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An RNA Signature Links Oxidative Stress To Cellular Senescence

Nowsheen Mullani

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Thèse de Doctorat de L'Université Pierre et Marie Curie
Ecole Doctorale Complexité du Vivant

Présentée Par
Nowsheen MULLANI

Pour obtenir le grade de
Docteur de l'Université Pierre et Marie Curie

Sujet de la thèse:
**An RNA Signature Links Oxidative Stress To Cellular
Senescence**

Soutenance le 27 Septembre 2019

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Abstract :

Oxidative Stress is one of the routes leading to cellular senescence. While the damages that reactive oxygen species inflict on proteins and DNA are well described, our insight on how transcription may participate in the onset of senescence is still limited. At a transcriptional level, oxidative stress results in accumulation of promoter RNAs (uaRNAs) and enhancer RNAs (eRNAs) as a consequence of defective release of the RNAPII from the chromatin a phenomenon known as RNAPII creeping. We observed that RNAPII creeping was also detected downstream of a small series of genes known to be regulated by HP1Y at the level of their termination. Exploring this phenomenon yielded an unexpected result in the sense that it revealed an inhibiting effect of hydrogen peroxide on the RNA exosome complex involved in degradation of polyadenylated RNAs. The creeping RNAPII results in the transcription of ALU sequences located in the neighborhood of promoters and enhancers and downstream of intron-less genes and of small series of intron-containing genes. As ALU sequences contain genome encoded A tracts, they should normally be degraded by the RNA exosome. Yet, as oxidative stress also inhibits this RNase activity, mRNAs containing serendipitously transcribed ALU sequences get stabilized and are detected in the cytoplasm and even polysome fractions. This phenomenon may participate in the onset of the interferon response associated with oxidative stress.

Keywords:

Cellular Senescence, Exosome, Integrator-NELF complex, Mitochondrial stress, Oxidative stress, SINEs, uaRNAs, eRNAs, SASPs RNA signature

Resumé de travaux :

Le stress oxydatif est l'une des voies menant à la sénescence cellulaire. Bien que les dommages causés par les espèces réactives de l'oxygène aux protéines et à l'ADN soient bien décrits, notre compréhension de la manière dont la transcription peut participer à l'apparition de la sénescence est encore limitée. Au niveau de la transcription, le stress oxydatif entraîne l'accumulation d'ARN promoteurs (ARN_{Au}) et d'ARN amplificateur (ARNs), conséquence de la libération défectueuse du RNAPII de la chromatine, un phénomène connu sous le nom de RNAPII crawling. Nous avons observé que l'exploration de RNAPII était également détectée en aval d'une petite série de gènes connus pour être régulés par HPIY au niveau de leur terminaison. L'exploration de ce phénomène a donné un résultat inattendu, en ce sens qu'il a révélé un effet inhibiteur du peroxyde d'hydrogène sur le complexe exosome d'ARN impliqué dans la dégradation des ARN polyadénylés. Le RNAPII rampant a pour résultat la transcription de séquences d'ALU situées au voisinage des promoteurs et amplificateurs et en aval de gènes sans intron et de petites séries de gènes contenant un intron. Comme les séquences ALU contiennent des séquences A codées par le génome, elles doivent normalement être dégradées par l'exosome de l'ARN. Cependant, comme le stress oxydatif inhibe également cette activité d'ARNase, les ARNm contenant des séquences d'ALU transcrites par hasard se stabilisent et sont détectés dans le cytoplasme et même dans les fractions de polysomes. Ce phénomène peut participer à l'apparition de la réponse à l'interféron associée au stress oxydatif.

Mots clés :

Sénescence cellulaire, Exosome, complexe intégrateur-NELF, stress mitochondrial, stress oxydant, SINE, ARNa, ARNs, signature de l'ARN des SASPs

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Chapter 01: Cellular Senescence: Causes & Consequences

Aging is a phenomenon defined by sequential loss of organ and tissue functions over a period of time (**Flatt T. 2012**). Cellular senescence is a process that is characterized by permanent cell cycle arrest that is the result of many different factors like oxidative stress, telomere shortening, activation of oncogenes, cell-cell fusions etc. For the first time, **Hayflick and colleagues** explained it, when they observed that normal human fibroblasts stopped proliferating after approx. 50 divisions and they seemed degenerated, though they appeared to be viable and active metabolically. Accumulation of senescent cells in a tissue or an organ over a period of time is the primary cause of organismal aging. Senescence is considered as a very good example of antagonistic pleiotropy of aging (**George C. Williams, 1957**) that proposes that organismal health deteriorates over time, partly because evolution prefers genetic arrangements that support reproductive vigor in early years at the expense of overall organismal health in later years of life, i.e., post-fertility period. One good example that highlights this phenomenon is the potent anti cancer mechanism that completely & permanently removes pre-neoplastic cells from cell cycle. However, on the flipside, it is considered as a major trigger of age-related diseases and aging itself.

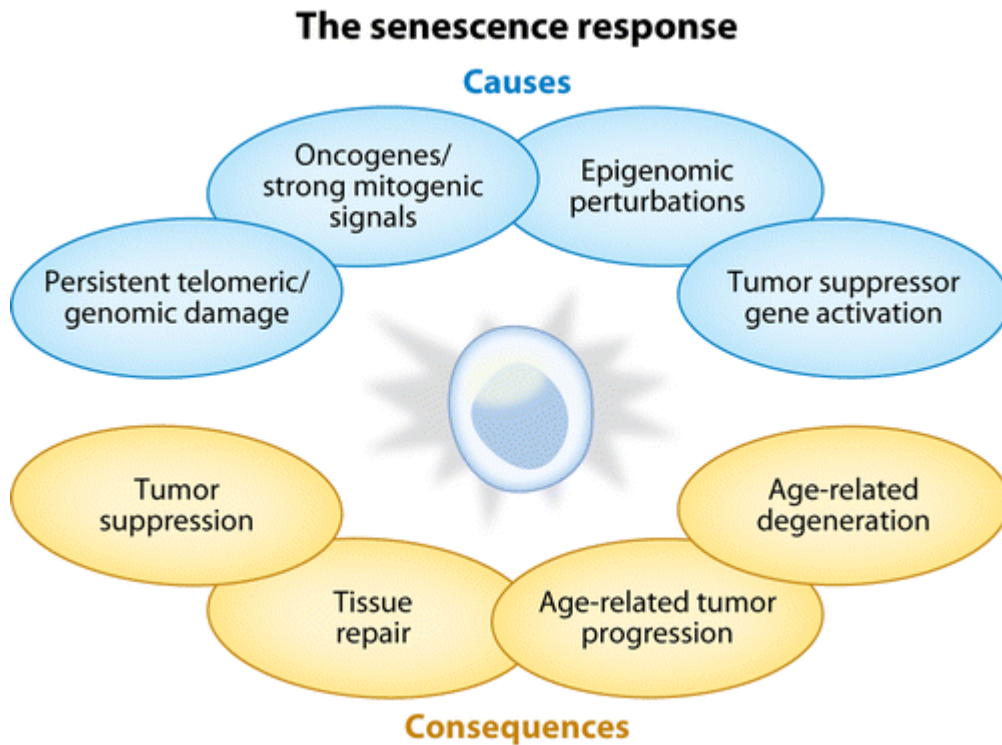


Figure 01: Effectors of senescence
(J. Campisi, 2013)

I. Different effectors of Senescence:

1.1: Telomere Shortening

It is now understood clearly which mechanism affects the replicative lifespan of normal dividing cells. It is because of the inefficiency of DNA polymerases to replicate the ends of the chromosomes as they require a labile primer for them to copy the DNA template (Levy MZ et al. 1992). This is termed as the end replication problem. Therefore the telomeres which are the sequences containing multiple repeats of nucleotides (TTAGGG), that cap the ends of linear chromosomes are shortened with each cell division (Allsopp RC et al., 1995).

However, telomere shortening is not a characteristic of cells that express telomerase, the reverse transcriptase, by the grace of which the repetitive telomeric DNA is replenished *de novo* (Collins K. 2000). There is a variation in the number of telomerase positive cells and the length of telomeres among different species. For example, mice have been reported to have 5-10 times longer telomeres than humans and many of the adult cells in mice are telomerase positive (Calado RT & Dumitriu B, 2013). In humans, on the other hand, cancer cells, certain types of adult stem cells, embryonic stem cells and a few somatic cells like activated T cells are telomerase positive cells.

Telomeres that are functionally efficient, prevent DNA repair machineries from recognizing the end replication problem, as Double Strand Break (DSB) which would incite rapid attempt to repair (Blackburn EH. 1991). Such repair followed by cell division potentially causes excessive genomic instability through cycles of chromosome breaks and fusion which are identified as major factors for causing cancer. Repeated cell cycles without telomerase results in the formation of short and dysfunctional telomeres. Such dysfunctional telomeres elicit a DNA damage response but suppress attempted DNA repair. This DDR arrests the cell cycle division through p53 tumor suppressor protein activation that results in senescence growth arrest. As discussed later in this chapter, DDR signaling also establishes and maintains SASP (Fumagalli M et al., 2012)

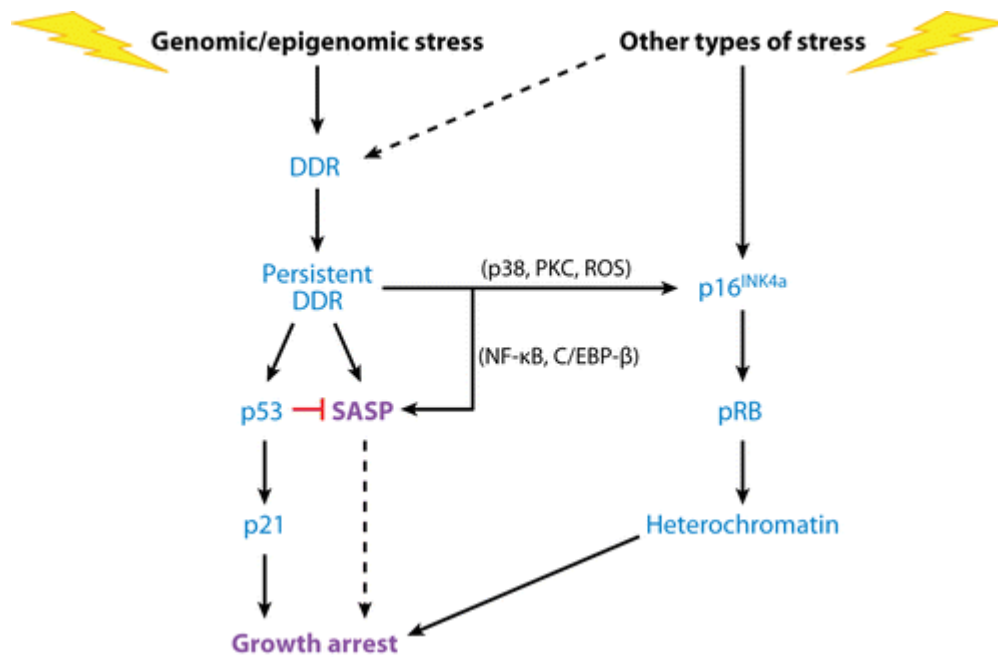


Figure 02: Regulation of Senescence growth arrest and Senescence

Associated Secretory phenotype (SASP) (J. Campisi, 2013)

1.2: Genomic Damage

Dysfunctional telomeres are one of the many potentially oncogenic stimuli, that result in a senescence phenotype (Nakamura AJ et al., 2008). Regardless of the genomic location many cells undergo senescence due to DDR. DNA double strand breaks (DSBs) such the ones induced by ionization radiation, topoisomerase inhibitors and cytotoxic chemotherapies potentially induce senescence in both tumor cells and their surrounding environment (Chang BD et al., 2002; Coppe JP et al., 2008; Novakova Z et al., 2010; Schmitt CA et al., 2002)

DNA lesions that are caused by oxidative stress may induce senescence. oxidative stress and several other DNA-damaging agents often cause DNA base

damage and/or single-strand breaks (Nogueira V et al., 2008; Parrinello S et al., 2003; Sedelnikova OA et al., 2010). During base excision repair mechanism or DNA replication, these base damages are converted to DSBs. The G-rich telomere DNA is notably vulnerable to oxidative stress, therefore accelerating telomere shortening. Therefore these cells senesce in response to direct or indirect DSBs.

The info on the type of genomic lesions that generate a senescence response is imprecise as yet but the most potent of these lesions are reported to generate persistent DDR signaling. This contrasts with the mild DNA damage that only results in a transient growth arrest and DDR signaling. However, a persistent DDR signaling is characterized by the existence of nuclear DNA damage foci that contain a variety of activated DDR proteins that include activated p53.(Fumagalli M et al., 2012, Sedelnikova OA et al., 2010, Rodier F et al., 2009, Rodier F et al.,2011)

1.3: Mitogens and Proliferation-Associated Signals

Mitogenic signals are other potent inducers of cellular senescence, consistent with its role in suppressing tumorigenesis. One of the best studied examples of senescence inducers is via strong, potent , imbalanced mitogenic signals, like H-RAS (H-RAS^{V12}) that results in chronic stimulation of mitogen-activated protein kinase (MAPK) signaling pathway (Serrano M et al., 1997) that leads to

senescence in normal cells, what is now termed as oncogene-induced senescence. Over expression or oncogenic forms of several other MAPK pathway components have since been shown to induce senescence. (**Braig M et al., 2006, Campisi J. 2005, Prieur A, Peeper DS. 2008.**). Also overexpressed growth factor receptors like ERBB2, chronic stimulation by cytokines such as interferon- β (**Moiseeva O et al., 2006**), loss of PTEN (which truncates growth factor signaling) (**Alimonti et al., 2010**), and several other forms of chronic or high- intensity mitogenic stimulation (**Blagosklonny MV. 2003, Deng Q et al, 2004, Takahashi A et al., 2006**) induce senescence.

How do these supra physiological external signals induce senescence? By inducing persistent DNA damage. Some oncogenes and strong mitogenic signals, induce persistent DDR signaling, possibly as a consequence of inappropriate replicon firing and replication fork collapse (which creates DNA DSBs).(**Bartkova J et al., 2006, Di Micco R et al., 2006, Mallette FA et al., 2007**). This mechanism cannot, however, explain all instances of senescence. For example, hyperactivation of p38MAPK, a stress-responsive MAPK pathway component, induces senescence by a DDR-independent mechanism (**Mallette FA et al., 2007**).

Likewise, activation of ATR, a DDR protein that responds to replication stress,

can induce senescence in the absence of actual DNA damage (**Toledo LI et al., 2008**). Whatever the initiating event, mitogenic signals ultimately engage the p53/p21 and/or p16^{INK4a}/pRB pathways (discussed below).

1.4: Epigenomic Damage

Since cellular senescence is such a dynamic process, it is only natural to expect widespread changes in chromatin organization that includes the formation of repressive heterochromatin at several loci proliferative genes (**Adams PD. 2009**). Any perturbations to the epigenome can elicit a senescence response as evidently observed, when broad acting histone deacetylase inhibitors, cause global chromatin relaxation, that induces senescence probably by de-repressing p16^{INK4a} tumor suppressor, that promotes the formation of senescence-associated heterochromatin. Notably, p16^{INK4a}, which is expressed by many senescent cells, is both a tumor suppressor and a biomarker of aging (**Ohtani N et al., 2004, Kim WY et al., 2006**). Other inducers, like suboptimal c-MYC or p-300 histone acetyltransferase (**Bandyopadhyay D et al., 2002**) activity also has been reported to induce senescence by perturbing chromatin organization. Finally, under some circumstances, epigenomic perturbations can elicit a DDR in the absence of physical DNA damage. For example, histone deacetylase inhibitors activate the DDR protein ATM (ataxia-telangiectasia-mutated), which initiates a DDR without DNA damage (**Bakkenist CJ et al., 2003, Pazolli E et al., 2012**).

1.5: Activation of Tumor Suppressors

By engaging either p53/p21 or p16INK4a/pRB tumor suppressor pathways or both generally induces cellular senescence. These pathways are complex and employ multiple upstream regulators, downstream effectors and modifying side branches. In addition, these pathways cross regulate each other. These both pathways, mainly induce the senescence response by changing the gene expression with the help of the transcription factors like p53 and pRB that are the master transcriptional regulators. Overexpression or persistent activation of p53, pRB, p21, or p16^{INK4a} is reportedly enough to induce a senescence growth arrest. These pathways, as expected, being so important also regulate several other aspects of senescent cells, not always all- other features of senescent cells.

Chapter 02: Role of Senescence Associated Secretory Phenotypes (SASPs) and Senescence Associated Heterchromatin Foci (SAHF) – A discussion

Over the previous years, there have been consistent, emerging reports that suggest the binary drawbacks of senescence, one of them being that, senescence in progenitor cells, causes a loss of tissue- repair capacity. The second being senescence-associated secretory phenotype (SASP) that are proinflammatory and matrix- degrading molecules produced by senescent cells

It has been speculated that cellular senescence may have evolved in parallel to apoptosis as anticancer mechanism despite the ill effects, due to the characteristic secretome of SASP, senescent cells & preneoplastic lesions can be recognized by the immune system and be eliminated by it. Recently, many reports pointed out to the beneficial outcome of cellular senescence being more than just tumor suppression, and directed towards wound healing/repair and embryogenesis. This is the tissue remodeling aspect of senescence, where these cells have shorter half-life, presumable because they are effectively cleared by the immune system.

There is convincing evidence for that alongside inducing degenerative pathology, senescence also drives the hyperplastic pathology. This was observed from xenograft studies obtained, when senescent and normal fibroblast cells were co injected into immunocompromised mice, it significantly stimulated the proliferation of mouse and human epithelial cancer cells (**Coppe JP et al., 2006, Krtolica A et al., 2001, Liu D, Hornsby PJ. 2007**). This stimulation is partly because of the soluble secretory factors produced by the senescent cells like components MMP3 (stromelysin) (**Liu D, Hornsby PJ. 2007**), which also promotes tumor cell invasion, and VEGF (**Coppe JP et al., 2006**), which promotes tumor-driven angiogenesis. Other SASP factors implicated in stimulating tumor cell growth are amphiregulin and the GROs (**Coppe JP, et al.,**

2010, **Yang G et al., 2006, Bavik C et al., 2006**) but there are a plethora of other candidates.

In addition to stimulating tumor growth in mice, SASP factors can stimulate malignant phenotypes in culture. One such phenotype is the epithelial-to-mesenchymal transition (**Laberge RM et al., 2012**) (Ref **Figure 02**). This morphological transition enables transformed epithelial cells to invade and migrate through tissues and is critical in the development of metastatic cancer. Senescent fibroblasts induce an epithelial-to-mesenchymal transition in premalignant epithelial cells and nonaggressive cancer epithelial cells in part through the secretion of IL-6 and IL-8 (**Coppe JP et al., 2008, Laberge RM et al., 2012, Hampel B et al., 2006**).

A prominent feature of the SASP is the ability to cause inflammation. Senescent cells, presumably by virtue of SASP-derived factors, can stimulate the infiltration of leukocytes (**Freund A et al., 2010, Kang T et al., 2011, Xue W et al., 2007**), which produce reactive toxic moieties that can cause DNA damage.

Even more ironic is the finding that senescent cells, particularly those that senesce in response to DNA-damaging radiation or chemotherapeutic agents, secrete factors that can protect neighboring tumor cells from being killed by those same chemotherapeutic agents (**Sun Y et al., 2012, Gilbert LA & Hemann MT 2010**). These chemoprotective SASP factors include WNT16B, IL-6, and TIMP-1 (tissue inhibitor of metalloproteinases-1). In contrast, at least some

SASP components can be chemosensitizing. For example, global suppression of the SASP (through NF- κ B inhibition) promoted resistance to chemotherapy in a mouse lymphoma model (**Chien Y et al., 2011**).

The effects of senescent cells within the tumor microenvironment are complex and highly dependent on physiological context. Especially within the context of DNA-damaging cancer therapies, it may be particularly important to consider adjuvant therapies aimed at eliminating senescent cells, both normal and tumor derived. Such therapies could enhance tumor cell killing by chemo- or radiotherapies by preventing the development of a senescence-driven, chemo-resistant niche. They could also inhibit cancer recurrence by preventing senescent cells from stimulating the proliferation of any residual cancer cells.

2.1: Formation of SAHF

Senescent cells have a typical characteristic large flat morphology and the expression of senescence-associated β -galactosidase (SA β -gal) activity, whose origin is quite unclear in the community (**Chen, Z. et al., 2005; Dimri, G. P. et al., 1995**). It is only natural to expect that the chromatin of senescent cells undergoes quite dynamic modifications and remodeling that are categorized as senescence-associated *heterochromatin foci* (SAHF).

They act mainly by repressing the expression of proliferation-promoting genes such as E2F target genes, like cyclin A, through pRB tumor suppressor proteins

therefore, finally irreversibly exiting the cell from the cell cycle. SAHF, since they are heterochromatin foci, they contain known heterochromatin forming proteins, HP1 and the histone variants macroH2A and other specific chromatin proteins, like HMGA proteins (Narita, M. et al., 2006). Also, previously it has been reported that a complex of histone chaperones, like *histone repressor A* (HIRA) and *antisilencingfunction1a* (ASF1a) also play a key role in the execution of SAHF (Zhang, R et al., 2005). Briefly, it begins by the formation of SAHF focus, which is a condensed chromosome. This however, depends on the ability of ASF1a to physically interact with histone H3 alongside its co chaperone HIRA (Tagami. H et al., 2004). In the cells entering senescence, interestingly only HP1 γ but not its relative counterparts are phosphorylated on serine 93, that contributes to the efficient integration of HP1 γ into SAHF.

As indicated previously, SAHF formation is a multi step process, it has been reported that in the earliest defined step, the histone chaperones HIRA and HP1, together are recruited to promyelocytic leukemia (PML) nuclear body that is a specific sub nuclear organelle. Generally, most human cells reportedly have 20-30 PML nuclear bodies that are typically 0.1 to 1 μ M in diameter and are enriched in the protein PML alongside other regulatory nuclear proteins (Sharp J. A. et al., 2002; Salomoni, P., and P. P. Pandolfi. 2002). PML bodies have been reportedly shown to participate both in cellular senescence and tumor suppression (deStanchina,E. et al.,2004; Ferbeyre,G. et al.,2000; Pearson,

M. et al., 2000). After HIRA's displacement to PML bodies, chromatin condensation starts becoming apparent and finally as H3K9Me starts assembling; HP1 & macroH2A are relocated to form SAHF. So the first step is the condensation of individual chromosomes, into what is called, a single SAHF focus. Second step is the interaction between histone chaperone ASF1& H3 histone alongside HIRA, for the formation of SAHF most likely by nucleosome assembly by H3/H4 complexes. The phosphorylation of HP1 γ on serine 93 in senescent cells is the third step (**Narita, M. et al., 2003; Zhang, R. et al., 2005**). However this modification is required for its accumulation of in SAHF but not for its localization into PML bodies. The noteworthy point here is that, neither copious amounts of HP1 proteins/ aggregation of macro H2A nor other known hallmarks of senescence like the expression of senescence-associated cell cycle exit and senescence-associated β -galactosidase activity are required for the formation of SAHF. This highlights a point that HP1 proteins may not initiate senescence but maybe play an integral role in the long-term maintenance of senescence phenotype. However, it is unclear as the identity of the kinases that initiate the phosphorylation of HP1 γ , the methylases that methylate H3 histones and other factors that are required for the deposition of macroH2A onto the SAHF.

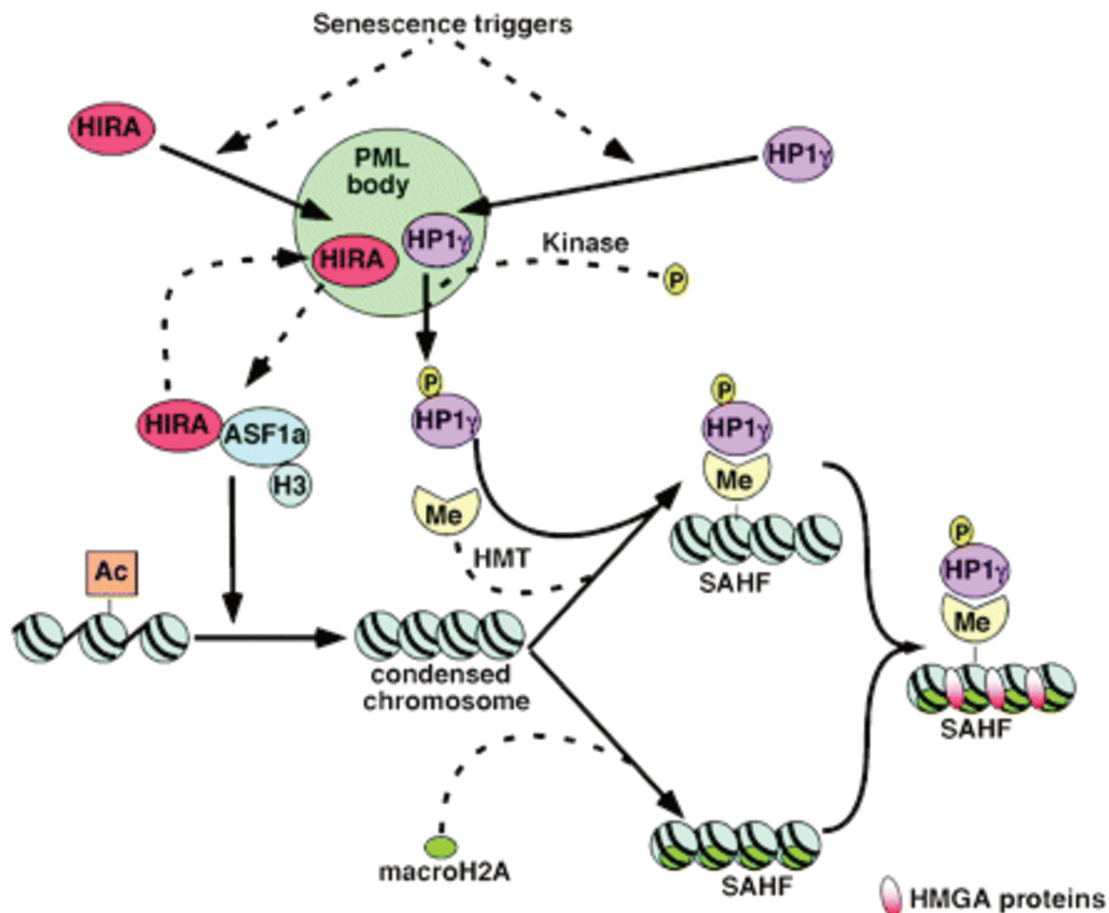


Figure 03: A model for the formation of SAHF

A stepwise model for the formation of SAHF in senescent human cells. This model indicates the key steps in SAHF formation, after initiation by senescence triggers. Dashed lines are steps which are currently poorly defined (see text for details). Abbreviations: HMT, histone methyltransferase; Me, methylated lysine 9 histone H3; Ac, acetylated histone. HMGAs proteins are shown in mature SAHF (56), but their point of entry is not known.

It is unclear as to what role does the translocation of HIRA and HP1 proteins to PML bodies serve. Though reportedly, PML bodies serve as the sites of assembly of macromolecules and protein modifications (Fogal, V. et al., 2000; Guo, A et al., 2000; Pearson, M. et al., 2000). Hence it is speculated that HP1 γ might be getting phosphorylated either inside or outside PML bodies and then targeted to SAHF (Zhang, R et al.,2005).

A noteworthy point here is that, the histone chaperone complex HIRA/ASF1a preferentially utilizes H3.3 as a deposition substrate (**Loppin B et al., 2005; Tagami. H et al., 2004**). Histone 3.3 has been reportedly shown to be significantly accumulating in pre senescent fibroblasts and non dividing differentiated cells and in some cases upto 90% of the H3 accumulates with majority being in inactive chromatin (**Bosch, A., and P. Suau. 1995; Borden, K. L. 2002, Brown, D. T., et al., 1985, Grove, G. W., and A. Zweidler. 1984; Krimer, D. B. et al., 1993; Ooi, S. L. et al., 2006; Pina, B., and P. Suau. 1987; Rogakou, E. P., and K. E. Sekeri-Pataryas.1999; Urban, M. K., and A. Zweidler.1983; Wunsch, A. M., and J. Lough.1987**). Unfortunately, there is no clear answer as to whether endogenous histone H3.3 is abundant in SAHF as there is only five amino acids that are different between H3.3 and H3.1.

Chapter 03: Cellular Senescence: Positive side of a demanding process

If the senescent phenotype is debilitating for the cell, then why did such a phenotype evolve? Especially, the characteristic secretome of the senescent cells like SASP? Experts in the field agree that it is to suppress tumorigenesis. They why not apoptosis? Why rely on the complicated inflammatory response, disrupting tissue structure & function and ironically promote malignant tumor phenotypes? Mounting evidence shows that there are many beneficial effects of

cellular senescence and the SASP.

3.1: Tumor Suppression

As extensively discussed before, there is no doubt that senescence growth arrest suppresses the development of cancer. But is SASP playing a role in this? Indeed certain SASP components have reportedly shown to orchestrate this growth arrest in an autocrine fashion.

IL-6, IL-8 & IGFBA7 (insulin-like growth factor-binding protein 7) bolster the senescence growth arrest caused by the oncogenic forms of cytoplasmic proteins RAS and BRAF, in human cells. These proteins participate in transducing growth factors and other extra cellular signals to the interior of cells. The genes encoding these two proteins are frequently mutated in human cancers. In the same manner, a potent mitogen, GRO α that has been reported as a component of SASP, causes senescence growth arrest by inducing oncogenic RAS, in human ovarian fibroblasts. Seemingly at least some SASP factors establish the oncogene-induced senescence response and in the case of IL-8 and IL-16, these SASP components act by employing a self sustaining intra cellular signaling loop that ultimately induces senescence by activating NF- κ B and C/EBP- β transcription factors

SASP factor, PAI-1 (plasminogen activator inhibitor 1 or SERPIN E1) bolsters senescence in mouse cells. However, in mouse cells, proliferative arrest is

observed in fibroblasts that are cultured in higher oxygen concentrations like 20% O₂ that is substantially higher than the physiological O₂. It has been reported in the same report that when mouse cells were grown at 3% O₂, the cells achieved proliferative arrest much later and at much higher passage numbers. Likewise, WNT16B is an important inducer of senescence in human fibroblasts growing in culture & also in mouse cells in vivo, by inducing the activation of RAS oncogene

With these findings it is evident that at least some of the SASP factors help to maintain the tumor suppressive growth arrest of senescent cells. However, it is to be noted that these SASP factors help establish the senescence rather than maintain the senescence once fully established

3.2: Immune Clearance

Since SASPs are proinflammatory in nature, it is not surprising that senescent cells attract the immune cells including damaging leukocytes of the innate and adaptive immune systems. One of the functions of these immune reactions is to kill and eventually clear all senescent cells. Another function would be the elimination of oncogene-expressing cells, both those cells that have undergone oncogene-induced senescence and those oncogene-transformed cells that have bypassed or escaped senescence (**Kang et al., 2011**). Given the proinflammatory nature of the SASP, it is not surprising that senescent cells can attract immune cells, including destructive leukocytes of the innate and adaptive immune

systems (**Kang T et al., 2011, Xue W et al., 2007, Chien Y et al., 2011**). One function of this immune reaction appears to be the killing and eventual clearance of senescent cells. Another function appears to be the stimulation of a local immune reaction to eliminate oncogene-expressing cells, both those cells that have undergone oncogene-induced senescence and those oncogene-transformed cells that have bypassed or escaped senescence (**Kang T et al., 2011**). Thus, in addition to suppressing tumorigenesis by implementing a cell-autonomous growth arrest, senescent cells can suppress cancer non autonomously by stimulating the immune system to target oncogene- expressing premalignant or malignant cells.

Among the cells that participate in the clearance of senescent cells are natural killer cells, macrophages, and T cells (**Kang T et al., 2011, Chien Y et al., 2011, Krizhanovsky V et al., 2008**). The SASP cytokines that are responsible for these immune responses are incompletely understood but are very likely numerous (**Xue W et al., 2007, Chien Y et al., 2011**). In addition, genomic damage—a common cause of cellular senescence—induces expression of the membrane-bound ligands for the major natural killer cell receptor NKG2D (**Gasser S et al., 2005**). Thus, senescent cells, in part by virtue of the SASP, appear to be programmed to mobilize the immune system to ensure their elimination.

If this is the case, why, then, do senescent cells increase with age and persist at sites of age-related pathology? One possibility is that age-related changes in the immune system make it less likely that senescent cells will be cleared efficiently. There is a striking, well-documented age-related decline in the adaptive immune system, particularly in the ability to mount functional T cell-mediated responses (**McElhaney JE, Effros RB. 2009**). This decline is largely responsible for the heightened susceptibility to infection in the elderly. There are also age-related changes in the innate immune system, although they tend to be less striking than the changes in adaptive immunity; moreover, the aged innate immune system is more likely to show a loss of proper regulation than a loss of function (**Shaw AC et al., 2010, Le Garff-Tavernier M et al., 2010**).

Another possibility is that, with age, senescent cells are produced at a higher frequency, perhaps owing to increased levels of damage, oncogenic mutations, and/or other senescence-inducing events. Indeed, aging tissues show a steady accumulation of cells that harbor DNA damage foci, similar to the foci that are found in senescent cells (**Sedelnikova OA et al., 2004, Wang C et al., 2009, Hewitt G et al., 2012**).

Finally, the SASP also includes proteins that can help senescent cells evade immune recognition and clearance (**Coppe et al., 2010, Freund A et al., 2010**). For example, as noted above, senescent cells secrete high levels of MMPs. These proteases can cleave both the cell surface ligands on natural killer target.

cells and the cell surface receptors on natural killer cells, thereby preventing natural killer cells from targeting and killing senescent cells. There may be a subpopulation of senescent cells that secrete unusually high levels of MMPs, and these cells increase with age. Alternatively, the aging tissue milieu may contain fewer inhibitors of MMPs or other proteases, thereby promoting immune evasion due to elevated protease action.

3.3: Tissue Repair

Recently, there have been reports that indicate that there are some beneficial effects of senescence response and the secreted SASP, that is the ability to promote optimal repair of damaged tissue. The effects of which are discussed below.

When an injury was induced in mouse model of acute liver injury, the senescent cells were cleared by the immune system (primarily by natural killer cells). But when the injury was performed on mice deficient in the p53/p21 and p16INK4a/pRB pathways, a.k.a mice that cannot launch a senescent response the injury was accompanied by a marked increase in fibrosis. These results explain the earlier stated findings that show that the presence of senescent hepatic stellate cells correlates with increased inflammation but reduced fibrosis.

In the same way, in the skin injury mouse model, the injury again induced cellular senescence in the resident fibroblasts. However, in this case, the

senescence response was induced by the binding of CCN1 a multicellular protein to its receptor, an integrin protein on the surface of target cells. This signaling reaction therefore induced both senescence growth arrest and the expression of SASP producing genes and interestingly in CCN1 mutants there was a defective binding of CCN1 to integrin on the surface of the cell and therefore the wounds were lacking in senescent cells and SASP gene expression. Therefore together these studies suggest that one of the noteworthy functions of senescent cells and their corresponding characteristic secretome is to promote wound healing after tissue injury. In the case of acute liver injury and cutaneous wounds, senescent cells limit the development of fibrosis.

Chapter 04: Oxidative Stress and Cellular Senescence

Commonly defined reactive oxygen species (ROS) include superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($\bullet OH$), and singlet oxygen (1O_2) they are generated as metabolic by-products by biological systems (Joseph et al., 2015; Barry, 2007). Vital cellular processes like activation of several transcriptional factors, protein phosphorylation, apoptosis, immune response and differentiation, are dependent on a regulated balance of ROS production and scavenging, inside the cellular environment (Hwang O, 2013). As expected, when there is more than necessary amounts of ROS inside the cell, it has a harmful effect on important structural & biochemical molecules like proteins, lipids and nuclei acids (Romá-Mateo et al., 2015) In the recent

years, humungous evidence started accumulating about the harmful effects of oxidative stress and its resulting manifestation as diseases/disorders like cancer, diabetes, metabolic disorders, atherosclerosis, and cardiovascular diseases (Valko et al., 2007).

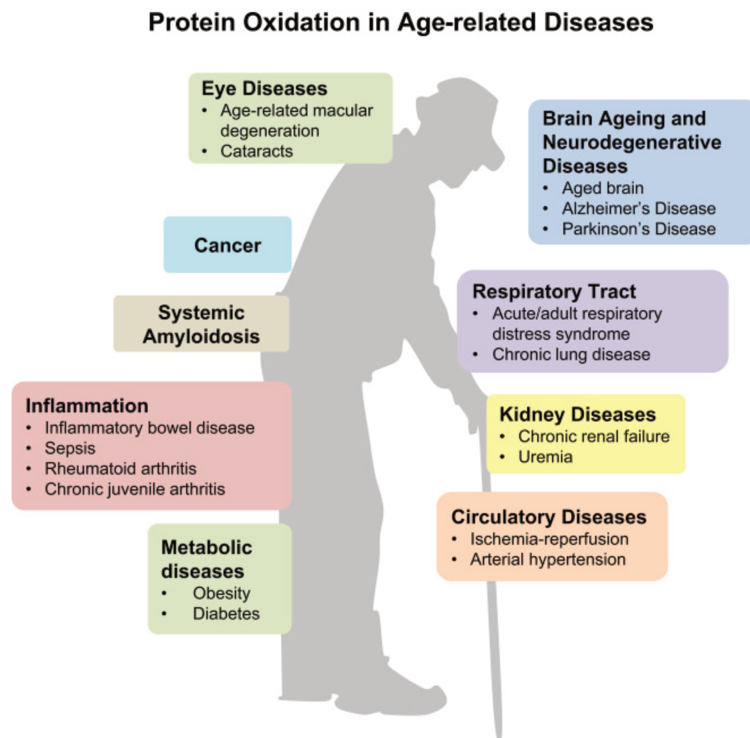


Figure 04: Adapted from Hohn et al., 2016

This figure shows the numerous diseases in which protein oxidation has been reported so far in the context of aging. Protein oxidation may be the cause or consequence of these diseases which affect nearly all organ systems.

The major producer of ROS during both physiological & pathological conditions is mitochondria. For example: $O_2^{\bullet-}$ can be formed by cellular respiration, by lipoxygenases (LOX) and cyclooxygenases (COX) during the arachidonic acid metabolism, and by endothelial and inflammatory cells (Bonomini F et al., 2008) Though these cell organelles are well equipped with their own scavenging mechanisms, it is important to note that it is just not

enough to efficiently clear the ROS produced by the mitochondria in a cell **(Singh et al., 1995; Ramond et al., 2013)**.

So how do the cells survive the amount of ROS that has been generated? They deploy antioxidant defensive system based on enzymatic components such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx).

The implications of oxidative stress and the ROS generated through this stress as a driver of aging has been a topic of interest in the community for a significant time.

The free radical theory of aging also called as the oxidative stress theory of aging, states that age-associated loss of function is majorly due to the accumulation of oxidative damage of macromolecules like lipids, DNA and proteins by reactive oxygen and nitrogen species (RONS). Though the exact mechanism by which the damage occurs is not clear, it is hypothesized that it is probably because increased levels of RONS lead to cellular senescence, that results in the production of SASPs, as mentioned before **(Beckman KB, 1998)**. Senescent cells acquire an irreversible senescence-associated secretory phenotype (SASP) involving secretion of soluble factors (interleukins, chemokines, and growth factors), degradative enzymes like matrix metalloproteases (MMPs), and insoluble proteins/extracellular matrix (ECM) components. RONS induce cellular senescence acting on various components of

SASP:

- regulation of mammalian target of rapamycin complexes' functions
- production of IL-1 α leading to a pro inflammatory state, which increases nuclear factor kappa-B (NF κ B) activity and epithelial–mesenchymal transition and tumor metastatic progression.
- induction of MMPs expression, which is associated with age-related and chronic diseases such as cancer, Alzheimer's, atherosclerosis, osteoarthritis, and lung emphysema.
- inhibition of FOXO (Forkhead box) proteins activity, which is involved in insulin/insulin-like growth factor-1-mediated protection from oxidative stress.
- reduction of sarco/endoplasmic reticulum Ca²⁺-ATPase activity leading to cardiac senescence

In particular, during T cell activation, the transient generation of ROS is necessary to stimulate ROS-dependent transcription factors like NF- κ B, JUN and FOS, and NFAT that in turn will stimulate transcription of pro-inflammatory cytokines (**Desdin Mico et al.,2018**).

Prolonged or excessive exposure to ROS is a cause of tissue damage (**Mittal et al., 2014**). At a molecular level, the damages that ROS inflict on proteins and DNA are well described. In contrast, our understanding on how oxidative stress affects RNA metabolism, including synthesis,

maturation, and degradation, is still limited.

Chapter 05: Introduction to RNA Degradation

5.1: RNA Degradation—Conserved Basic Features

RNA degradation is one of the most prevalent and conserved activities among different organisms in all kingdoms of life. There are emerging reports that even though there exists a general complexity of the process of RNA degradation there are substantial similarities between bacteria, archaea and eukaryotes, thus conveying its prolonged importance.

There are three well known classes of intracellular RNA degrading enzymes (ribonucleases or RNases): endonucleases that cut RNA internally, 5' exonucleases that hydrolyze RNA from the 5' end, and 3' exonucleases that degrade RNA from the 3' end. Endo and 3' exonucleases have long been characterized in all domains of life, whereas 5' exonucleases were, until recently, believed to be absent from bacteria (**de la Sierra-Gallay et al., 2008, Mathy et al.,2007**).

Though genomes encode a plethora of RNases that have various activities, mutation in a single RNA degradation enzyme does not result in a complete block of RNA degradation, in both eukaryotes and bacteria, with a few important exceptions. This indicates the presence of multiple enzymes that recognize the same target RNAs. Presumably, this feature of redundancy enhances the overall efficiency and robustness of degradation pathways. There are many enzymes and cofactors that are involved in RNA processing and

degradation that are multifunctional. In yeast, for example, both the 5' exonuclease Rat1 and the 3' exonucleases of the exosome complex not only target and degrade RNAs transcribed by RNA polymerases I, II, and III but also function in RNA-processing reactions that generate the mature termini of stable RNA species. Similarly, in bacteria the same factors participate in RNA maturation and in the degradation of both stable RNAs and messenger RNAs (mRNAs) (Deutscher 2006). Such dual functions require that a single enzyme can precisely process some RNA species to generate defined ends while retaining the capacity to degrade other RNAs entirely—even the same RNAs under different circumstances.

This functional multiplicity of ribonucleases that specifically identifies target aberrant RNAs and RNA-protein complexes, this is frequently conferred by cofactors that are already characterized in both bacteria and eukaryotes.

5.2: Cofactors for RNA Degradation

5.2.1: Helicases

RNA Helicases can undergo extensive movement upon ATP binding and hydrolysis and translocate along nucleic acids potentially unwinding secondary structures or removing proteins/RNA bound to them. On a flipside, they might be acting as “Place mats” , i.e., they might remain in a temporarily fixed position acting as signal or recruiter for the degradation machinery (Reviewed in Cordin et al., 2006, Rajkowitsch et al.,2008).

5.2.2: Polymerases

Polymerases might have evolved along with exonucleases to help them to initiate degradation process close to stable stem structures, as the polymerases provide a stable “launching pad” for 3’ exonucleases. The best example in eukaryotes is TRAMP polyadenylation complexes. They are reported to act as major cofactors for the exosome complex in budding yeast *Saccharomyces cerevisiae* (reviewed in **Houseley et al., 2006**) and the fission yeast *Schizosaccharomyces pombe* (**Buhler et al., 2008**). The TRAMP complexes are characterized by a poly(A) polymerase (Trf4 or Trf5 in budding yeast—Cid14 in *S. pombe*), a zinc-knuckle putative RNA-binding protein (Air1 or Air2 in budding yeast), and an RNA helicase (Mtr4 in budding yeast).

TRAMP acts on defective nuclear RNAs by tagging them with a short poly(A) tail, this TRAMP-exosome combination serves as a potent surveillance system of different RNAs and RNA protein complexes. Polyadenylation as a marker for RNA degradation by exosome is akin to polyubiquitinylation in protein degradation by proteasome. (**Lorentzen and Conti, 2006**). There have been reported some 3’ exonucleases that reverse as RNA polymerases that include bacterial polynucleotide phosphorylase (PNPase) or the archaeal exosome (**Mohanty and Kushner, 2000; Portnoy et al., 2005**).

5.2.3: Chaperones

Chaperones promote RNA-RNA and RNA-protein interactions, thus regulating

the degradation of many RNAs. One good example is the degradation of human histone mRNAs through recruiting Lsm 1-7 complexes (**Mullen and Marzluff, 2008**). These are closely related, ring shaped complexes that are present in Eukaryotes alongside Lsm 2-8 and Hfq in bacteria. (**Beggs, 2005**).

5.3: Roles of Small RNAs

Different small RNAs (sRNAs) can interact functionally and be regulated by poly(A)-stimulated degradation (**Urban and Vogel, 2008**) There have been reportedly many functional similarities between bacterial endogenous RNAs and eukaryotic miRNAs that generally interact by regulation of mRNA translation termed prokaryotic silencing RNAs (psiRNAs) or crRNAs that are transcribed from CRISPR (clustered regularly interspaced short palindromic repeat) loci.

These CRISPR loci integrate short multiple regions that match the sequences of invading viruses and thus transcribed into long pre-crRNA transcripts that finally result in individual crRNA/psiRNAs. These RNAs provide defense by what is called the Cascade complex (CRISPR-associated complex for antiviral defense (**Barrangou et al., 2007, Brouns et al., 2008, Hale et al., 2008**) by directing the endogenous cleavage of homologous viral RNAs, which is apparently similar to eukaryotic small-interfering RNAs (siRNAs) that direct site-specific cleavage by the RNA-induced silencing complex (RISC).

5.4: Degradation of Different Types of RNA in Eukaryotes

Different classes of RNA degradation can be potentially discriminated as follows:

- **Processing:** Generally, all RNA species are synthesized as bigger precursors and therefore should undergo 3' and in many cases 5' processing by nucleases. In addition to that many excised spacer fragments must be degraded alongside, mRNA precursors. These maturation pathways are integral for processing of all classes of RNA and

removal of the discarded material probably dominates total RNA degradation.

- **mRNAs and non-protein-coding RNAs (ncRNAs):** The turnover of mRNAs is a key factor for the control of gene expression and an apparent key factor in mRNA metabolism. At a similar pace, there are large classes

of unstable ncRNAs that undergo continuous “Constitutive” degradation, which makes them distinct from other stable RNA species.

- **Quality control:** Surveillance pathways are constantly active on all classes of eukaryotic RNA, always identifying and degrading defective RNAs and RNA-protein complexes. These pathways however are hard to assess because they only appear defective RNAs or ribonucleoprotein particles (RNPs), which are apparently found rarely. Exceptions exist like RNAs with premature translation termination codons (PTCs) that are

generated by alternative splicing or by programmed genome rearrangements in some specific cell types, and serve as targets for nonsense mediated decay (NMD) pathways.

5.5: Types and of functions of RNA Polymerases

Even though the three different RNA polymerases in the eukaryotic cells have very different products. Transcripts generated by RNA polymerases I, II, and III can all be targets for 3' degradation by the exosome and its TRAMP cofactor, or for the Rat1 5' exonuclease (Xrn2 in humans).

The different RNA polymerases and their functions are briefly described as follows

5.5.1:RNA polymerase I

RNA polymerase I produces polycistronic RNA encoding 3 different eukaryotic ribosomal RNAs (rRNA), which are generated by endonuclease cleavages and exonuclease trimming, during which, the ETS & ITS (external and internal transcribed spacer regions) are removed and degraded. Since there is a high production of ribosomes (approximately 2000 min^{-1} in budding yeast), of the pre-rRNA spacers ($\sim 3 \times 10^6 \text{ nt min}^{-1}$) presumably accounts for a significant degradation of total cellular RNA. TRAMP and exosome complexes largely degrade the defective ribosomes but it is currently unclear how aberrant ribosomes are targeted.

5.5.2: RNA Polymerase III

Pol III is known to produce multiple small stable RNAs like RNA Pol III produces multiple small stable RNAs, including tRNAs, the 5S rRNA, the U6 snRNA, and the RNA component of signal recognition particle (SRP). They exhibit a far simpler processing than rRNAs as the mature 5' ends are at the TIS and 3' end are produced simply by trimming. However, tRNAs are an exception as they undergo 5' cleavage by RNase P and has a 3'-terminal CCA added by a dedicated polymerase. Nuclear surveillance of the RNA pol III complex is via poly(A) addition by the TRAMP complex and 3' degradation by the exosome as Evidently observed for 5S rRNA, U6 snRNA, the RNA component of SRP, and pre-tRNAs (Copela et al., 2008, Kadaba et al., 2006).

5.5.3: RNA Polymerases IV and V

In plants, two additional RNA polymerases have been characterized (Wierzbicki et al., 2008). Termed RNA Pol IV and V (or nuclear RNA polymerase D and E) these appear to function specifically in siRNA-mediated gene silencing, with RNA Pol IV generating the siRNA precursors and RNA Pol V generating ncRNA targets for the siRNAs.

5.5.4: RNA Polymerase II

RNA Pol II produces messenger RNA precursors (pre-mRNA) and precursors to

numerous stable RNAs including small nuclear RNAs (snRNAs) that function in pre-mRNA splicing, small nucleolar RNAs (snoRNAs) that function in ribosome synthesis, and miRNAs that regulate mRNA translation and stability, as well as many other ncRNA transcripts. Transcription by RNA Pol II is also accompanied by co transcriptional 5'-end capping that protects against 5' exonucleases as depicted in Fig 4 (Staley and Guthrie, 1998).

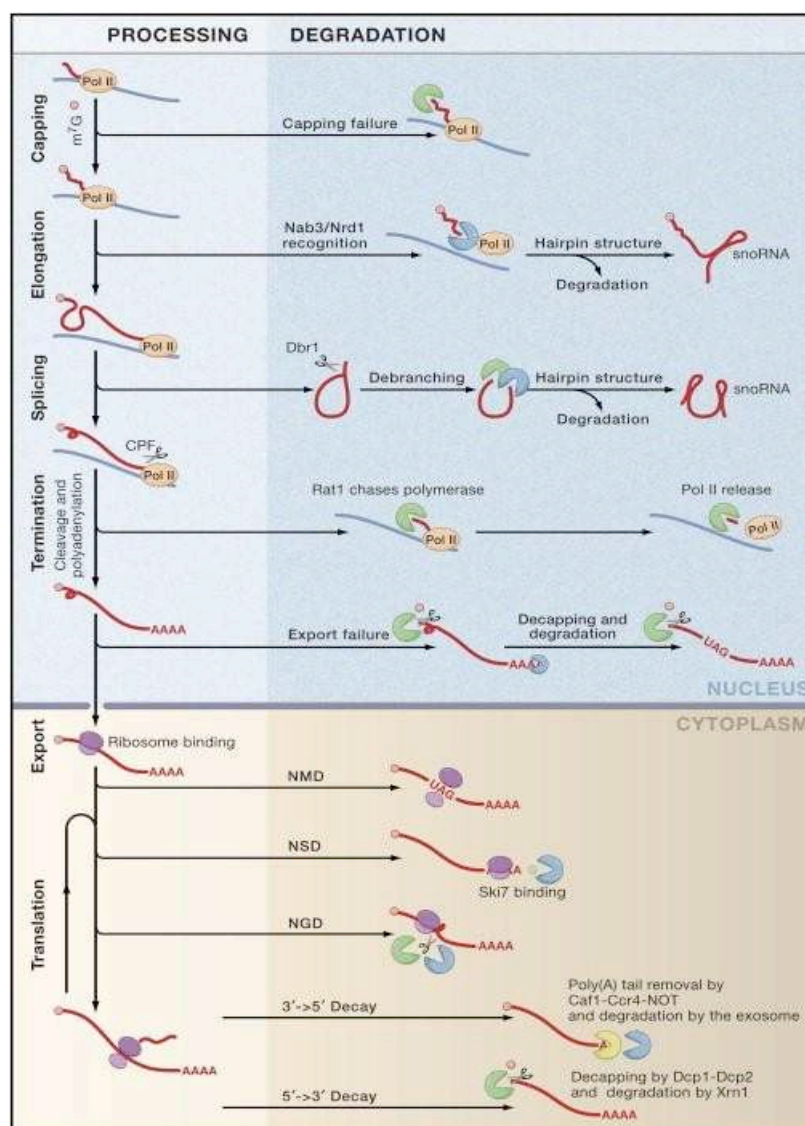


Figure 5: Processing and Degradation of RNA Polymerase II Transcripts
(Houseley & Tollerevey 2009)

A polymerase II also brings about precursors to the very small RNA species, like, miRNAs, siRNAs, and piRNAs, which in some specific scenarios target mRNAs and other RNAs for degradation. There are two related RNP complexes, the cytoplasmic RISC and the nuclear RNA-induced transcriptional silencing complex (RITS) that function as the effectors of miRNA and siRNA. Each of these complexes direct site-specific cleavage of target RNAs that considerably complementing to miRNA or siRNA, that are mediated by the “slicer” activity of an Argonaute protein (**Ref Fig 5**). The RISC complex also targets mRNAs that only show partial complementarities to the miRNA or siRNA, binding to the sequences in the 3'-untranslated region, resulting in decreased translation and increased 5' and 3' degradation (**Eulalio et al., 2008, Wu and Belasco, 2008**).

The siRNAs and miRNAs themselves undergo active degradation by the 3'-exoribonuclease Eri1, which negatively regulates the activity of miRNA/siRNA-mediated gene repression by degrading siRNA-containing duplexes (**Ref Fig 5**). Eri1 also functions in rRNA processing (**Ansel et al., 2008, Gabel and Ruvkun, 2008**), and for two DEAD-box helicases that act together with Drosha in pri-miRNA processing (**Fukuda et al., 2007**), suggesting that miRNA-processing system arises from pre-existing RNA-processing factors. In plants, small RNA *degrading nucleases* (SDNs) are the counterparts of Eri1. They are related but distinct family of single-strand-specific nucleases that limits miRNA levels and is important for normal development (**Ramachandran and Chen,**

2008) (Ref Fig 5). Both the Eri1 and SDN families are widely conserved among eukaryotes and seemingly play an important role in these organisms.

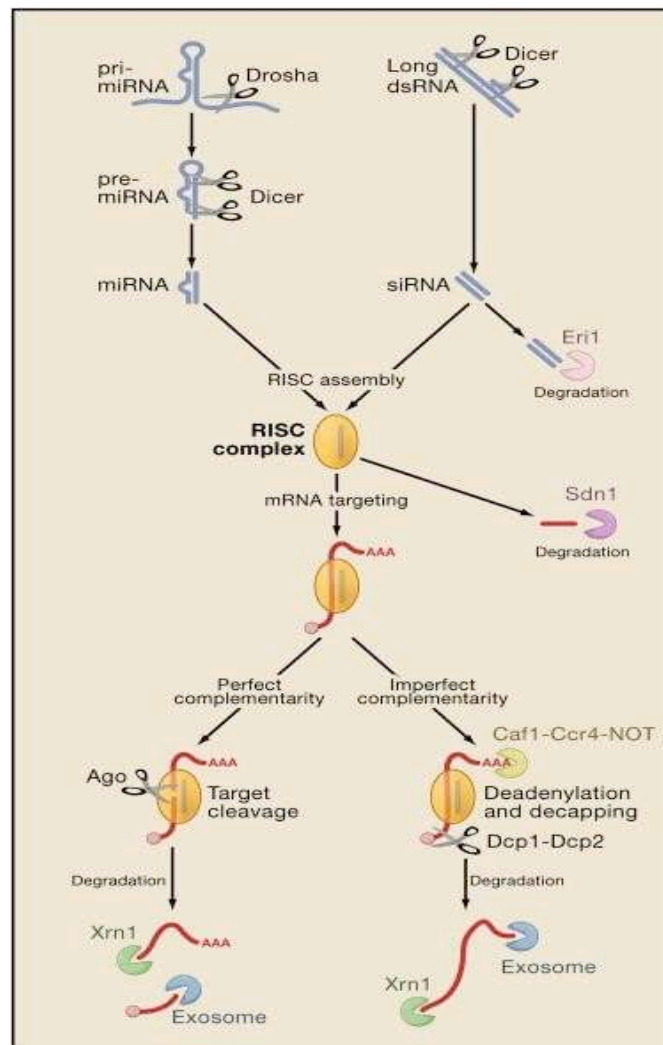


Figure 6: siRNA- and miRNA-Directed RNA Degradation (Houseley & Tollerevey 2009)

5.5.4.i: RNA Polymerase II and NELF complex

The elongation step of RNA polymerase II (RNAPII) mediated transcription is widely being recognized as a critical and important control point for the expression of various gene involved in diverse biological processes including neuronal fate determination during embryonic development (Guo et al., 2000;

Zorio et al., 2001) gene expression of HIV (Garber and Jones. 1999; Karn, J. 1999; Kim et al., 1999; Mancebo et al., 1997; Zhu et al., 1997), transcriptional regulation of heat shock genes (Andrulis et al., 2000; Kaplan et al., 2000; Lis et al., 2000) and replication/transcription of hepatitis delta virus (Yamaguchi et al., 2001). In all these examples studied, the role of three transcription elongation factors like DRB (5,6-dichloro-1- β -d-ribo furanosyl benzimidazole) sensitivity inducing factor (DSIF), NELF (negative elongation factor), and positive transcription elongation factor b (P-TEFb) has been discussed.

NELF (negative elongation factor) is a complex that has four-protein subunits- namely (NELF-A, NELF-B, NELF-C/NELF-D, and NELF-E) that impacts transcription by RNA polymerase II, negatively, by pausing about 20-60 NTs downstream from the transcription start site (TSS) (Adelman K & Lis J. T. 2012).

NELF-A subunit is encoded by the gene WHSC2 (Wolf-Hirschhorn syndrome candidate 2). The gene COBRA1 encodes NELF-B protein that reportedly interacts with BRCA1. It is currently, unclear, whether or not NELF C and NELF D are peptides resulting from the same mRNA with separate translation initiation sites possibly only differing in an extra 9 amino acids for NELF C at the N-terminus, or the peptides from different mRNAs entirely. However, a single NELF consists of NELF-C or NELF-D but not both at the same NELF-E

is also known as RDBP. (Narita Takashi et al., 2014; Yamaguchi Yuki et al., 1999).

The other two transcriptional factors that stabilize paused Pol II are the 5,6-dichloro-1- β -d-ribofuranosyl benzimidazole (DRB) sensitivity-inducing factor (DSIF), composed of subunits SPT4 and SPT5; Positive transcription elongation factor b (P-TEFb), which contains the kinase CDK9 and the predominant cyclin subunit CYCT, release the paused polymerase. P-TEFB phosphorylates Pol II, DSIF and NELF. DSIF can both positively and negatively affect RNAPII transcription. It has been reported that DSIF and its homologues are conserved from bacteria to humans while NELF is conserved among metazoans. HIV-1 virus also reportedly uses Pol II pausing to recruit viral factors such as Tat and also to promote transcriptional elongation through P-TEFb (Gilchrist DA, et al. 2008). NELF homologues reportedly exist in some metazoans like insects and vertebrates but not in plants, yeast and nematode (worms) (Narita Takashi et al., 2014).

5.5.4.ia: Working model of NELF

Soon after transcription initiation starts, RNAPII is under either positive or negative control of DSIF, NELF and P-TEFb. DSIF and NELF physically associate with RNAPII and result in transcriptional pausing. Though DSIF binds to RNAPII directly and stably, (Wada et al., 1998; Yamaguchi et al., 1999), it seems to have an insignificant effect on the catalytic activity of

RNAPII (**Yamaguchi et al., 1999**). Previously, it has been pointed out that NELF does not significantly bind to DSIF & RNAPII separately but NELF preferentially binds to the complex of (**Yamaguchi et al., 2002**). This association likely triggers transcriptional pausing. P-TEFb, positively regulates RNAPII transcriptional elongation by preventing the action of DSIF and NELF (**Renner et al.,2001, Yamaguchi et al., 1999**). P-TEFb is the protein kinase, and it is speculated that its primary target is the C-terminal domain (CTD) of RNAPII (**Price et al., 2000**). Most reports, suggest that P-TEFb-dependent phosphorylation of the CTD is responsible for the release of DSIF and NELF from RNAPII, thereby reversing the inhibition (**Bourgeois, C et al., 2002; Ping et al., 2001 Yamaguchi et al., 1999**)

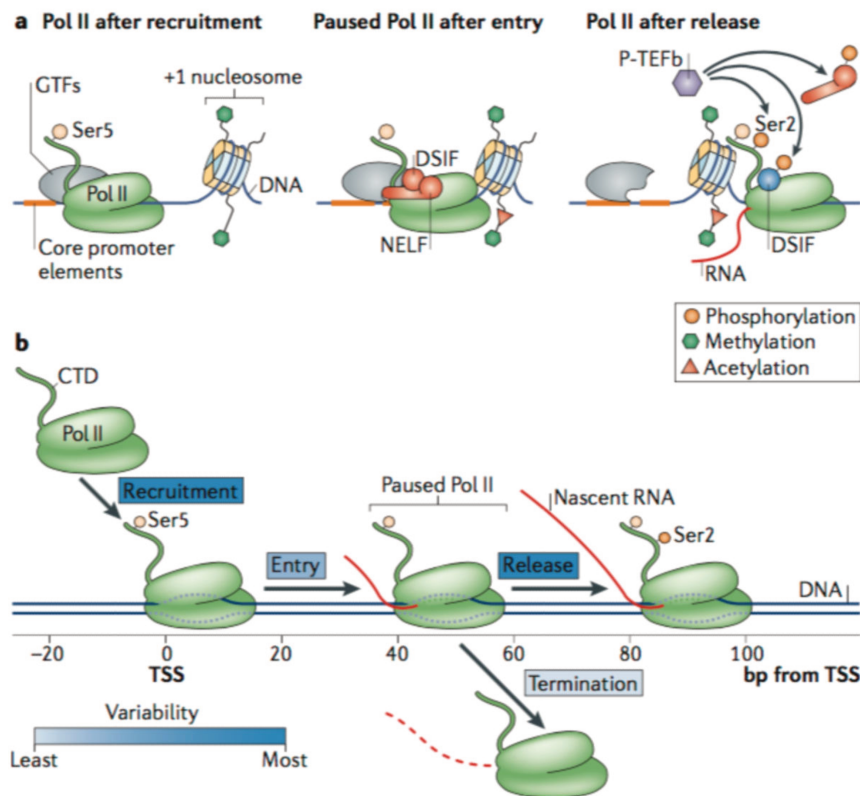


Figure 7: RNA polymerase II recruitment, initiation and gene entry, pausing and release (adapted from Jonkers and Lis Page 21 *Nat Rev*)

a) RNA polymerase II (Pol II) is associated with promoters at, and just downstream of, the transcription start site (TSS). Recruitment of Pol II by general transcription factors (GTFs) results in a pre-initiation complex (PIC). After rapid Pol II initiation and entry into the pause site, Pol II pausing by negative elongation factor (NELF) and DRB-sensitivity-inducing factor (DSIF) occurs, supported by the core promoter elements and the +1 nucleosome. Positive transcription elongation factor-b (P-TEFb) intervenes in the release of paused Pol II by phosphorylating NELF, DSIF and the carboxy-terminal domain (CTD) of Pol II. DSIF becomes a positive elongation factor after phosphorylation. **b)** The transcription cycle is predominantly regulated near the TSS, at the stages of recruitment of Pol II to promoters, and release from the promoter-proximal pause site. These steps are most variable in terms of speed (as indicated by the dark blue shading of the boxes defining the steps).

5.5.4.ii: *RNA Polymerase II and Integrator complex (INT)*

As previously discussed in this section RNA Polymerase II (RNAPII)-mediated transcription is tightly controlled at several steps, from the recruitment and assembly of the entire transcription apparatus, to the initiation, elongation and termination of transcription. Previously we discussed about the NELF complex regulating the elongation process of transcription. However, in the last decade,

a novel multi subunit complex has been discovered as one of the components of the RNAPII-mediated transcription machinery for most regulated genes, named Integrator (INT) (**Baillat & Wagner 2015**) In the initial studies of affinity purification of INT complex identified twelve subunits (IntS1 to IntS12) and thoroughly demonstrated its association with the C-terminal domain (CTD) of RPB1, which is the largest subunit of RNAPII (**Baillat et al., 2005**). In humans, these proteins are annotated from IntS1-S12, in numerical order on the basis of gel migration, with IntS1 having the largest predicted molecular mass of 244 kDa and IntS12 being the smallest one with 49 kDa (**Chen & Wagner 2010**). To date most of the INT subunits are yet to be fully characterized and structurally more clearly depicted.

5.5.4.iii: Role of INT in enhancer RNA (eRNA) transcription

Since its first description, INT complex has been described more in the 3' end formation of noncoding uridine-rich small nuclear RNA (snRNA). However, in the last years, genome wide analyses and other studies have brought into light that INT may play a rather important role in development and certain diseases. Also, INT controls RNAPII pause-release at diverse gene classes, working alongside the NELF-DSIF-P-TEFb complexes. (**Baillat & Wagner 2015; Gardini et al., 2014; Skaar et al., 2015; Stadelmayer et al., 2014**)

Enhancers are the main regulator elements of the genome allowing cell-type and cell-state specificities of gene expression (**Buecker & Wysocka 2012; Li**

& Notani 2016) . Enhancers are broadly transcribed, resulting in the production of enhancer-derived RNA, or eRNA (**Lam et al., 2014; Li & Notani 2016**) In recent years, INT complex was shown to encourage the biogenesis of eRNA transcripts derived from distal regulatory elements (enhancers) that are known to be involved in tissue- and temporal-specific regulation of gene expression in metazoans (**Lai et al., 2015**).

Lai et al., 2015, demonstrated the requirement of IntS11 catalytic activity in the regulation of eRNA induction. This study also supports the role of INT in 3'-end cleavage of eRNA primary transcripts leading to transcriptional termination. Therefore, in the absence of INT complex, eRNAs remain bound to RNAPII resulting in the accumulation of their primary transcripts. Interestingly, the induction of eRNAs along with the gene expression requires the catalytic activity of INT. Further evidences are required for explaining this mechanism in a detailed way. In a previous study eRNAs were found to facilitate the release of the NELF complex from paused RNAPII and subsequently promotes its entry into a productive elongation stage. When the underlying action mechanism was investigated, eRNAs were proved to be acting as a decoy for NELF, which supposedly is a common strategy of eRNAs to regulate gene expression, and exhibited no function in the chromatin looping between the enhancer and the promoter (**Schaukowitch et al., 2014**). Thus, the same complex could be involved in both activation and termination, two

divergent roles that occur at different places during the transcription cycle.

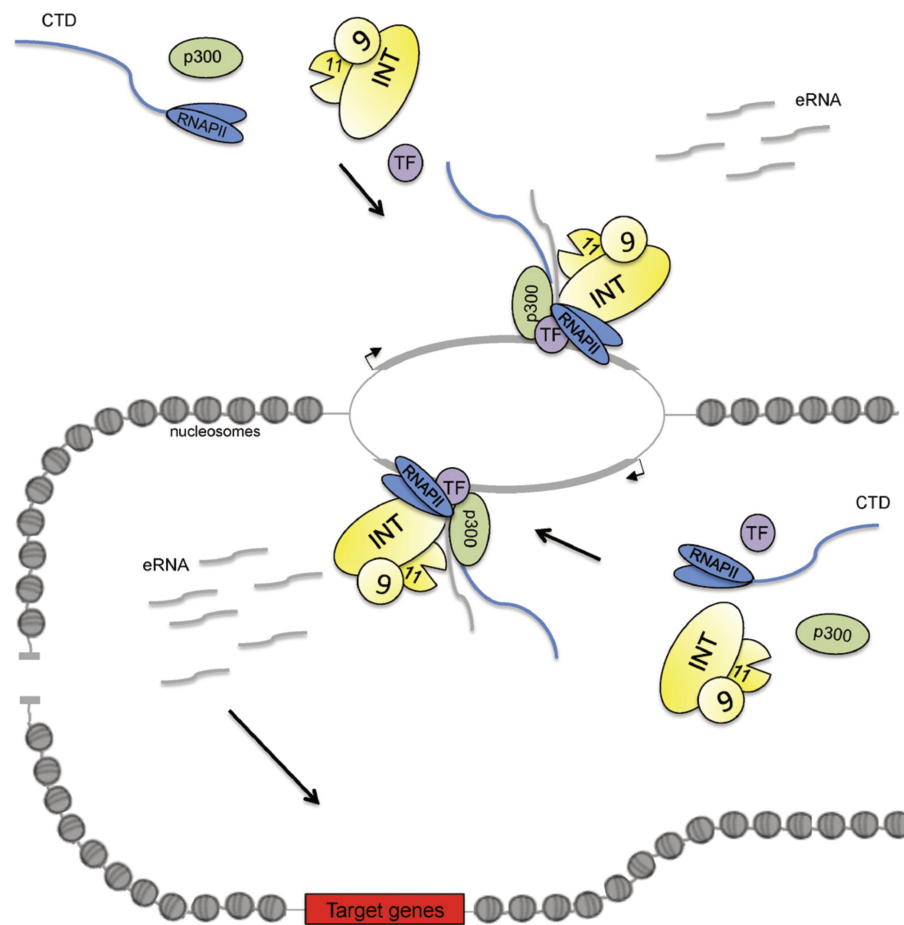


Figure 08. Model for INT involvement in eRNA transcription. (adapted from Reinzo & Casamassimi 2016)

RNAPII (colored in blue) and INT (yellow) are involved in the bi-directional transcription of non polyadenylated eRNAs. INT complex navigates with RNAPII on the enhancer to promote endonucleolytic cleavage of the nascent transcript, promoting the release of the mature eRNAs (Zhu et al., 1997). Schematically, the fig. shows the loop occurring between the enhancer and the promoter of the distal target gene that is being activated.

5.5.4.iii: RNA Polymerase II and Non coding RNAs (ncRNAs)

Recent reports have shown that yeast and human cells transcribe almost their entire genomes therefore implying that there is an existence of huge mass of cryptic ncRNAs which are supposedly transcribed by RNA polymerase II (reviewed in Amaral et al., 2008). The evidence for the existence of a complex population of nuclear RNAs in mammalian cells has been reported through

metabolic labeling performed many years ago indicated that majority of newly synthesized transcripts are retained and degraded in the mouse nucleus resulting in the formation of heterogeneous nuclear RNA (hnRNA) population (see, for example, **Brandhorst and McConkey, 1974**). Over the course of time, these observations though earlier discounted were later supported by deep sequence analyses (**Birney et al., 2007, Han et al., 2007, Maeda et al., 2006**).

NcRNAs reportedly fall into different categories. Some could be basic transcriptional noise that is generated throughout the genome due to the inability of transcriptional promoters to efficiently identify true promoters. Studies imply that there are short, cryptic antisense transcripts, generated at a high level at the promoter regions that are lucrative targets for the exosome in humans cells (**Core et al., 2008, He et al., 2008, Preker et al., 2008, Seila et al., 2008**) and yeast (**Davis and Ares, 2006**) (**Neil et al., 2009**) and it is suspected that it may also be the case for transcription termination region, that may or may not prove to be functionally important. In addition to these sporadic transcripts, both yeast cells and human cells seem to have ncRNA that are transcribed from apparently dedicated promoters. Such ncRNA genes could be located either within intergenic regions or antisense to protein-coding genes. A commonly found feature in all of these ncRNAs is that they all share high instability, that seems to explain how they escaped detection and analysis. A good example is the yeast GAL1-10 cluster that was found to be present at the

extremely low abundance of 1 molecule per 14 cells (**Houseley et al., 2008**). ncRNAs' degradation is best understood in yeast, where distinct features like redundancy and co-transcriptional association of the surveillance machinery are key players in rapid degradation. The first is redundancy; individual RNAs can be targeted for degradation by the exosome complex by several different cofactors (**Milligan et al., 2008**). The second is co transcriptional association of the surveillance machinery. Nrd1-Nab3, that are the exosome cofactors, and the TRAMP complex associate with at least some nascent transcripts (**Carroll et al., 2007, Houseley et al., 2007, Vasiljeva et al., 2008**), hence pre targeting the ncRNAs for degradation as soon as they are synthesized. Recent data slightly hints that a third feature that promotes rapid ncRNA degradation could be endonuclease cleavage.

Chapter 06: Why Is RNA Degradation so Efficient? - A sneak peek into RNA degradation by exosome.

The remarkable feature of most characterized eukaryotic RNA degradation pathways is their striking efficiency. In yeast mutants that contain ribosome synthesis defects, the pre-rRNAs are generally degraded with almost undetectable intermediates—kilobases of RNA with dozens of associated proteins apparently just disappear despite their very high rates of synthesis. Degradation of ncRNAs is also reportedly so efficient that their widespread

existence is only now being brought to the forefront. Why is it that RNA degradation is strikingly efficient? Possible explanation for the high prevalence of exonuclease activities than endonucleases is the presence of a variety of different types of small RNA (such as miRNAs, siRNAs, and piRNAs) exerting potent effects on gene expression at multiple steps from chromatin structure to translation and mRNA turnover. There might have been strong and persistent accumulation of random RNA fragments particularly from highly expressing RNAs. As expected, loss of TRAMP-mediated RNA degradation reportedly allows inappropriate entry of rRNA & tRNA fragments into the RNAi pathway in fission yeast (**Buhler et al., 2008**).

Eukaryotic exosome core, that is discussed in details below, associates with hydrolytic exonucleases Rrp44/Dis3 (which is related to *E. coli* RNase R) and Rrp6/PM-Sc1100 (which is related to *E. coli* RNase D) unlike Bacterial PNPase and the archaeal exosome that include three active sites for phosphorolytic exonuclease activity. The hydrolytic exonuclease activity is more favourable than PNPase or the archeal exosome, owing to the fact that, hydrolysis is more thermodynamically favoured hence is efficient in degrading through stable RNA-protein (RNP) – without generating degradation intermediates that could enter RNAi pathway. It is a possibility that RNAs that accumulate sans degradation can disrupt DNA replication and other activities, by forming RNA-DNA hybrids, as it has been proposed for telomeric ncRNAs in yeast strains that are defective for the 5' exonuclease Rat1 (**Luke et al., 2008**).

6.1: RNA Exosome complex

The exosome complex or PM/Sc1 complex is multiprotein intracellular complex that can degrade various types of RNA molecules. Exosome complexes are found both in archaea and eukaryotic cells while in bacteria a rather simpler complex called the degradosome does the job. RNA exosome was first discovered as an RNAase in *S. cerevisiae* in 1997 (Mitchell et al., 1997). In 1999, a yeast equivalent of PM/Scl complex was discovered, which had been already described in the human cells as an autoantigen in autoimmune disease. (Illmang et al., 1999).

The core of the exosome contains a 6-membered ring-like scaffold to which other proteins are attached. In the eukaryotic cells, the exosome complex can be found in the cytoplasm, nucleus and especially in the nucleolus. There are however, different proteins interacting with the exosome complex in these compartments regulating the RNA degradation activity of complex to substrates specific to these cell compartments. The different substrates of the exosome are messenger RNA, ribosomal RNA & many species of small RNAs. The exosome exhibits an exo ribonucleolytic function and endo ribonucleolytic function, which means the RNA is degraded starting at one end (3' end) and also cleaves RNA within the molecule itself.

As briefly mentioned above, several proteins in the exosome complex are targets for autoantibodies in patients with specific autoimmune diseases like

PM/ScI overlap syndrome and some anti metabolic chemotherapies for cancer work by blocking the activity of the exosome. In addition, mutations in exosome component 3 reportedly are a cause of ponto-cerebellar hypoplasia and spinal motor neuron disease.

6.1.2: Structure of RNA Exosome

The core of the exosome complex consists of a ring structure consisting of six proteins that all belong to the same class of RNases, the RNase PH-like proteins (**Schilders et al., 2006**). In archaea there are PH-like proteins called Rrp41 and Rrp42, that are present in an alternating order, three times. Eukaryotic exosome complexes however have six different proteins that form the ring structure. (**Lorentzen et al., 2005; Shen & Kiledijan 2006**). Of these six eukaryotic proteins, three are similar to the archaeal Rrp41 protein and the other three proteins are more similar to archaeal Rrp42 protein (**Raijmakers et al., 2002**).

On the top of this ring are three proteins that have an S1 RNA binding domain (RBD). The difference between eukaryotes and archaea is that in eukaryotes, three different "S1" proteins are bound to the ring, whereas in archaea either one or two different "S1" proteins can be part of the exosome (**Walter et al., 2006**)

In bacteria, a separate RNase PH protein exists that is involved in transfer RNA processing, has been shown to adopt a similar six-membered ring structure, but here, it consists of 6 identical protein subunits (**Harlow et al., 2004**). The RNase PH-like exosome proteins, PNPase and RNase PH all of them belong to

the RNase PH family of RNases and they exhibit phosphorolytic exoribonucleases, which means that they employ inorganic phosphate to remove nucleotides from the 3' end of RNA molecules (**Schilders et al., 2007**).

6.1.3: Associated proteins

Besides these nine core exosome proteins, two other proteins, Rrp44, and Rrp6 (in yeast) or PM/Sc1-100 (in human) which are both hydrolytic RNases often associate with the complex in eukaryotic organisms are associated with the exosome proteins. In addition to being an exoribonucleolytic enzyme, Rrp44 also has endoribonucleolytic activity, which resides in a separate domain of the protein (**Lebreton et al., 2008; Schneider et al., 2009**) The protein PM/Sc1-100 is most commonly part of exosome complexes in the nucleus of cells, but can form part of the cytoplasmic exosome complex as well (**Raijmakers et al., 2004**)

6.1.4: Regulatory proteins

Apart from these two tightly bound protein subunits, many other regulatory proteins, that may either regulate the activity or specificity, interact with the exosome complex in the cytoplasm and nucleus both. In the cytoplasm, the exosome interacts with AU rich element (ARE) binding proteins like KRSP and TTP), which either promote or prevent degradation of mRNAs. The nuclear exosome associates with RNA binding proteins for e.g. MPP6/Mpp6 and

C1D/Rrp47 in humans/yeast that are required for processing certain substrates **(Schilders et al., 2007)**.

6.1.5: Protein complexes

In addition to single proteins, other protein complexes like the cytoplasmic Ski complex, which includes an RNA helicase (Ski2) and is involved in mRNA degradation **(Wang et al., 2005)** interacts with the exosome. In the nucleus, the processing of rRNA and snoRNA is mediated by the TRAMP complex, (mentioned in section 5.2.2), which contains both RNA helicase (Mtr4) and polyadenylation (Trf4) activity **(Lacava et al., 2005)**

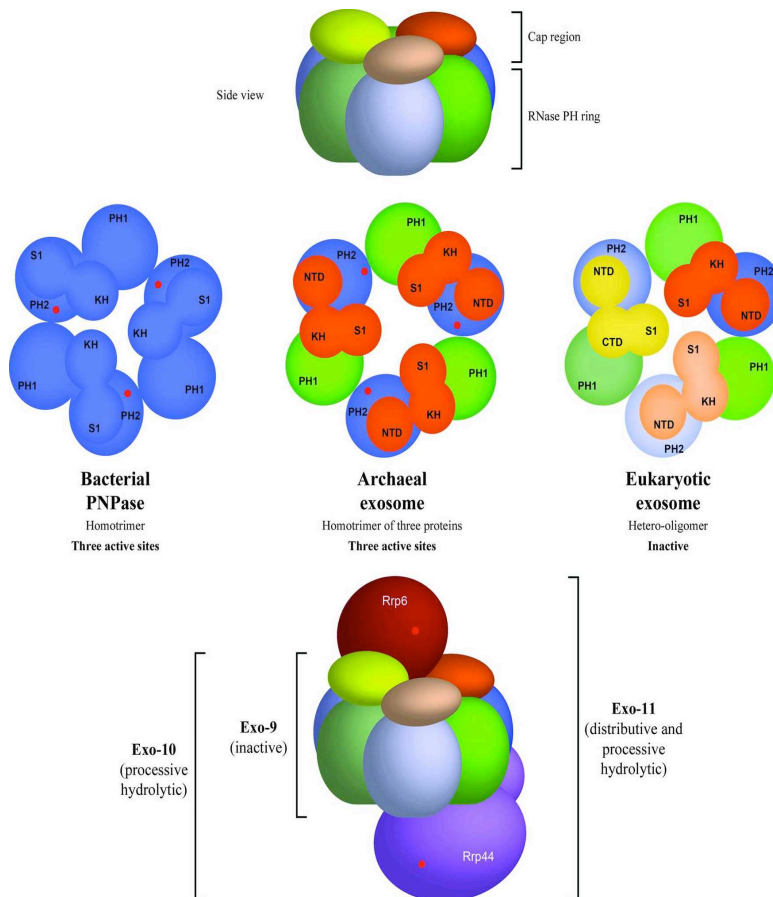


Figure.09: Structural organization of RNase PH complexes. (Adapted from Makino, D. L. and Conti, E. 2013)

The top panel shows a side view of their ring arrangement, with the S1/KH domains, also called the cap region, on top. The middle panel illustrates side-by-side the evolutionary architectural conservation of the RNase PH complexes. In bacterial PNPase, one chain contains two RNase PH domains and one S1/KH region, forming a homotrimer with three phosphorolytic active sites. The archaeal exosome evolved into three distinct subunits, carrying RNase PH subunits, Rrp41 and Rrp42, and a cap protein, which could be either Rrp4 or Csl4. This complex comprises a homotrimer of three different proteins that, similarly to the bacterial PNPase, has three phosphorolytic sites. The eukaryotic exosome, however, is composed of nine different subunits that are still somewhat related in sequence to the archaeal Rrp41-like subunits (Rrp41, Rrp46 and Mtr3), the archaeal Rrp42-like subunits (Rrp45, Rrp43 and Rrp42) and the cap proteins (Rrp4, Csl4 and Rrp40). As a consequence of this increase in structural complexity, the eukaryotic exosome core is catalytically inactive. Its catalytic function arises from the association of a tenth subunit, Rrp44 (violet; bottom panel), a processive hydrolytic exoribonuclease. In the nucleus of yeast cells, an eleventh component, Rrp6 (red; bottom panel), binds to the exosome, providing a second exoribonucleolytic site to the entire complex.

At the level of transcription, oxidative stress causes a very rapid accumulation of the RNA polymerase II (RNAPII) at promoters and enhancers, accompanied by an accumulation of enhancer RNAs (eRNAs) and upstream antisense RNAs (auRNAs). This phenomenon referred to as “creeping RNAPII” is currently

explained by defective transcription termination, via a mechanism involving loss of association and function of the **negative elongation factor NELF**, the function of which was discussed in detail above (**Nilson et al., 2017**). The accumulation of RNAPII at enhancers was suggested to be the mechanism allowing maintenance of chromatin in an open state. Another report brings evidence for a more extensive activation of long non-coding RNA (lncRNA) expression, with several RNA species accumulating in the cytosol and interacting with the ribosomes (**Giannakakis et al., 2015**).

6.2: Brief Perspectives I- NELF, Integrator & Exosome complex & their link to mitochondrial metabolism

In our study, by examining publicly available data, we show that RNA species affected by exposure to hydrogen peroxide remarkably matches those described as substrates of the Integrator complex and/or of the nuclear RNA exosome. The Integrator complex is associated with the carboxy-terminal domain (CTD) of the RNAPII and controls termination of several classes of short transcripts. It processes endonucleolytic activity and was initially described for its role in proper maturation of small nuclear RNAs (snRNAs) (**Chen et al., 2010**). Later, this complex was also associated with the proper termination of eRNAs (**Lai et al., 2015**).

Some evidence also suggests a role for the Integrator in termination of non-polyadenylated auRNAs that accumulate at some genes upon depletion of

INTS11. Finally, it must be noted that several studies have documented an interaction between the integrator complex and NELF (Yamamoto et al., 2014). Also interestingly we observed that examination of cells with chronic defects in mitochondrial metabolism also show this effect (inactivation of the RNA exosome). Hence, it is interesting for the reader to understand the relation between mitochondria, ROS production & subsequent mitochondrial stress response that these cell organelles employ during chronic stress.

Chapter 07: Connecting the dots-Oxidative Stress and Mitochondrial Stress response

The cellular stress response related to the mitochondria is called **mitochondrial unfolded protein response (UPR^{mt})**. The UPR^{mt} arises when there is an accumulation of misfolded or unfolded proteins in mitochondria beyond the limit of chaperone proteins to handle them. The UPR^{mt} can ensue in mitochondrial matrix or in the mitochondrial inner membrane. In the UPR^{mt}, the mitochondrion reportedly upregulates chaperone proteins or activates antioxidant enzymes and mitophagy through Sirtuin SIRT3 (Pellegrino MW et al., 2013; Papa L & Germain D, 2014).

It has been reported that the UPR^{mt} can be activated by mitochondrial electron transport chain mutations that extend the life span of *Caenorhabditis elegans* (nematode worms) (Durieux J et al., 2011). Supplementation with nicotinamide or nicotinamide riboside increases NAD⁺ that in turn activates UPR^{mt},

reportedly increases the lifespan in nematode worms. This nicotinamide riboside supplementation has also been reported to activate UPR^{mt} in mice (**Mouchiroud L et al., 2013**)

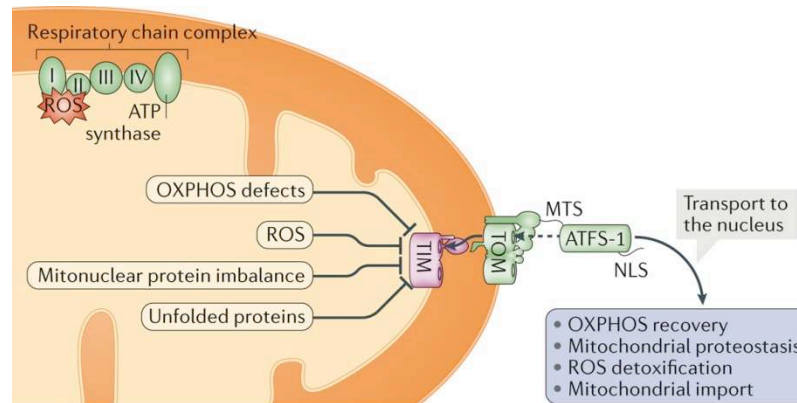


Figure 10: Pathway of mitochondrial unfolded protein response;
adapted from Shpilka & Haynes 2017

Oxidative phosphorylation (OXPHOS) perturbation, reactive oxygen species (ROS), protein imbalance in the mitonuclear membrane and the accumulation of misfolded proteins impair mitochondrial protein import efficiency and activate ATFS-1 (activating transcription factor associated with stress) that is imported into healthy mitochondria via mitochondrial-targeting sequence (MTS) and degraded. If mitochondrial import efficiency is hampered, ATFS-1 is transported to the nucleus and what is called mitochondrial unfolded protein response (UPR^{mt}) is activated. In the nucleus, ATFS-1 induces a plethora of genes like those that promote recovery of the OXPHOS complexes, protein import components, NLS, nuclear localization sequence; TOM, translocase of the outer membrane & TIM, translocase of the inner membrane as well as those genes re-establish mitochondrial proteostasis by upregulating chaperones and proteases and detoxify ROS.

7.1. ROS and mutations in mitochondrial DNA mutations

Mitochondria are the only organelles in cells, besides the nucleus, that contain their own DNA (called mitochondrial DNA) and their own machinery for synthesizing RNA and proteins (**Douarre et al., 2012**) and mtDNA makes up approximately 1% of total cellular DNA and reportedly particularly susceptible to ROS attack associated with oxidative stress (**Hollensworth et al., 2000**).

Persistent mitochondrial DNA damage leads to mutations in the mitochondrial genome (**Hollensworth et al., 2000**) and gives rise to further mitochondrial dysfunction, which induces and the diseases especially the neurodegenerative disease.

7.2: Brief Perspectives II- uaRNAs, eRNAs & SINEs

As mentioned above, creeping RNA Pol II, results in the accumulation of upstream antisense RNAs (uaRNAs) and enhancer RNAs (eRNAs). There have been numerous reports indicating that SINEs (Short Interspersed Nuclear Elements) exhibit enhancer like activity especially by the genes showing a regulatory function (**Policarpi et al., 2017**). Therefore, there is a necessity here to introduce the structure and transcription of these nuclear elements.

Chapter 08: Short interspersed nuclear elements (SINEs) - an introduction

Short interspersed nuclear elements (SINEs) are non-autonomous, non-coding transposable elements (TEs) that range upto 100 to 700 base pairs in length (**Kramerov D. and Vassetzky N. 2012**). They belong class of retrotransposon which are defined as DNA elements that amplify themselves throughout eukaryotic genomes, more often than not through RNA intermediates.

The internal regions of SINEs remain highly conserved and originate from tRNA. SINEs are often lineage specific; hence they serve as good markers for

divergent evolution between species. SINEs are reported to play an important role in certain types of genetic diseases in humans and other eukaryotes.

In essence, short interspersed nuclear elements are genetic parasites, which evolved early on in eukaryotes to utilize protein machinery from organism as well as to use the machinery from similarly parasitic genomic elements. Since eukaryotes have been able to integrate SINEs into different signaling, metabolic and regulatory pathways they impart great genetic variability. They reportedly play a particular role in the regulation of gene expression like chromatin re-organization, regulation of genomic architecture and the creation of RNA genes. SINEs are incredibly useful tool in phylogenetic analysis as they have different lineages, mutations, and activity among eukaryotes.

8.1. Internal structure

SINEs are essentially characterized by different sections of their sequence. SINEs do not necessarily possess a head, a body, and a tail. The head, is at the 5' end of SINEs and is an evolutionarily derived from ribosomal RNAs and tRNAs, synthesized by RNA Polymerase III, The 5' of the AluSINE is derived from 7SL RNA, a sequence transcribed by RNA Polymerase III that codes for the RNA element of SRP, an abundant ribonucleoprotein (**Kriegs J et al. April 2007**). The body of SINEs possess an unknown origin but often share homology with a corresponding LINE which thus allows SINEs to parasitically co-opt

endonucleases coded by LINES (which recognize certain sequence motifs). The 3' tail of SINEs is composed of short simple repeats of varying lengths that serve as the sites where two (or more) SINEs combine to form a dimer (**Okada N. et al. 1997**). SINEs that have a head and tail are called simple SINEs whereas SINEs that have a body or are a combination of two or more SINEs are complex SINEs (**Kramerov D. and Vassetzky N. 2012**).

8.2. Transcription:

Short-interspersed nuclear elements are transcribed by RNA polymerase III. SINEs, possess an internal promoter and thus are transcribed differently than most protein-coding genes, like tRNAs (**Kramerov D. and Vassetzky N. 2012**). In other words, short-interspersed nuclear elements have their key promoter elements within the transcribed region itself. Though transcribed by RNA polymerase III, SINEs and recruit different transcriptional machinery and factors compared to the genes possessing upstream promoter.

Also, from our datasets, we observe that SINEs might be playing a functional role in induction of inflammatory pathway and this could be how senescent cells activate the immune response.

RESULTS:

I. Cells exposed to H₂O₂ and senescent cells share a common RNA signature:

Cellular senescence has been associated with increased expression of numerous noncoding RNAs. To further explore this phenomenon, we carefully re examined publically available RNA-seq data from WI38 human fibroblast either proliferating or WI38 cells driven into senescence by expression of RAF. This drew our attention to an accumulation in the senescent cells of upstream antisense promoter RNAs (auRNAs) in the absence of increased expression of gene itself. The promoter of H6PD gene is an illustration of this phenomenon (**Fig 1A**). In total, the accumulation was detected clearly only at approximately 50 promoters, possibly because of the insufficient depth of the RNA-seq data and the high instability of this class of non coding RNAs (Sup. Table 1). Yet, quantification at a series of 5260 promoters not overlapping with coding regions of any gene allowed us to estimate the overall increase in accumulation of auRNA in senescent cells to approximately 10% (**Fig 1B and Sup1B**).

Accumulation of auRNA has previously been reported in cells exposed to oxidative stress. This was confirmed by examining an RNA-seq data series from either BJ or MRC5 cells exposed to H₂O₂ for 30mins, or 2h or 4h as indicated. (see example of the H6PD gene in Fig 1A, bottom lanes and the

profiles in **Sup. Figure 1**). Since oxidative stress is one of the main triggers of senescence, we examined both the data from senescence cells and those from the cells exposed to H₂O₂ for additional similarities. This allowed us to identify a small number of discrete sites with striking resemblances in their transcriptional behavior. First, we noted that senescence and oxidative stress conditions both display increased read counts over the 3' UTR of the histone gene HIST2H2BE (**Fig 1C**). A similar increase in read counts was also detected at a region overlapping an enhancer upstream of MIR52A (**Fig 1D**). Finally, we detected inefficient maturation of several U snRNA gene products (**Fig 1E and 1F**). Altogether, these observations suggested that patterns in transcription, maturation, degradation of RNA species are conserved between the very early phases of oxidative stress and cells having entered a definitive stage of growth arrest.

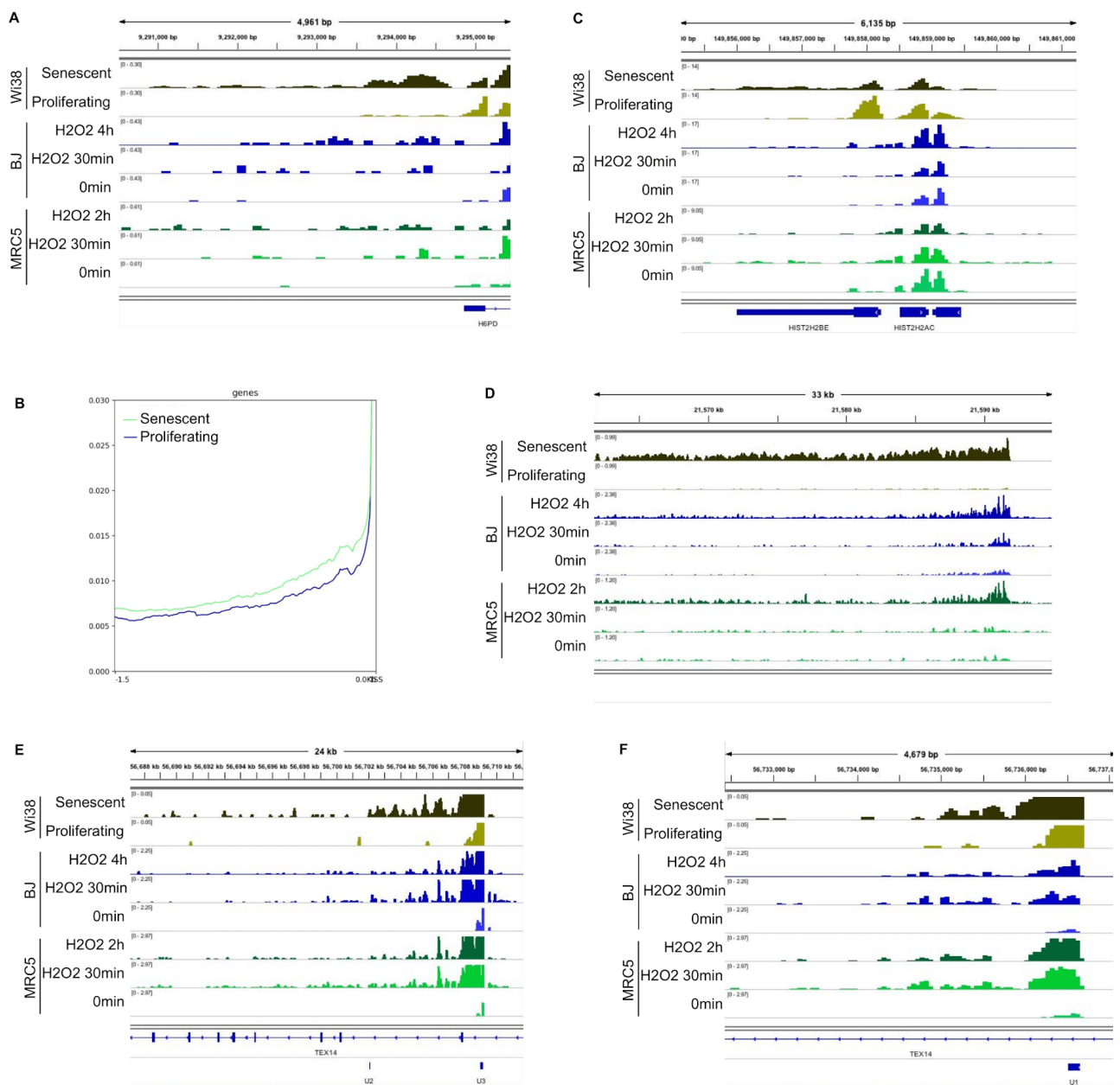
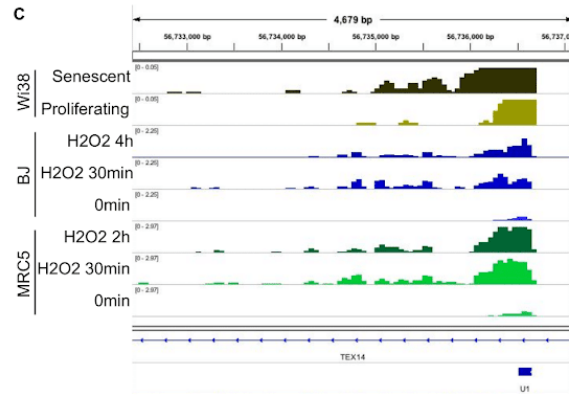
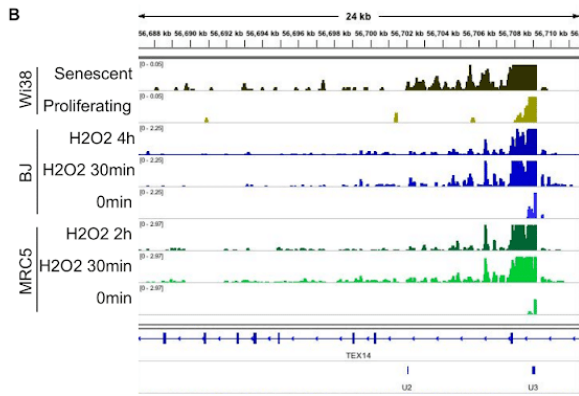
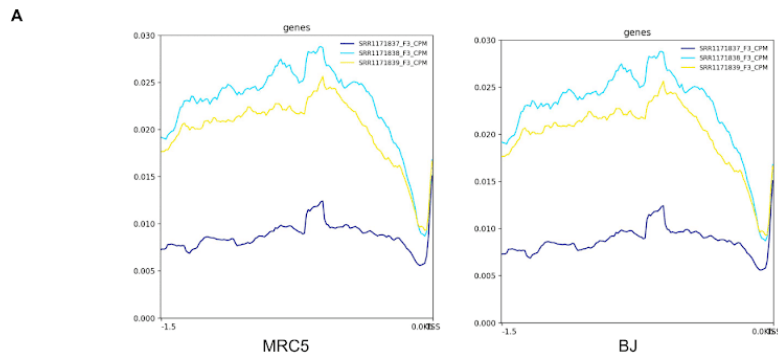
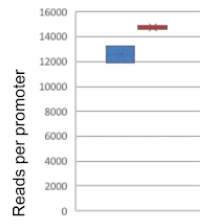


Figure 1: Comparison of the creeping polymerase in oxidative stress induced and senescence. **A.** IGV track showing the upstream region of KLF6 in oxidative stress induced cells and senescence cells dataset. **B.** Profile of the 1500bp region upstream promoter of genes in senescence cells dataset for a list of 5260 genes with the characteristic to not have any other gene in the 10kb upstream of their promoters. **C.** IGV track showing the downstream region of histone genes HIST2H2AC and HIST2H2BE. **D.** IGV track showing RNA accumulation on enhancers. **E,F.** IGV track of the accumulation of RNA downstream of Us snRNA inside TEXT14 gene.

Figure 1

Supplementary Figures



sup. Figure 1

II. Indications of reduced RNA turnover in cells exposed to hydrogen peroxide and senescent cells:

As 3'UTR regions frequently harbor sequences involved in mRNA stability, the increased accumulation of reads over the 3' end of HIST2H2BE was highly suggestive of reduced efficiency of machineries involved in RNA decay. In parallel, the poor maturation of U snRNAs was rather suggestive of an impairment of Integrator activity, a complex involved in the 3' end cleavage of several types of non coding RNAs. To explore these possibilities, we compared the RNAseq dataset of senescent vs. proliferating cells with RNA seq data from HeLa cells that were depleted of either INTS11 (for

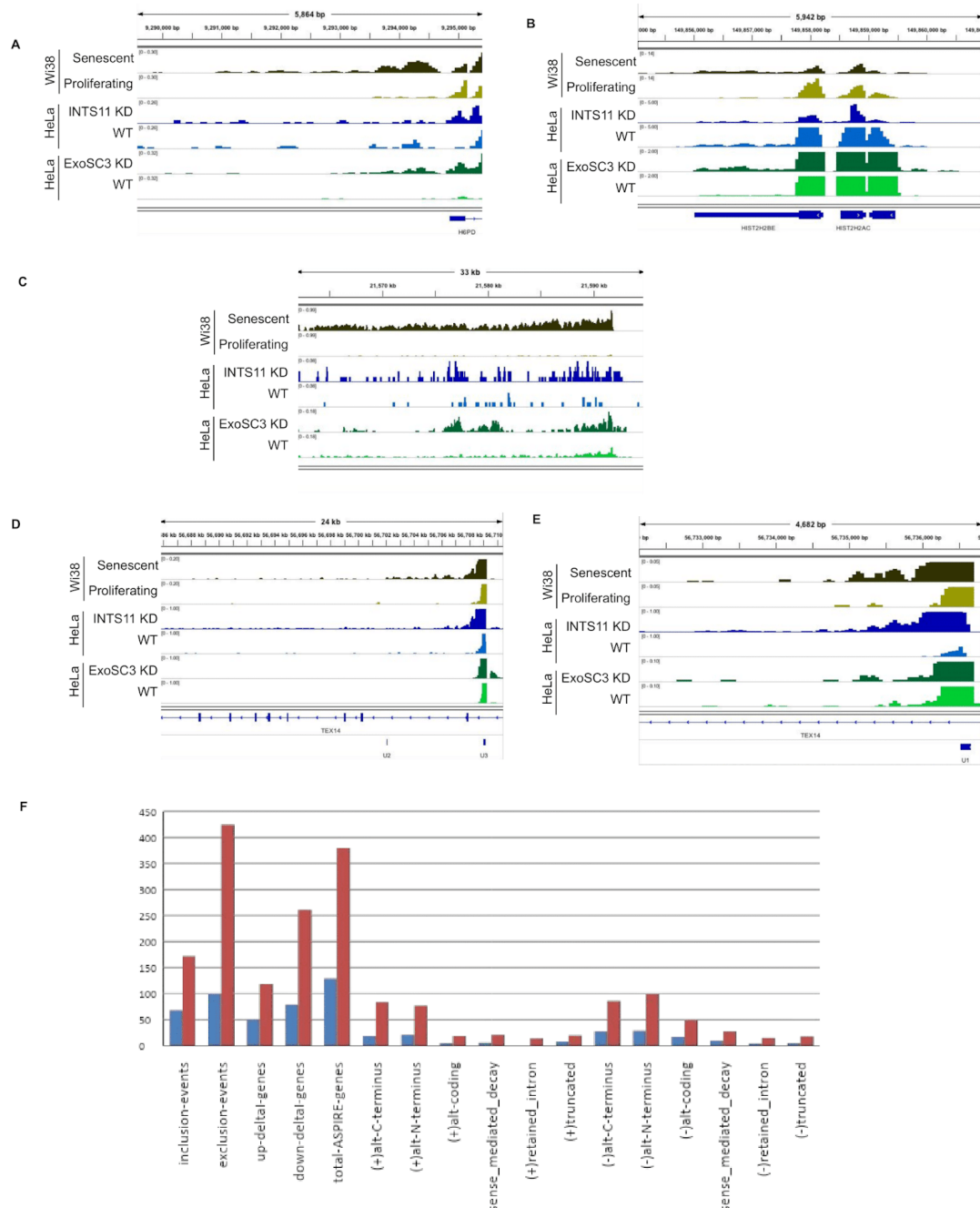
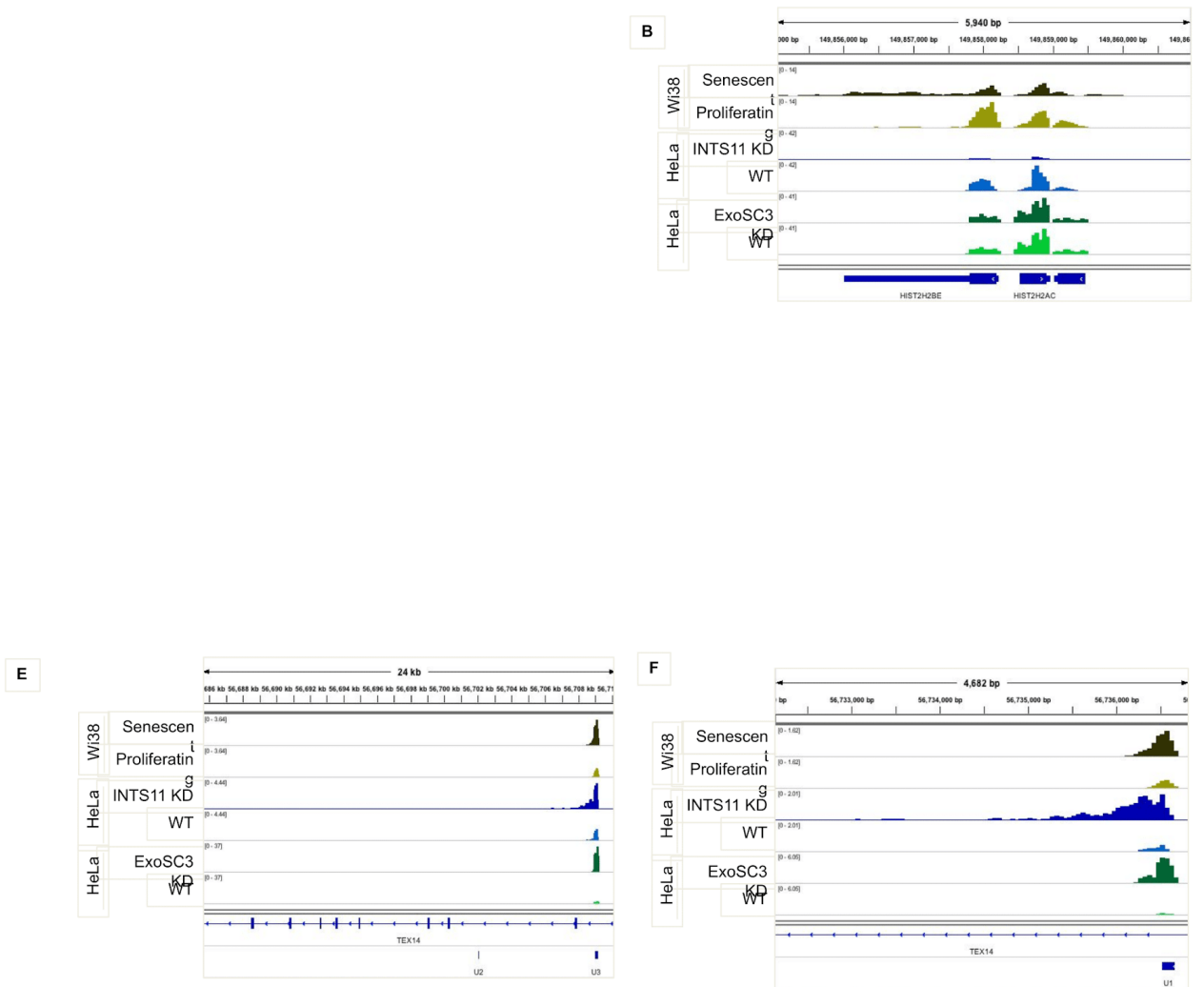


Figure 2: Impact on the RNA turnover by the senescence, the knockdown of Exosc3 or the knockdown of Ints11. **A.** IGV track showing the RNA accumulation in the upstream region of H6PD. **B.** IGV track showing the downstream region of histone genes HIST2H2AC and HIST2H2BE. **C.** IGV track showing RNA accumulation on enhancers. **D,E.** IGV track of the accumulation of RNA downstream of Us snRNA inside TEXT14 gene. **F.** Histogram showing the accumulation of any kind of alternative event of splicing over the time after induction of oxidative stress by ROS in human fibroblasts. **Blue:** Human fibroblasts 4 days after induction of oxidative stress. **Red:** Human fibroblasts 10 days after induction of oxidative stress

Supplementary Figures



Sup Figure 2

inactivation of the Integrator complex) or ExoSC3 (for inactivation of RNA exosome activity). From all 3 datasets, we observed an increase in accumulation of auRNAs on a plethora of genes, which is in concordance with the initial reports that examined data separately (**see example of the H6PD gene in Fig 2A**). Likewise, the enhancer locus upstream of MIR52A showed a similar accumulation of reads in the 3 datasets. In contrast, the UsnRNA maturation defect observed in the senescent cells was best mimicked by inactivation of INTS11, while stabilization of the 3'UTR of HIST2H2BE was clearly detected only upon ExoSC3 knock down.

Together, these observations were highly suggestive of reduced RNA decay in the senescence cells. Furthermore, the beacon gene that we examined were suggestive of reduced activity of the Integrator complex and of the RNA exosome. The reduced activity of the Integrator complex was consistent with earlier data showing that oxidative stress causes reduced activity of NELF (**Nilson et al., 2017**), a negative regulator of elongation also required for the activity of the integrator (**Stadelmayer et al., 2014**).

On the other hand, a reduced activity of the RNA exosome was more unexpected. This prompted us to globally examine senescence cells for mis-spliced RNA species, a primary target of the nuclear exosome. To that end, we identified another sets of publicly available RNA-seq data from IMR90 cells driven into senescence by expression of oncogenic RAS for 0, 4, or 10 days. The multiple time points and the availability of 4 replicates for each

time point made this data suitable for global analysis of splicing products. This approach revealed that induction of senescence resulted in accumulation in a large range of alternatively spliced isoforms, a phenomenon strongly suggestive of reduced mRNA turnover (**Fig 2E**). As the IMR90 data was of relatively low depths, it did not allow for detection of auRNAs.

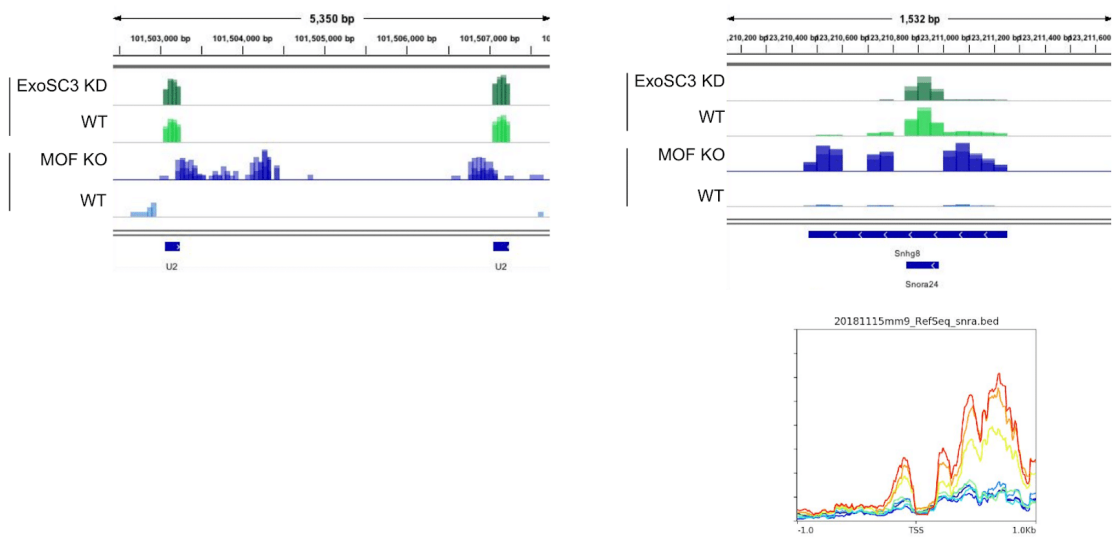
Together, these observations allow to suggest that both senescence and possibly also oxidative stress is associated with reduced activity of the Integrator complex and of the nuclear RNA exosome, resulting in the accumulation of certain RNA species like auRNAs and eRNAs, in increased stability of some mRNAs like the one encoded by HIST2H2BE, and in poor maturation of U snRNAs. We believe that this phenomenon has been difficult to observe due to the technical challenge of RNA isolation from senescent cells, that, because of their large size, are always few on a plate, while also being poorly metabolically active. We further suggest that the few loci that we have identified and that seem easy to monitor in a variety of cellular models, could be good markers to monitor both oxidative stress and cellular senescence by RT-qPCR.

III. Bidirectional crosstalk between mitochondrial stress and RNA exosome

The data described above suggested a role for the RNA exosome in the cellular response to oxidative stress. As hydrogen peroxide (**H₂O₂**) is highly reactive

Supplementary Figures

B



Sup Figure 3

wear-off rapidly, we searched publicly available RNA-seq data repositories for studies addressing mitochondrial defects. In that context, we particularly examined data from cardiomyocytes from mice inactivated for MOF, a histone acetylase regulating transcription and respiration in mitochondria. Inactivation of this gene has catastrophic consequences for tissues with high-energy consumption, triggering hypertrophic cardiomyopathy and cardiac failure in murine hearts; cardiomyocytes show severe mitochondrial degeneration and deregulation of mitochondrial nutrient metabolism and oxidative phosphorylation pathways (**Chatterjee et al., 2016**). Consistent mitochondrial suffering and subsequent oxidative stress, MOF inactivation reproduced the increased accumulation of uRNAs, alike what we observed in human senescent cells and what was reported previously for human cells exposed to hydrogen peroxide. To illustrate this, **Fig 3D** shows the example of *Klf6* gene, while **Fig3E** shows the profile of uRNA accumulation from the compilation of a series of 1200 genes with similar expression levels. Likewise, MOF inactivation resulted in accumulation of non-matured U snRNA, reproducing another RNA signature of senescence.

Consistent with this reduced RNA decay, we noted in the MOF KO cells, a significant decrease in the expression of several subunits of the Integrator complex and of the RNA exosome, particularly ExoSC3 (**Fig 3C**). In parallel, and consistent with MOF inactivation driving cells into growth arrest, we noted a clear increase in the expression of *Cdkn1a* (**Fig 3A**).

The reduced expression of Exosome subunits in the MOF KO cells prompted us to question the effect of ExoSC3 inactivation on long term cellular physiology. To that end, we examined a dataset from mouse embryonic stem cells depleted from ExoSC3 for 3 days. As expected and previously described, these cells recapitulated the increased accumulation of uRNAs. The effect on U snRNA maturation was less clear, in agreement with this process being mostly dependent on the Integrator complex. Interestingly, GO term analysis of genes up regulated upon long-term ExoSC3 inactivation showed a highly significant enrichment in genes associated with the p53 pathway and we pinpointed an increased expression of both Cdkn1a and Cdkn2a. Consistent with this, downregulated genes were highly enriched in genes associated with DNA replication and cell cycling. Examining of GO terms for cellular compartments finally highlighted a significant enrichment in mitochondrial genes among the genes downregulated by the inactivation of MOF. This is consistent with an earlier report showing mitochondrial suffering in patients with pontocerebellar hypotrophy linked to a mutation in the ExoSC3 gene (**Schottmann et al., 2017**).

Together, these observations are suggestive of a bidirectional crosstalk between oxidative stress and RNA degradation for the induction of growth arrest. In this model, oxidative stress causes reduced activity or expression of RNA decay enzymes, while reduced RNA decay seems to induce mitochondrial stress, that in turn generates oxidative stress. Possibly, this

mechanism may be a fundamental engine when cells are driven into senescence.

IV. Serendipitous transcription of SINEs is best tolerated at T cell enhancers:

Further examination of the MOF KO data revealed a surprising accumulation of A-tracts within the reads. This was a possible consequence of the reduced efficiency of the nuclear RNA exosome involved in degrading non-coding polyadenylated RNA species. Alternatively, A tracts are abundant in retrotransposons of the SINE family and are required for the replicative cycle of these elements. Examination of sites of auRNA accumulation clearly showed that this accumulation resulted in the serendipitous production of transcripts covering SINE sequences. Inspired by several studies suggesting a function for SINE and Alu (their human counterparts), we were prompted to examine usage these sequences in regulatory elements. To reach an overview of the participation of SINEs in transcription initiation throughout human tissues, we extracted all regions annotated as active promoters or enhancers in the 127 tissues mapped by the NIH Roadmap Epigenomics Mapping Consortium (Roadmap Epigenomics Consortium *et al*, 2015). These regions will be referred to as PEs for “Promoter or Enhancer” regions.

To estimate the similarity between each of the 127 sets of PEs and regions annotated SINEs in RepeatMasker, we used the Jaccard index defined as the size of the intersection divided by the size of the union of the sample sets (**Fig**

4B).

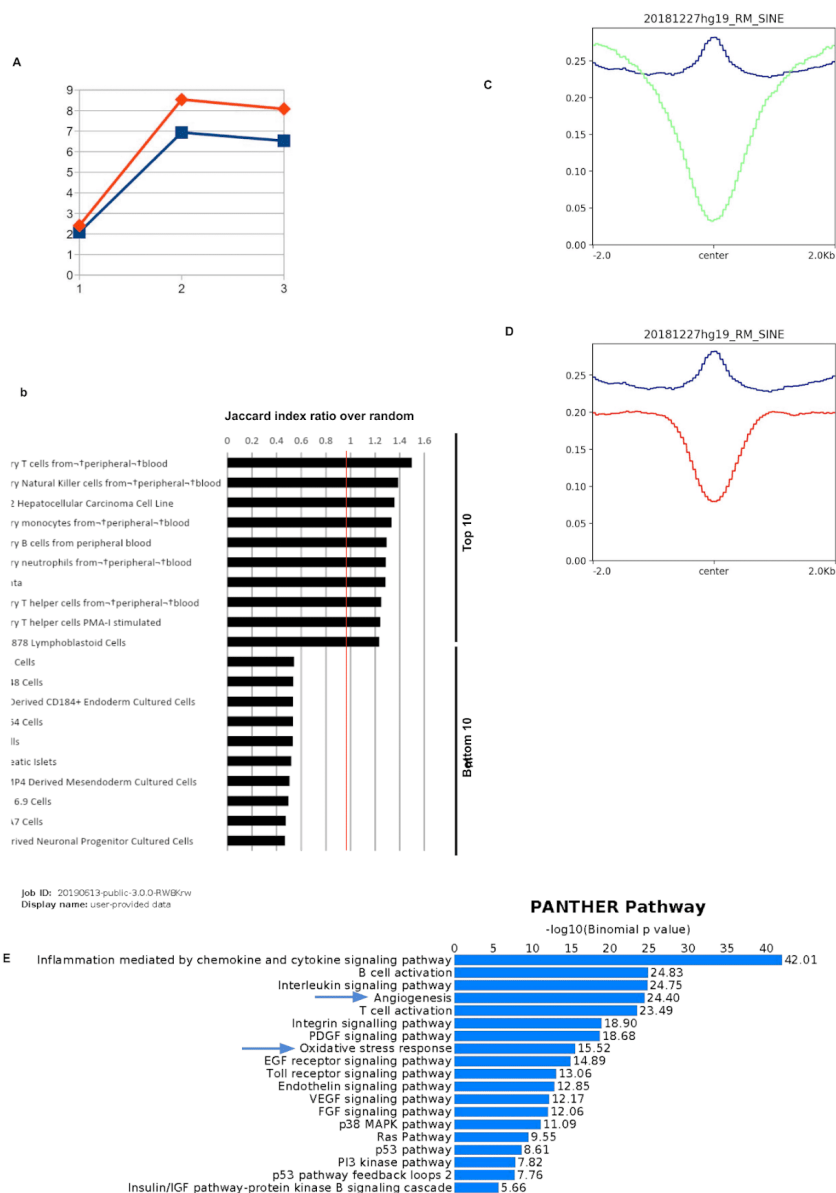


Figure 4: Role of SINEs as T cells enhancers. **A.** Comparison of the accumulation RNAs (number of reads normalized for 1 gene) in the 3kb upstream regions of the promoters that contains genome encoded at least 20A tract (Red line) versus 3kb upstream regions of the promoters that not containing any A tract (Blue line) over the time after oxidative stress induction in MCR5 cells. The 1 value from bottom axis correspond to non treated cells, the 2 value from bottom axis correspond to cells treated for 30 minutes with H₂O₂ and the 3 value from bottom axis correspond to cells treated for 2 hours with H₂O₂. **B.** Comparison of regions annotated “SINE” in RepeatMasker with the regions annotated as enhancers in the 15 core marks model of the Epigenomic Roadmap consortium. For each tissue, the Jaccard index comparing enhancers to SINEs is divided by the average Jaccard index (1,000 iterations) obtained when comparing enhancers to randomly selected genomic locations. Only the 10 highest and the lowest score are shown. **C.** Comparison of the profiles of regions annotated as enhancers with regions annotated as promoters around the center of the SINEs in T cells. Annotation took from the Epigenomic Roadmap consortium. **D.** Comparison of profiles of the regions annotated as enhancers by the Epigenomic Roadmap consortium between T cells and H1derived neuronal progenitor cells. **E.** PANTHER pathways analysis from genes located 100 kb or less from a region annotated “SINE” in RepeatMasker overlapping an annotated TSS from the Famtom5 catalogue.

Supplementary Figures

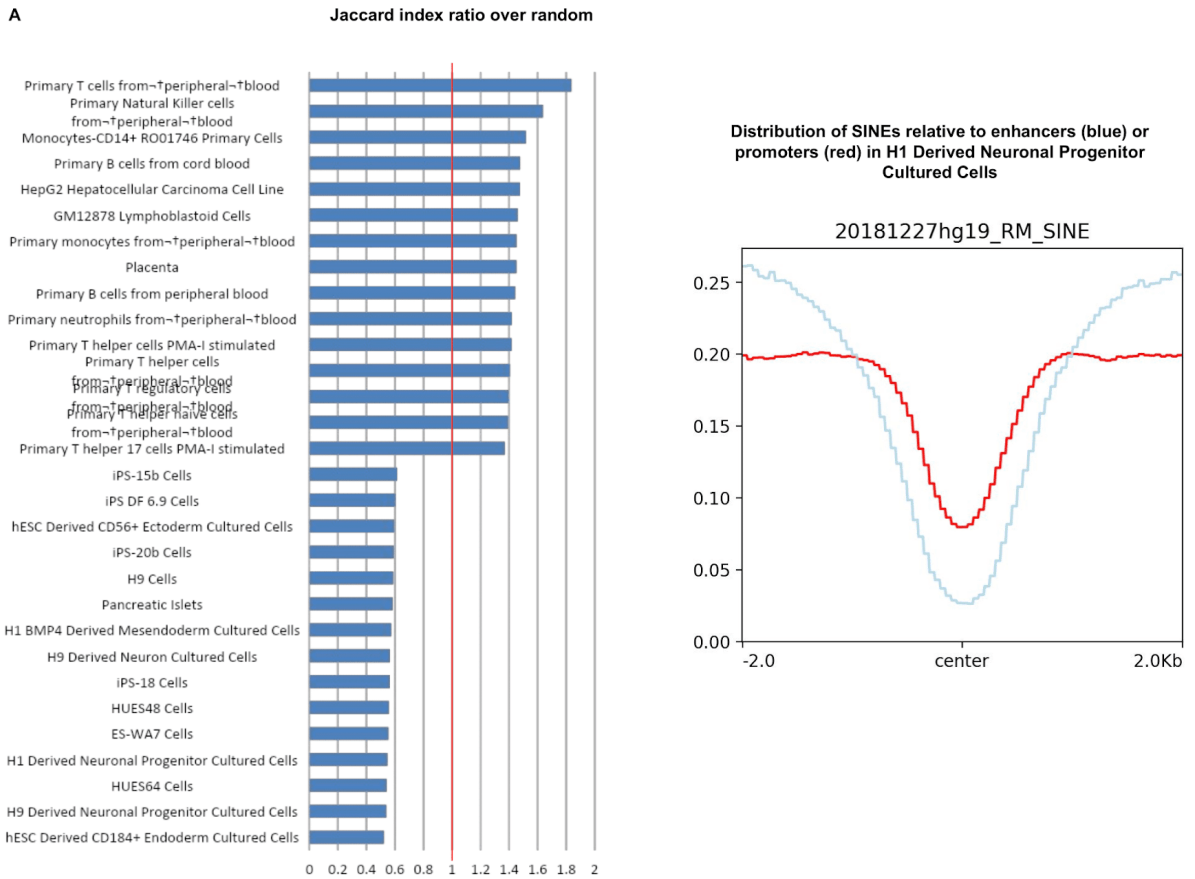


Figure 4

As the total number of PEs varies among the tissues, we also calculated the Jaccard index between the PEs from each tissue and randomly selected SINE-free regions (average of thousand iterations). The score shown for each tissue is the Jaccard index (PEs vs. SINEs) divided by the Jaccard index (PEs vs. random). The approach showed that in most tissues, the overlap between PEs and SINEs is smaller than that expected by chance (ratios are below, with the exception of a range of immune cells (T cells, B cells, Nk cells etc). To further understand the phenomenon, we focused our attention on the top and the bottom score tissues, respectively primary T cells from peripheral blood, and H1-derived neuronal progenitor cultured cells. For these, we segregated enhancers from promoters and examined separately the distribution of SINE sequences relative to these regulatory elements. This showed that in both extreme tissues, promoter corresponded to valleys of SINEs. In contrast, enhancers were peaks on SINEs in T cells while they remained valleys in the H1-derived neuronal progenitor cultured cells.

These observations were strongly suggestive of the importance of avoiding transcription of SINEs sequences during normal promoter activity. However, the data also suggested that tissues involved in cellular defense abundantly produce SINE RNAs in the form of eRNAs.

To increase the robustness of this observation, we took the reverse approach and questioned the function of genes located in the neighborhood of enhancers likely to produce SINE-encoding eRNAs. We performed an intersection

between SINEs and positions annotated as sites of transcription initiation (TSS) in at least one of the 975 CAGE libraries of the Fantom5 consortium. We then used GREAT to identify genes located less than 100Kb away from these enhancers and to perform a GO term analysis on these genes(Fig 4E). This approach clearly identified inflammation and T and B cells as benefitting from these enhancers.

Finally, we questioned whether the presence of SINE sequences and their associated A-tracts would be play a role in the increased stability of uaRNAs accumulating in cells exposed to oxidative stress from Figure 1. To explore this, we selected two sets of promoters either containing A tracts in the 2Kb upstream of the TSS or being devoid of such sequences. Interestingly, we observed a greater increase in the accumulation of auRNAs at promoters containing A tracts when the cells were exposed to hydrogen peroxide. This effect was observed with both cell lines, BJ and MRC5.

Together, these observations are in favor of a function for SINE sequences in auRNAs and possibly in eRNAs

V. T cell activation is associated with a limited accumulation of exosome RNA-targets:

Reactive oxygen species (ROS), including hydrogen peroxide, function as second messengers in T cell receptor (TCR) signaling (Yarosz et al., 2018).

Therefore, we investigated whether creeping RNAPII and reduced RNA

turnover were inherent to T cell activation. To determine that, we treated

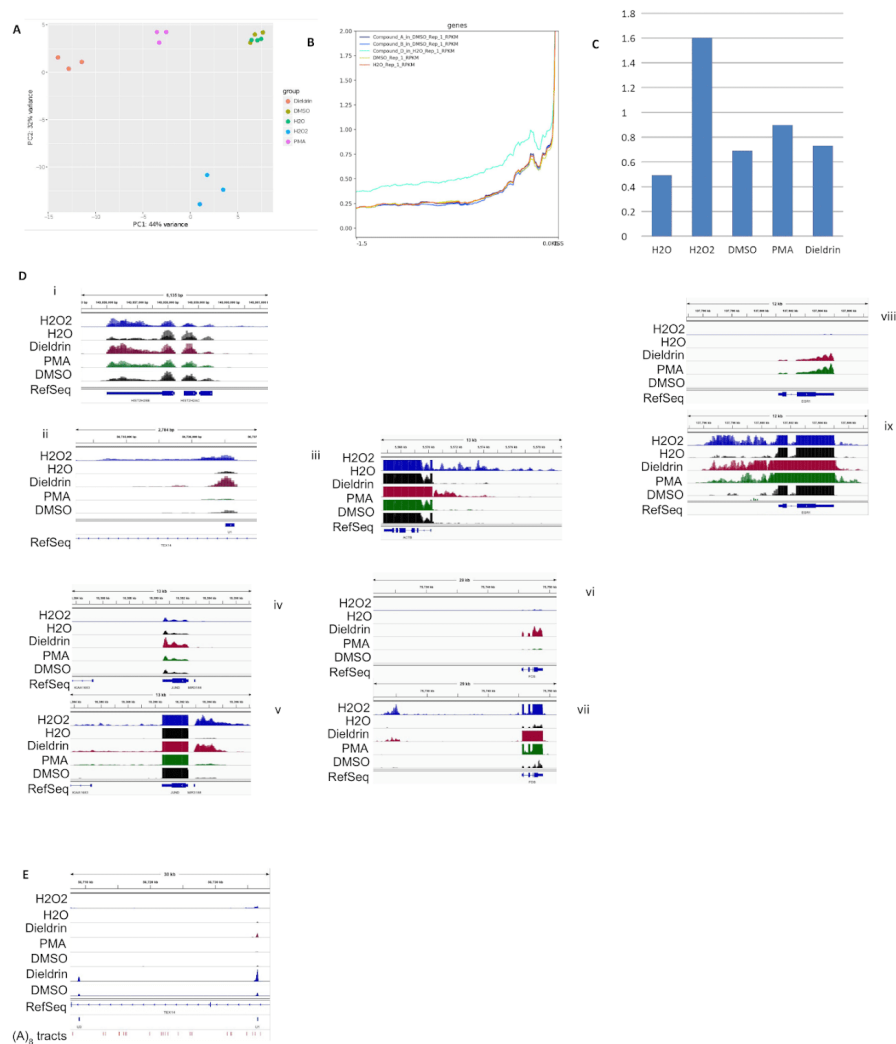
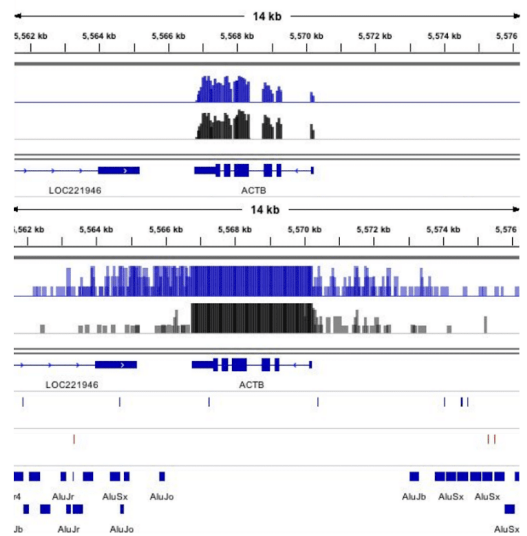
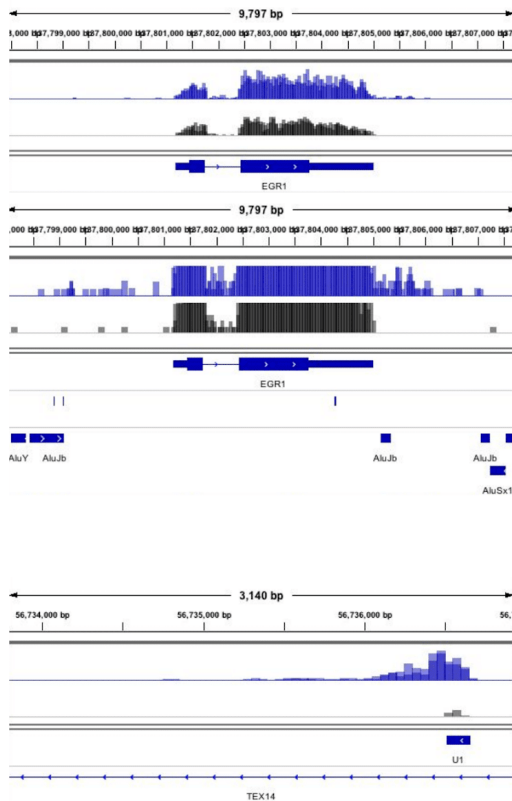


Figure 5

Figure 5: induction of the oxidative stress phenotype to Jurkat cells by the treatment by H2O2. **A.** Principal component analysis (PCA) based on gene expression between cells treated with Dieldrin, PMA, H2O2, DMSO or H2O. **B.** Profile of the upstream region of promoters for a list of 5600 genes with any other gene in the 10kb upstream of their promoters. **Dark blue:** Jurkat cells treated with Dieldrin. **Blue:** Jurkat cells treated with PMA. **Light blue:** Jurkat cells treated with H2O2. **Green:** Jurkat cells treated with DMSO. **Red:** Jurkat cells treated with H2O. **C.** Proportion of the reads per thousand that are containing a 30A tract motif between each treatment. **D i.** IGV track of the accumulation of RNAs downstream of the HIST2H2AC and HIST2H2BE histone genes in H2O2 treated cells. **D ii.** IGV track of the accumulation of RNAs downstream of the U1 snRNA which is inside the TEX14 gene in H2O2 treated cells. **D iii, iv.** IGV track of the upstream region of the gene ACTB in H2O2 treated cells. **D v,vi.** IGV track of the upstream region of the gene JUND in H2O2 treated cells. **D vii, viii.** IGV track of the accumulation of RNAs on the enhancer located upstream of the FOS gene in H2O2 treated cells. **D ix,x.** IGV track showing the increase number of RNA upstream of the EGR1 promoter in H2O2 treated cells. **E.** IGV track of the accumulation of RNAs downstream of the U1 snRNA surrounding by A tract and the absence of RNA accumulation in the case where U3 is not surrounded by A tract.

Supplementary Figures

Interferon-treated B cells



Jurkat T cells with either PMA/ dieldrin, or hydrogen peroxide. Principal component analysis (PCA) confirmed that the H₂O₂ treatment segregated away from the progressive T cell activation reach with PMA and dieldrin (**Fig 5A**). The PCA also established that the H₂O and the DMSO baselines were very similar and not overlapping with any coding regions of genes on 10kb before the gene promoters. However, we see in the H₂O₂ treated cells an increase in the accumulation of auRNAs compared to the other treatments (**Fig 5B**), This is consistent with the “creeping polymerase” phenomenon causing transcription of regions containing SINEs (and therefore genome-encoded polyA tracts). Moreover, the proportion of read containing a 30A tract is largely superior in the case of H₂O₂ treated cells in comparison to the other conditions (**Fig 5C**). This suggests, an increase of the transcription of the (SINEs/Alus). This transcription should not have happened in normal promoter activity but since cells were treated with H₂O₂, SINEs sequence seems to exhibit a gain in function maybe as eRNA. Consistent with polyadenylation participating in auRNA termination, these transcripts were not eliminated by the polyA selection and accumulated at many but not all expressed genes. Interestingly, at EGR1 there was an accumulation of these auRNAs in both H₂O₂ and in the dieldrin treated cells but it was more significant in the case of the H₂O₂ treated cells compared to the dieldrin treated cells even the if the activation of the genes is greater in these cells (**Fig 5 panels iii, viii, ix**).

The RNA-seq data also allowed us to detect enhancer-like the one upstream of the FOS gene where we can see a huge accumulation of RNAs (**Fig 5 panels vi,vii**) This type of accumulation of RNAs can also be seen in the 3' UTR regions of some histone genes (**Fig 5 panel i**). As described previously, oxidative stress induced by H₂O₂ was also able to increase the accumulation of RNAs downstream of the miR (**Fig 5 panel iv,v**). In the similar context, we also examined loci encoding UsnRNAs. As the cDNA libraries were constructed with poly(A) selection, we expected to detect only improperly matured UsnRNAs that were inappropriately polyadenylated or containing genome-encoded A-tracts. One of the U1 genes hosted by TEX14 satisfied those criteria and showed some accumulation of immature U1 in the dieldrin-activated, although lesser than what was detected in the H₂O₂- treated cells (**Fig 5D panel ii, 5E**). Altogether, these experiments suggested that the acute oxidative stress associated with T cell activation is accompanied with accumulation of some of the same RNA species observed upon exogenous oxidative stress, while appearing under tight regulation and mostly associated with genes stimulated by T cell activation.

VI. Gaining insight into the partners of HP1 γ during senescence

Among the three different HP1 proteins (α,β,γ), HP1 γ plays the most important role during the formation of senescence associated

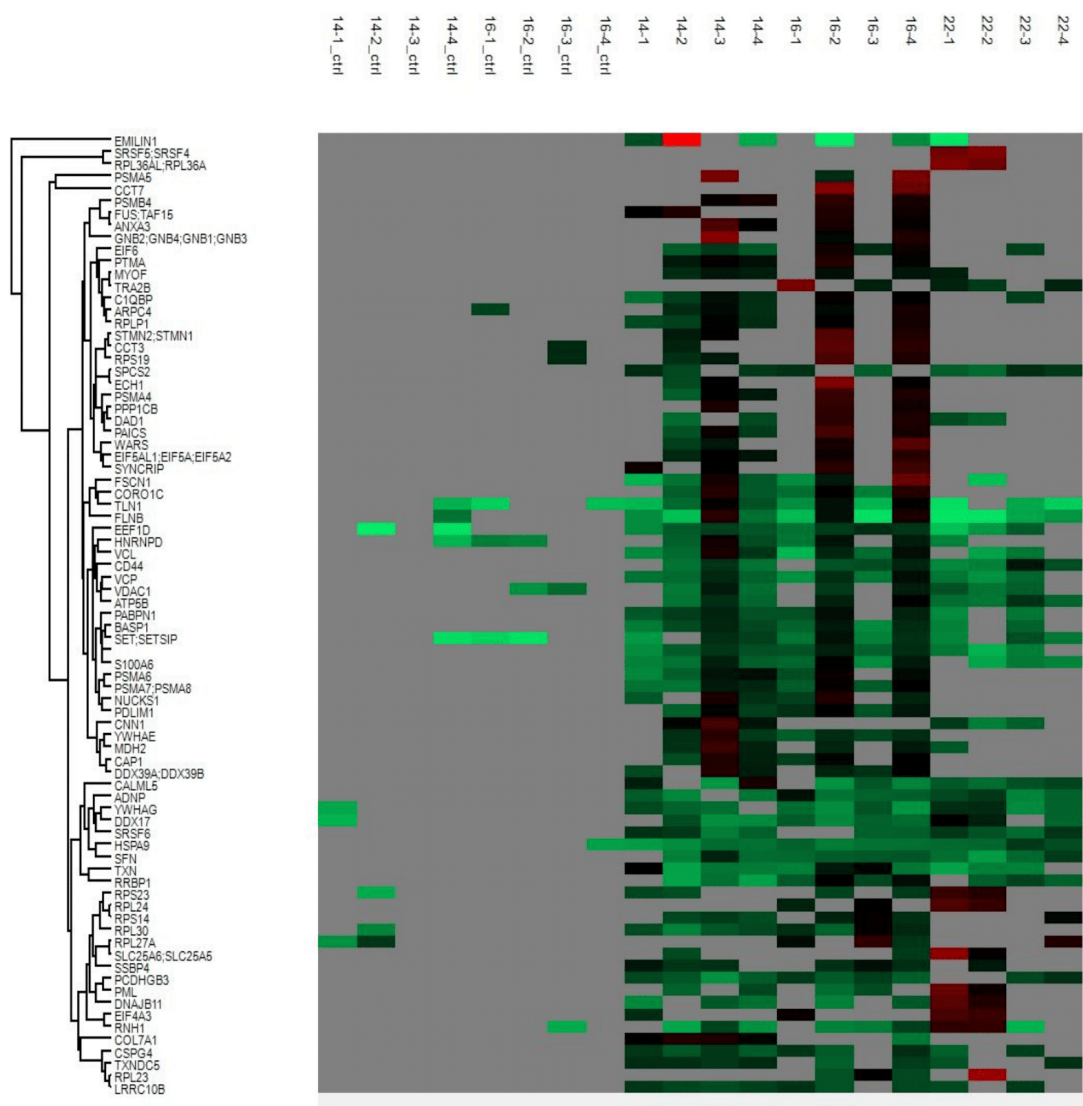


Figure 6. Heat map representing all the partners of HP1 γ expressing abundantly in senescent cells (P22) vs proliferating cells (P14 & P16)

heterochromatin foci (SAHF), by undergoing phosphorylation at serine 93 (Zhang et al., 2007). Also, recently, there have been reports showing that HP1 γ , binds to the hexameric motifs of SINE repeats and regulates their alternative splicing (Rachez et al., 2019). Hence we wanted to understand what are the other proteins that maybe interacting with HP1 γ when a cell is undergoing senescence. For which, IMR90 cells were collected at cell passages P14, P16 and P22 (n=4) in their cell cycle progression, that we annotated as early phase, proliferating and pre senescent cells based on the population kinetics that was performed in the lab. Then we co-immunoprecipitated the nuclear fractions of the whole cell protein lysates with anti HP1 γ antibody onto protein A beads, then performed mass spectrometry analysis, on them. We observed among all the commonly annotated partners of HP1 γ , there were also a few interesting candidates like PML (Promyelotic leukemia) and RNH1 (Ribonuclease/Angiotensin inhibitor 1), that were abundantly interacting with HP1 γ in the later passage of P22 compared to P14 and P16. PML has been widely reported to be colocalizing with HP1 γ in senescent cells and also suspected to be closely interacting with HP1 γ and other chromatin condensation chaperones like ASF1 & HIRA to form SAHF (Zhang et al., 2007). So far, there has been little evidence of the interaction RNH1 with HP1 γ ; hence it's quite interesting to see that it is expressed abundantly in senescing cells. However, since RNH1 is reportedly expressed all over the cell equally abundantly, when we

performed western blot analysis to confirm the interaction of RNH1 with HP1 γ in similar conditions as that of MS samples, we observed the signal to be equally present across all the selected passages (P14, P16 & P22).

In conclusion, consistent with earlier reports, PML seems to be interacting with HP1 γ in senescing cells but we could not confirm it through western blot due to the perceived inefficiency of the antibody and also that PML- HP1 γ interactions require a more sensitive approach than western blot to observe. Also, we identified interesting interaction of RNH1-HP1 γ whose function is yet to be realized.

Discussion & Perspectives

Senescent cells have been reported to accumulate with age and have been associated extensively with the phenomenon of aging and age related pathologies and this accumulation of senescent cells is suspected to be because of the impaired immune cell clearance in the tissues (Ovadya et al., 2018). But it is unclear yet as to how the senescent cells trigger the inflammatory response.

In this study, we have observed that when different cell types were subjected to oxidative stress, both the integrator complex and the exosome seem to be affected. The integrator complex depends on the NELF complex (as discussed in the introduction) that is reportedly destabilized under oxidative stress induced by H₂O₂ treatment. As we observed the accumulation of mis-spliced RNAs and increased accumulation of reads containing A tracts, we suspect that the MTR4 dependent targeting complex of the exosome is affected. This led us to ask the question whether this inactivation of RNA exosome is causality or a well-orchestrated response to oxidative stress? Oxidative stress (OS) is an imbalance between the production of reactive oxygen species and the cell's ability to detoxify them. It results in both DNA base damage as well as strand breaks. However, base damage is reportedly mostly indirect and caused by reactive oxygen species (ROS) generated, e.g. O₂⁻ (superoxide radical), OH (hydroxyl radical) and H₂O₂ (hydrogen peroxide) and has been implicated in many diseases like ADHD, cancer, myocardial infarction, atherosclerosis, heart

failure, Alzheimer's , Parkinson's, Autism, vitilligo and fragile X syndrome. (Joseph N et al., 2015, Hayat M, 2014, Valko M et al., 2007, Singh N et al., 1995, Ramond et al., 2013, James SJ et al., 2004). Within the cell, mitochondrial structures are reportedly more susceptible to oxidative stress than any other cell organelle. Oxidative stress induces mitochondrial DNA (mtDNA) mutations that form a basis for different human pathologies. It is also one of the well-documented routes to induce cellular senescence. MOF, as mentioned previously, is important for regulating mitochondrial transcription and respiration and the MOF depleted mouse cardiomyocytes show reduced expression of ExoSc3. In these cells we observed upregulation of P21, possibly as a consequence of the prolonged exposure to oxidative stress. Alongside, depletion of ExoSc3 results in extensive downregulation of mitochondrial encoded genes, especially PCK2, and again, upregulation of p21. Together, this points out that there might be a bidirectional cross talk between mitochondrial stress and reduced exosome activity, mitochondrial stress causing oxidative stress that affects RNA decay that in turn nurtures the mitochondrial stress. This feedback loop between oxidative stress and exosome dysfunction might be playing a role in the onset of cellular senescence as we consistently observed the accumulation of RNA exosome substrates in senescent cells in humans.

Accumulation of upto 10% of anti sense transcripts have been observed in senescent cells. They are considered as an indirect consequence of chromatin remodelling in these cells. Alongside this, there have been reports indicating the

involvement of lncRNAs in the formation of SASPs. (**Lazorthes, S. et al. 2015**).

In the present study, we also tried to identify a common denominator between the RNAs accumulating in cells exposed to oxidative stress. This led us to realize that the reduced RNA decay combined with the previously described phenomenon of “creeping polymerase” previously described as a consequence of oxidative stress resulted in serendipitous transcription of SINE sequences, rare at promoters, while abundant in intergenic regions. SINE sequences can function as enhancers of translations. Alternatively, as some strand asymmetry is also observed in the ExoSC3 mutant HeLa, it is possible that the genome-encoded A tracts present in SINE sequences may participate in stabilizing the product of the “creeping polymerase” in the absence of an active exosome complex. Finally, SINE transcripts are likely to trigger a defense response probably to shut down translation by promoting interferon response, because it has been reported that Ro60 bind to Alu SINE sequences and target them for editing, while edited RNAs are activators of the interferon pathway (**Hung et al., 2015**). In one go, the cell activates defense genes and stabilizes ncRNAs transcripts by providing them with genome-encoded polyA tails. At note, the A tails comes from the sense SINEs, while the Ro60 binding and the interferon activation comes from the antisense SINEs. Our original report on H₂O₂ treated JURKAT cells also provide evidence for increased accumulation of ncRNAs in the polysomes. Some genes seem to be particularly prone to have

stabilized auRNAs. Further studies will be required to determine what they have in common. There have been reports in yeast indicating that, mutations in the RNA exosome increases sensitivity of oxidative stress (**Tsanova et al., 2014**). All these observations inspire us to hypothesise that the combination of increased elongation of antisense promoter RNAs and of reduced decay of these RNAs is a carefully orchestrate response to external cues, aiming at producing RNA species that will function as adjuvants to the innate immune response.

How does that relate to senescence? We hypothesise that, since senescent cells show a similar profile of the accumulation of uaRNAs as was observed in oxidative stress, they would initiate the amplification loop described in **Figure 07**. If the loop is not interrupted, the cell may be driven into senescence.

Interestingly, it has been reported that the expansion of senescence from one cell to another and finally to the whole tissue or organ is in part carried out by autocrine/paracrine signaling of senescence associated secretory phenotype (SASPs) reportedly consisting of inflammatory cytokines, growth factors, and proteases (**Malaquin N et al.,2016**).This lead us to suggest that reduced RNA exosome activity and the alongside accumulation of SINE- containing transcripts, act paracrinely on the adjacent cells, initiating senescence by triggering an interon response and possibly mitochondrial dysfunction. This would suggest that senescence is induced not just by the known secretory phenotype consisting of proteins but also due to undegraded uaRNAs or better yet, the secretroy phenotype with cytokines, interferons etc., is a result of these

undegraded RNAs where SINEs are used to activate the inflammatory pathway **(Ref: Fig 07)**.

Further studies are required to understand whether it's a deliberate path a cell assumes to protect itself from becoming cancer prone or an effect of some other aspect that is yet to be uncovered. But, we suggest that, apart from the multiple markers of senescent cells, at a molecular level, we can also include the accumulation of RNA exosomes to be a plausible marker to define that a cell is senescent.

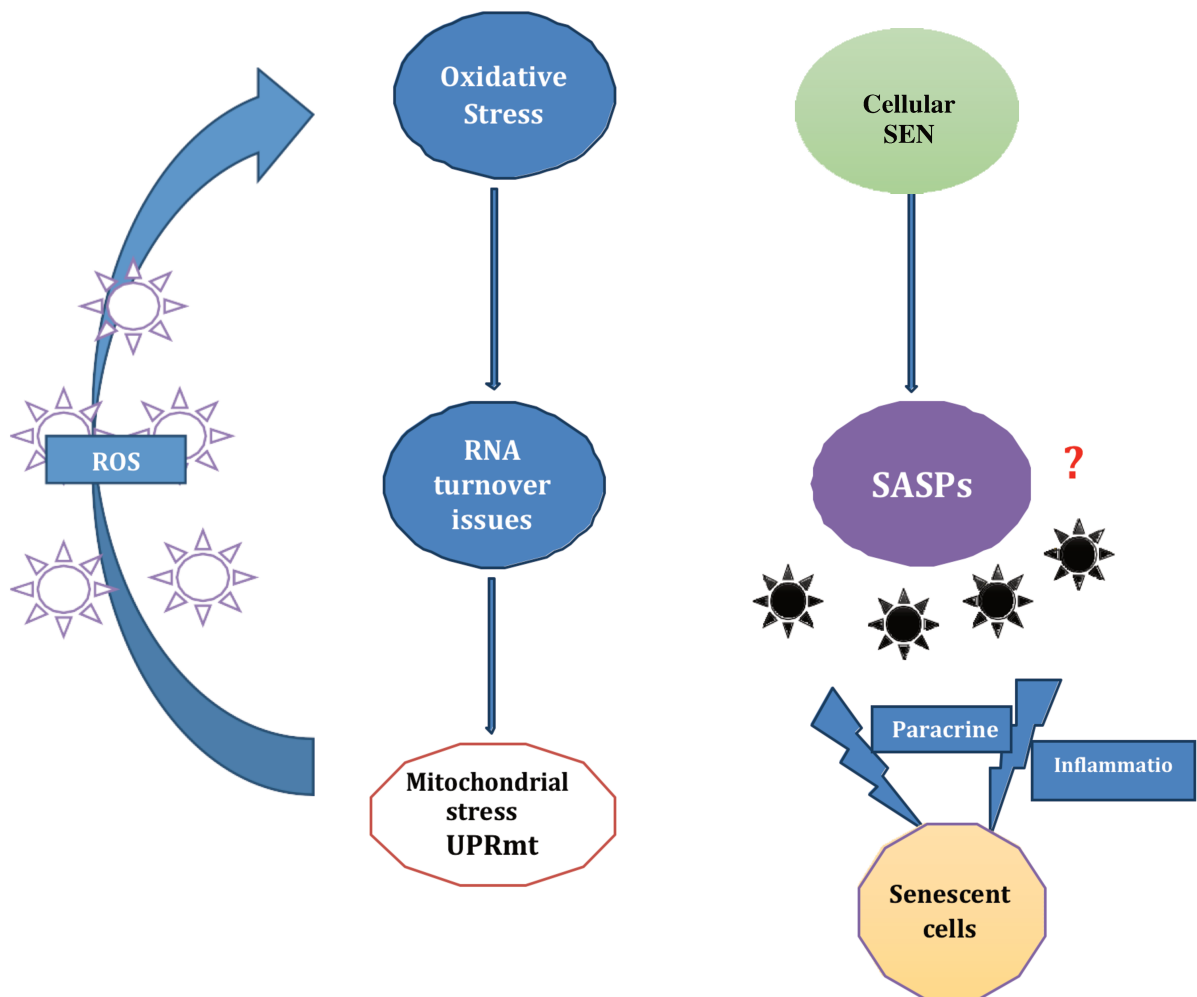


Figure 07: Schematic model for understanding the role of uaRNAs in senescent cells
 When a cell or an organism is subjected to oxidative stress, it causes impairment in the RNA degradation process. This results in the accumulation of undegraded RNAs that ultimately up regulate the mitochondrial stress response which in turn produces even more reactive oxygen species that further amplify

Alongside SASPs, Senescence Associated Heterochromatin Foci (SAHF), are specialized domains of facultative heterochromatin that are thought to push the cell into irreversible growth arrest. It involves different elements like chromatin condensation chaperones like HIRA, ASF1 and heterochromatin proteins like (HP1 α , β and γ). Of which HP1 γ is most important as it reportedly gets phosphorylated at its serine 93 and interacts with the molecular chaperones (HIRA & ASF1) and deposited onto PML (Promyelotic leukemia) to form

SAHF (**Zhang et al., 2007**). We observed from our mass spec data, that PML was indeed interacting with HP1 γ as the cells were progressing into senescent stage. Apart from that we also observed that another interesting factor RNH1 (Ribonuclease/Angiotensin inhibitor 1) was also increasingly interacting with HP1 γ in pre senescent cells. Overexpressing RNase H1 reportedly suppressed the yeast from entering early senescence (**Yu et al., 2014**). Could that be, that the pre senescent cells mildly overexpress RNH1 in order to abrogate senescence? Because in the same report, a strong correlation between TERRA (NcRNA of telomere) and telomere length in non telomerase expressing yeast cells, stating that, telomerase- associated TERRA induces type II recombination in normal yeast cells by suppressing early senescence (**Yu et al., 2014**). There is a need for further investigation to gain deeper understanding of the function and interaction of RNaseH1 with telomeres in cellular senescence and augmenting the protective role of this senescence against cancer. And if it is interacting with any other proteins like HP1 γ or PML in any manner, to perhaps enhance the process of formation of SAHF.

Materials and Methods:

Cell culture

Jurkat E6-1 cells purchased from ATCC were cultured in RPMI-1640 with 10% decompemented fetal bovine serum (FBS) and 100 U ml⁻¹ penicillin–streptomycin at 37°C in a 5% CO₂ incubator. Cells were treated with phorbol myristate acetate (PMA) or dieldrin at 100 µM for the indicated times, with wortmannin (10 mM), BX795 (6 µM), Akt1/2 kinase inhibitor (40 µM), H89 (40 µM), U0126 (12 µM), FK506 (100 nM), and PD98059 (25 µM) for 1 h and with 200 µM Cl-amidine for exactly 16 h in complete cell culture medium.

NanoLC-MS/MS protein identification and quantification

IMR90 cells were collected at different passages (p14, p16, p22) and nuclear lysates were collected by the protocol established by **Rachez et al., 2012**. Immunoprecipitation was carried out with Anti-HP1γ Antibody, clone 42s2 by Millipore (05-690) and protein G beads (Life technologies). A small fraction of beads was used to validate the maximum binding of HP1γ onto the beads, by western blot against different IP fractions like input, unbound and IP-IgG. This procedure was repeated for four replicates. After a thorough confirmation, the beads were snap frozen in 50mM ammonium bicarbonate and prepared for MS analysis as follows.

S-Trap™ micro spin column (Protifi, Hutington, USA) digestion was performed on IP eluates according to manufacturer's protocol. Briefly, 5% SDS was added to the samples.

Proteins were alkylated with the addition of iodoacetamide to a final concentration of 50mM. Aqueous phosphoric acid was added to a final concentration of 1.2%. Colloidal protein particulate was formed with the addition of 6 times the sample volume of S-Trap binding buffer (90% aqueous methanol, 100mM TEAB, pH7.1). The mixtures were put on the S-Trap micro 1.7mL columns and centrifuged at 4,000g for 30 seconds. The columns were washed five times with 150µL S-Trap binding buffer and centrifuged at 4,000g for 30 seconds with 180 degrees rotation of the columns between washes. Samples were digested with 4µg of trypsin (Promega) at 37°C overnight.

Negative controls were digested directly on beads. The beads were suspended in ammonium bicarbonate 50 mM, reduced with TCEP 100 mM and alkylated with iodoacetamide 50 mM. The proteins were digested with 1 µg of trypsin (Promega) at 37°C overnight. The peptides were recovered and the beads were washed once with ABC to retrieve remaining peptides. After elution, peptides were finally vacuum dried down. Samples were resuspended in 35 µL of 10% ACN, 0.1% TFA in HPLC-grade water. For each run, 5 µL was injected in a nanoRSLC-Q Exactive PLUS (RSLC Ultimate 3000)

(*ThermoScientific, Waltham MA, USA*). Peptides were loaded onto a μ -precolumn (Acclaim PepMap 100 C18, cartridge, 300 μm i.d. \times 5 mm, 5 μm)(*Thermo Scientific*), and were separated on a 50 cm reversed-phase liquid chromatographic column (0.075 mm ID, Acclaim PepMap 100, C18, 2 μm)(*Thermo Scientific*). Chromatography solvents were (A) 0.1% formic acid in water, and (B) 80% acetonitrile, 0.08% formic acid. Peptides were eluted from the column with the following gradient 5% to 40% B (38 minutes), 40% to 80% (1 minutes). At 39 minutes, the gradient stayed at 80% for 4 minutes and, at 43 minutes, it returned to 5% to re-equilibrate the column for 16 minutes before the next injection. One blank were run between each replicates to prevent sample carryover. Peptides eluting from the column were analyzed by data dependent MS/MS, using top-10 acquisition method. Peptides were fragmented using higher-energy collisional dissociation (HCD).

Briefly, the instrument settings were as follows: resolution was set to 70,000 for MS scans and 17,500 for the data dependent MS/MS scans in order to increase speed. The MS AGC target was set to 3.10^6 counts with maximum injection time set to 200ms, while MS/MS AGC target was set to 1.10^5 with maximum injection time set to 120ms. The MS scan range was from 400 to 2000 m/z. Dynamic exclusion was set to 30 seconds duration.

High-throughput RNA sequencing and bioinformatics

Stranded libraries of cDNA were prepared by random priming followed by either enrichment in poly(dA) with an oligo(dT) resin or depletion from ribosomal RNA. A minimum of 30-Gb sequencing was obtained for each sample with reads of 150 bases. All poly(dA)-enriched libraries were sequenced on a same flowcell.

All the RNA-seq raw fastq files were downloaded from the GEO NCBI database with the SRA toolkit (<http://ncbi.github.io/sra-tools/>). The files were retrieved from GSE55172, GSE68401, GSE72501, GSE77784, GSE81662, GSE85085 and GSE100535. The fastq files were firstly checked with fastQC (**Andrews et al, 2010**) and when needed the reads were cleaned of adapter sequences and low quality sequences with cutadapt (v2.3). (**Martin, 2011**).

The mapping were done with the STAR aligner (v2.6.0b) (**Dobin et al, 2013**) (parameters: `--out Filter Mismatch Nmax 1 --out SA Mmult Nmax 1 --out Multimapper Order Random--outFilter Multimap Nmax 30`) to the reference human genome (hg19 homo sapiens primary assembly from Ensembl) for the GSE55172, GSE68401, GSE72501, GSE81662, GSE85085 and to the reference mouse genome (mm9 *Mus musculus* primary assembly from Ensembl) for the GSE77784 and GSE100535). The files were converted to the BAM format with

samtools (v1.7) (**Li et al, 2009**) then converted to bigwig format with the bam Coverage tool (default parameters) from Deeptools (v3.1.3) (**Ramírez et al, 2016**). All observations were done using (the Integrative Genomics Viewer) IGV (**Robinson et al, 2011**). R (v3.4.3) and the package DESeq2 (v1.18.1) (**Love et al, 2014**) were used to make the differential gene expression analysis and principal component analysis (PCA). P-values from the differential gene expression test were adjusted for multiple testing according to the Benjamini and Hochberg procedure. Only genes with an adjusted p-value lower than 0.05 were considered differentially expressed.

GO term analysis were performed on these differentially expressed genes with Enrichr (**Kuleshov et al, 2016**). Reads inside upstream gene regions were quantified with feature Counts (v1.6.1) from the Subread suite (**Liao et al, 2014**). The profiles were produced with the compute Matrix reference-point (parameter: --referencePoint TSS for the observations of the regions upstream of the genes or --referencePoint TES for the observations of the regions downstream of the histones genes, snRNA and snHG genes) and plotProfile tools (parameter: --perGroup) from the Deeptools suite (v3.1.3) (**Ramírez et al, 2016**). Reads inside SINE sequences and chromatin states were quantified with featureCounts 1.6.1 from the Subread package (**Liao et al, 2014**). Identification of genes in the neighborhood of SINEs was carried out with GREAT (McLean et al, 2010).

Jaccard indexes were calculated with bedtools (v2.27.1) (Quinlan et al, 2010), while profiles were generated with deepTools (v3.0.1). Panels with chromatin states were screen captures from NIH Roadmap Epigenomics Mapping Consortium(https://egg2.wustl.edu/roadmap/web_portal/chr_state_learning.html#core_15state).

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