already revealed as a target of other viruses. For example the Hantavirus nucleocapsid peptide (N) protects and sequesters mRNA caps in P bodies. These stored caps are then used during the initiation of viral mRNA synthesis (Mir et al, 2008).

These observations are compatible with the hypothesis that Tax, by interacting with INT6, UPF1 or both, inhibits processing of NMD-prone mRNAs. We observed an interaction of Tax with the phosphorylated form of UPF1 (a form that interacts with degradation factors and which is localized in the P-bodies). We found that Tax expression stabilizes phospho-UPF1, thus inhibiting the correct recycling of this core NMD factor and probably triggering its observed accumulation in the P-bodies. A reasonable hypothesis would be that the reduced NMD efficiency observed in presence of Tax results from the decrease in available dephosphorylated UPF1 – the form active in NMD – triggered by the viral protein. However, analysis of several Tax mutants indicates that the effect of the viral protein on NMD cannot be completely attributed to its interaction with UPF1. Indeed, the Tax M47 mutant – which binds UPF1 slightly more efficiently than wt Tax but does not associate with INT6 – was found not active in NMD inhibition and did not affect the P-bodies. This indicates that the association of Tax with INT6 is important for functional inhibition of the NMD. Given the numerous interactions of Tax with cellular proteins and the number of pathways this viral protein has been demonstrated to have an impact on (reviewed in: (Boxus et al, 2008), it is highly possible that its interaction with UPF1 could target other activities of UPF1 than NMD. Indeed, UPF1 has been shown to participate in other decay mechanisms such as SMD or histone mRNA decay (functions of NMD factors outside the core NMD pathway have been reviewed by Nicholson et al: (Nicholson et al, 2010). Our findings open new possibilities to complete the complex picture of effects connected with the expression of Tax during viral infection. So far our observations support an effect of Tax on at least two different steps of the NMD. .

1.9. NMD: an antiviral mechanism?

Our data also demonstrate that HTLV-1 is targeted by the NMD pathway and that Tax significantly decreases NMD activity. The viral mRNAs expression downstream of activated transcription is also regulated at the posttranscriptional level. We have shown that Tax increases ~2.6 times the half-life of the *hbz* mRNA. One could presume that NMD constitutes a barrier for viral infection since many viruses present polycistronic transcripts which, during translation of the first ORF, would have an unusually long 3'UTRs which may elicit NMD. Moreover viruses often use alternative splicing to maximize coding potential, possibly creating spliced variants with EJC components remaining downstream of the stop codon used by the 5'ORF. NMD has been shown to be inhibited by infection with the polio virus, which cleaves and inactivates the eukaryotic initiation factors eIF4GI and eIF4GII and poly(A)-binding protein (PABP) (Carter et al, 1995).

The regulation of the splicing pattern is another way to prevent the NMD. The HIV-1 genome is spliced in a way that genes are always spliced out sequentially in a 5'to 3'order. Most of cellular mRNAs lack introns in the 3'UTR which are a signal eliciting the NMD. In the case of HIV-1 the splicing of a 3′ intron is tightly inhibited unless the 5′ intron is removed. This avoids generating transcripts where splicing of 3' genes leads to deposition of EJCs downstream of the termination codons of the 5'genes (Bohne et al, 2005).

Some viruses also specifically avoid NMD by encoding protective elements within their long 3'UTRs. An example is another retrovirus, the Rous Sarcoma Virus, which contains an RNA stability element (RSE) downstream of the *gag* gene stop codon. The RSE is thought to interact with unknown cellular factors to allow viral mRNAs to avoid destruction by the NMD pathway and promote proper translation (Withers & Beemon, 2010). Deletion of this sequence causes a dramatic shortening of the half-life of unspliced RSV mRNA in a translation- and UPF1-dependent manner, whereas insertion of the RSE protects PTC-containing NMD substrates from degradation.

Collectively, these findings illustrate how different viruses can disturb the posttranscriptional quality control or the translational machinery itself. Although the NMD has been associated before with the host-pathogen interactions of retroviruses and other viruses, including some oncoviruses (Amor et al, 2010; LeBlanc & Beemon, 2004), we demonstrate for the first time that a viral protein actively interferes with this pathway and that HTLV-1 is part of these

NMD-targeted-viruses. It would be tempting to test if the NMD core machinery is a target of other viruses, especially other retroviruses.

The observation that many viruses inactivate the NMD pathway raises the question as to whether NMD is part of the innate intracellular antiviral response. Although the hypothesis is tempting, another more conservative explanation can be proposed. Viral genomes are characterised by a high density of information in a relatively small space and by atypical structures and mechanisms, in all likeliness to maximise the number of viral copies that can assemble from a single infected cell. These peculiarities mean that viral mRNAs often differ sensibly from normal cellular mRNAs and may thus be recognized as abnormal by cellular mRNA quality control mechanisms. Thus, NMD and other mRNA quality control mechanisms, or any more general cellular processes targeted by viruses, may not necessarily have evolved as a barrier to viral infection, but instead be normal cellular mechanism that evolved to perform functions in non-infected cells which viruses only target because the only evolutive pathway they have found to package genetic information at a higher density than the host cells was to develop atypical structures that are recognized as abnormal by host cells.

1.10. Impact of Tax-mediated NMD inhibition on the stress response.

Viral infection is one of the factors conducting to a stress response in the cell. NMD has also been shown to degrade transcripts that participate in the adaptive response of cells to their microenvironment (Gardner, 2008; Mendell et al, 2004). NMD activation/inhibition is also an important regulatory step in the **U**nfolded **P**rotein **R**esponse (UPR) to the ER stress response (Gardner, 2010). A feature of ER stress is the upregulation of *xbp-1*. Interestingly this type of stress is commonly induced after viral infection, (Ni et al, 2009). Since NMD inhibition leads to the expression of truncated and potentially misfolded proteins, Tax could be a key player in the activation of these pathways, thus explaining the up-regulation of *xbp-1* mRNA.

Another interesting example comes from hypoxic cells where the eIF2α initiation factor is phosphorylated. The phosphorylation of eIF2α is known to promote translational and transcriptional up-regulation of genes important for the cellular stress response. It has been observed that the mRNAs of several of these stress-induced genes are NMD targets. eIF2α phosphorylation also represses NMD, thus stabilizing these mRNAs. This supports the idea that the inhibition of NMD increases the cellular stress response. Thus paradoxically the phospho-eIF2α-mediated inhibition of the NMD pathway is an important adaptive behaviour

of the cell in response to stress during tumorigenesis (Fels & Koumenis, 2006; Gardner, 2008).

In response to environmental stress and viral infection, mammalian cells postpone the translation of most cellular mRNAs and usually form foci termed stress granules (SGs). The temporally silenced mRNAs are gathered in the SGs and can undergo normal translation once the stress agent is eliminated, or are alternatively re-routed for degradation in other cytoplasmic compartments such as P-bodies. Subsequent to viral infection phosphorylation of eIF2α leading to translational repression has often been observed. However we observed that eIF2α is not phosphorylated in the presence of Tax (data not shown). This suggests that the action of Tax on the NMD pathway provokes an abnormal stress response. This goes along with the involvement of Tax in the cellular stress response defect that was recently identified due to its impact on the formation of stress granules after stress exposure (Legros et al, 2011). However in our experimental approach we did not test the effect of viral infection but only of Tax overexpression. These two events are likely to have different effects on the cellular stress response. Importantly these findings even strengthen our conclusion that Tax directly targets the NMD core machinery and does not provoke NMD inhibition via translational arrest which is one of the events following eIF2α phosphorylation.

1.11. Deregulation of NMD by the Tax protein

The HTLV-1 Tax protein has been described as a potent transactivator of provirus expression, but also as an immortalising protein with pleiotropic activities (reviewed in: (Matsuoka & Jeang, 2007)). In this report, we show a novel effect of this viral factor on the inhibition of the NMD pathway. This was observed both with the β-globin model system and with endogenous genes such as GADD45α, ATF4, SLIT2 and BAG1. In line with these functional effects, Tax has been observed to co-precipitate with UPF1, UPF2, UPF3b and INT6 in cells with an integrated and transcriptionally active provirus. In the case of UPF2, the association with Tax was observed to be mediated by UPF1.

This consequence of the NMD inhibition in HTLV-1 cells would be in line with the described mutagenic effects of Tax during the infection. For instance a recent study evaluates that the NMD-sensitive telomerase mRNAs were upregulated in **M**arek's **D**isease **V**irus (MDV) T-cell lymphoma (Amor et al). In our report, we limited the analysis to the study of mRNA from

genes already described to be stabilised after silencing of UPF1, UPF2 and INT6 (Mendell et al, 2004; Morris et al, 2007; Wittmann et al, 2006). Future systematic studies of NMD-sensitive transcripts increased by Tax should help to define how this activity of Tax contributes to cell transformation. Regarding this novel Tax function, we also observed that Tax associates with NMD-prone mRNAs. This is true for Gl NS39 reporter mRNA as well as endogenous mRNAs such as GADD45α, whereas Tax did not bind MAP3K14 whose half-life is not increased by Tax. At this step it is not clear whether the association intervenes very early after transcription or if it occurs after the pioneer round of translation once UPF1 has been phosphorylated. It is possible that a weak interaction occurs early, similar to what happens with UPF1 (Hogg & Goff, 2010; Hwang et al, 2010), and that this interaction is then stabilized after UPF1 phosphorylation.

Among the deregulated genes analysed, ATF4 (also called CREB-2) has often been associated with the biology of HTLV-1. The expression of this gene, like many in the genome, is regulated by the NMD not due to the presence of PTCs, but to that of three uORFs. Tax, by inhibiting the NMD pathway, stabilises ATF4 mRNA (the half-life is increased ~3 times) which would be expected to result in higher translation. ATF4 protein up-regulation by NMD inhibition is a well described pathway (Gardner, 2008). ATF4 has been involved in transactivation of the LTR, meaning that the NMD inhibition could result in an increased HTLV-1 genome transcription. However the role of ATF4 during HTLV-1 infection could also be related to its capacity to heterodimerize with AP-1 family factors to activate AP-1 responsive genes, which has already been associated with T cells transformation (Ameri & Harris, 2008).

Final conclusions

Our studies shed new light on the initiation of the translation of cell cycle-coupled, canonical histone mRNAs. In this work we identify INT6 as a new key player necessary for efficient histone mRNA translation. It is likely that the involvement of INT6 in this process is the result of its physical interactions with SLIP-1 and SLBP.

The second part of my project allowed the strengthening of the previous findings concerning the implication of INT6 in the NMD mechanism. This study also suggested a potential

explanation as to how the interaction between Tax and INT6 may contribute to the oncogenic process triggered by Tax. In conclusion, although NMD has already been associated with the host-pathogen biology of retroviruses and other oncoviruses, we report here for the first time that a viral protein actively interferes with this pathway and that HTLV-1 is part of the class of viruses the replication of which requires NMD inactivation.

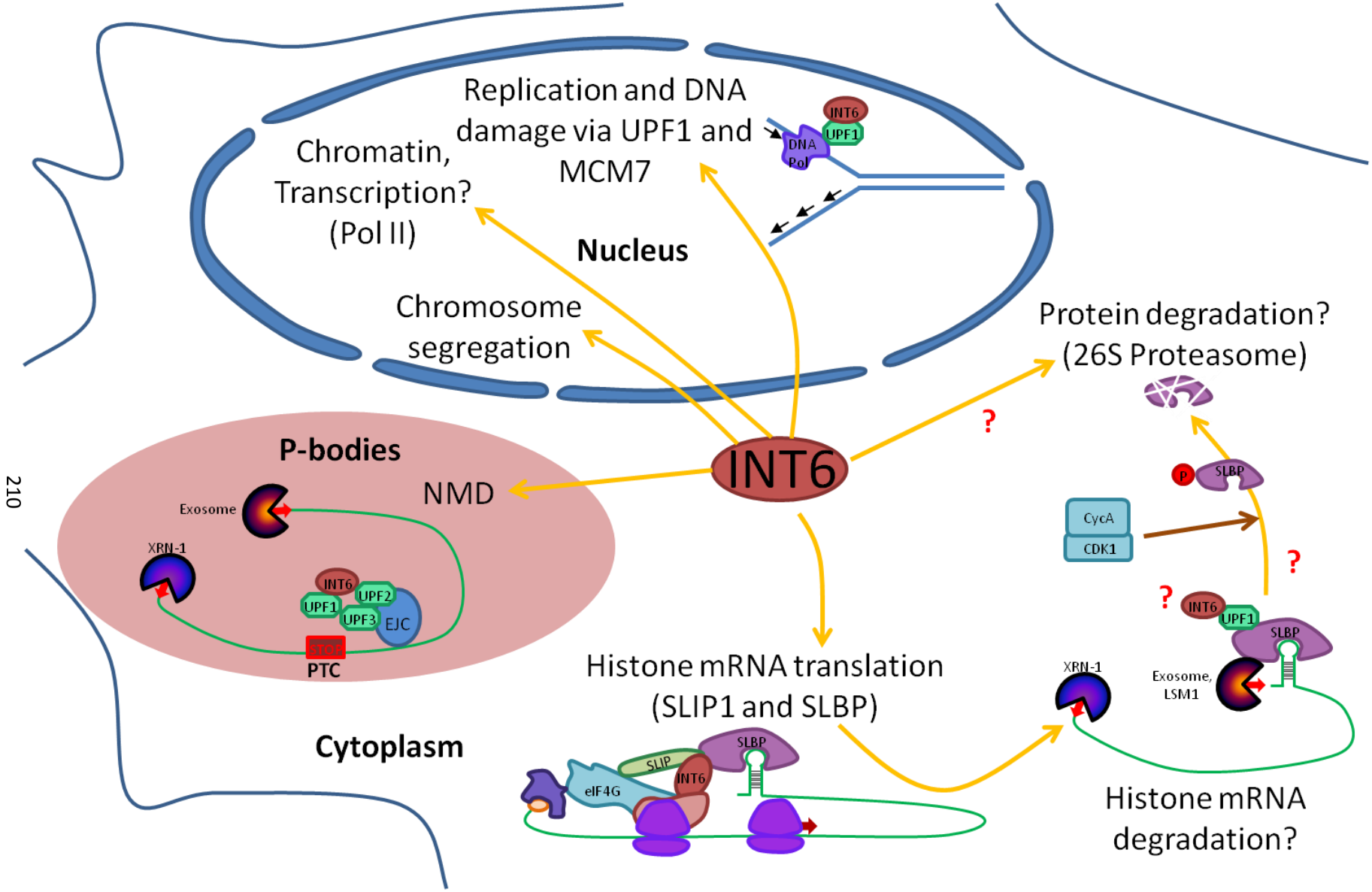

Figure 23. Different action of INT6 in a metazoan cell.
INT6 present in the cytoplasm and in the nucleus participates in divers pathways. The actions of INT6 are indicated by yellow arrows. The question marks indicate possible functions INT6 may exert in cells due to its interactions with other partners.

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