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Fonctions des protéines p53 et H3.3 dans la suppression des tumeurs

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'Nothing in Biology Makes Sense Except in the Light of Evolution'

Theodosius Dobzhansky

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Résumé en Français

Introduction

La protéine H3.3 est une histone variante de H3, qui diffère des histones canoniques H3.1 et H3.2 par seulement 5 ou 4 résidus aminés respectivement. Elle est codée par 2 gènes, *H3f3a* et *H3f3b*, et est essentielle chez les mammifères. Comme toutes les histones variantes, l'expression de H3.3 est indépendante de la réplication, elle peut donc être incorporée dans la chromatine à chaque étape du cycle cellulaire. Pour cette raison, H3.3 est majoritaire dans les nucléosomes des cellules post-mitotiques comme les neurones. H3.3 est impliquée dans de nombreux processus cellulaires, comme la spermatogénèse, la formation du pronucleus male après la fécondation, mais aussi dans la formation de l'hétérochromatine au niveau des rétrovirus endogènes, des télomères et des centromères où elle joue un rôle majeur dans le maintien de la stabilité et de l'intégrité du génome. H3.3 marque également les promoteurs des gènes actifs ainsi que les enhancers suggérant un rôle positif dans la régulation de l'expression des gènes. La manière exacte dont H3.3 fonctionne dans l'ensemble de ces processus reste encore inconnue (Buschbeck et Hake 2017 ; Hamiche et Shuaib 2013 ; Szenker, Ray-Gallet et Almouzni 2011:3 ; Talbert et Henikoff 2010). Notre laboratoire a montré que la protéine DAXX est une protéine chaperonne impliquée dans la déposition de H3.3 dans les régions hétérochromatiniennes, comme les péricentromères, les télomères et les éléments transposables (Drané et al. 2010).

Récemment, l'avènement du séquençage à grande échelle des génomes des cellules tumorales a révélé que l'histone H3.3 est mutée dans de nombreuses tumeurs, et notamment dans 30% des gliomes pédiatriques de haut grade (pHGG), avec jusqu'à 80 % des gliomes pontiques intrinsèques diffus (DIPG) portant la mutation K27M, et 36 % des gliomes non cérébraux portant les mutations K27M ou G34R/V (Schwartzentruber et al. 2012 ; G. Wu et al. 2012). Dans les 2 cas, des mutations somatiques hétérozygotes récurrentes du gène *H3f3a* ont été identifiées comme « motrices » du développement de ces maladies, mais les mécanismes moléculaires sous-jacents restent à établir (Vogelstein et al. 2013). Il est intriguant de constater qu'au sein d'une même tumeur, environ 30 % des mutations K27M sont associées à des mutations dans ATRX/DAXX et 60 % à des mutations du gène suppresseur de tumeur *TP53* codant pour la protéine p53 (Schwartzentruber et al. 2012). Parallèlement, les mutations G34R/V sont systématiquement associées à des mutations de p53 et ATRX/DAXX, mais aussi à des mutations de PDGFRA (Schwartzentruber et al. 2012). Bien que les tumeurs présentant des mutations de H3.3, p53 et ATRX/DAXX contiennent une plus grande variabilité du nombre de copies d'un gène (Schwartzentruber et al. 2012), une association claire entre l'augmentation des altérations du nombre de copies d'un gène et des mutations de *H3f3a* seules n'a pas été démontrée. Étant donné que les mutations de *H3f3a* sont trouvées sous forme hétérozygote et que ces gliomes présentent des taux de mutation de p53 similaires à ceux retrouvés dans d'autres types de gliomes, il est suggéré que les mutations H3.3K27M et H3.3G34R/V

pourraient agir comme des mutations « motrices », les mutations de p53 survenant en second lieu (Khuong-Quang et al. 2012; Schwartzentruber et al. 2012).

H3.3 est nécessaire à l'extinction des rétrovirus endogènes (ERVs) dans les cellules souches embryonnaires de souris (ESCs) (Elsässer et al. 2015). Des études récentes rapportent que les mutations de H3.3 K27M et K36M induisent une perturbation de la déposition des marques chromatiniennes antagonistes H3K27me3/me2 et H3K36me3/me2, qui conduisent à une dé-répression des ERV (Krug et al. 2019, Chaouch et al. 2021 ; Stekelenburg et al. 2022). En outre, plus d'un tiers des sites de liaison de p53 sont retrouvés au niveau des régions répétées situées aux extrémités (LTR) des ERVs (Wang et al. 2007), et les cellules cancéreuses déficientes en p53 sont prédisposées à surexprimer leurs ERVs. La concomitance des mutations de H3.3, p53 et ATRX/DAXX retrouvées au sein des pHGG indiquent un lien fonctionnel entre ces protéines. Nous faisons l'hypothèse que les mutations de H3.3 et de p53 affectent le paysage chromatinien et l'expression des rétrovirus endogènes. L'objectif principal de ma thèse est de disséquer les mécanismes moléculaires par lesquels H3.3 et p53 contrôlent l'expression des ERVs et d'aborder le rôle potentiel de ces derniers sur l'activité suppresseur de tumeur médiée par p53.

Résultats

Notre laboratoire ayant identifié précédemment que la chaperonne d'histone DAXX forme un complexe stable avec le facteur de remodelage de la chromatine ATRX pour cibler H3.3 au niveau des foyers hétérochromatiques (Drané et al. 2010), nous avons purifié le complexe DAXX endogène à partir de fibroblastes embryonnaires de souris (MEFs). De façon surprenante, nous avons identifié le facteur de transcription suppresseur de tumeur p53 associé à la machinerie de déposition de H3.3, ainsi que le marqueur d'hétérochromatine spécifique HP1a, et en en accord avec (Elsässer et al. 2015), l'ubiquitine ligase Trim28. Nous avons par ailleurs étudié l'effet des mutations H3.3 K27M et G34R sur l'expression du génome dans les ESCs, et montré que les mutations oncogènes de H3.3 induisent une dérégulation transcriptionelle des gènes ainsi qu'une surexpression des ERVs. Ces résultats sont en accord avec notre hypothèse de travail qui suppose un lien fonctionnel entre p53 et la machinerie de déposition de H3.3. (DAXX/ATRX/TRIM28) dans le contrôle de l'expression des ERVs. Il est probable que la machinerie de déposition de H3.3 soit responsable de la répression des ERVs en établissant une hétérochromatine dynamique, alors que le facteur de transcription pléiotropique p53 recrute des facteurs de remodelage de la chromatine favorisant l'expression des retrovirus endogènes.

Pour élucider les mécanismes moléculaires qui sous-tendent le lien fonctionnel entre p53 et le complexe répresseur H3.3 au niveau des ERVs, nous avons d'abord analysé le transcriptome des ESCs, des MEFs, du rein, du foie et du cortex en présence et en absence de p53 en utilisant un modèle murin KO pour p53. De manière intéressante, nous montrons que p53 est nécessaire à l'expression des ERVs de la famille MMERGLN-int spécifiquement (ERVs-GLN). En effet, seule cette famille est réprimée en absence de p53 à la fois dans les ESCs, les

MEFs, le rein et le foie. Dans le cortex, la famille GLN n'est pas exprimée dans la situation sauvage. En 1993, une procédure de sélection immunitaire appliquée sur une librairie d'ADN génomique de souris a permis de détecter p53 au niveau des séquences répétées situés aux extrémités (LTRs) des ERVs de la famille GLN (Zauberman et al. 1993). Nous avons confirmé par des expériences de ChIP-seq que p53 se lie spécifiquement aux LTRs des ERVs-GLN dans les ESCs et dans les MEFs. Collectivement, nos résultats montrent que p53 active spécifiquement les ERVs-GLN en se liant directement aux LTR de cette famille, et que cette fonction de p53 est maintenue depuis le développement embryonnaire précoce (dans les cellules souches embryonnaires pluripotentes et différentiées) jusqu'aux tissus adultes (dans le foie et le rein).

Les ERVs étant fortement exprimés dans les cellules souches, celles-ci constituent un excellent modèle *in vitro* pour étudier les mécanismes moléculaires à l'origine de la répression des ERVs de la famille GLN en absence de p53 au cours du développement précoce des mammifères. Avec l'objectif de caractériser les profils épigénétiques des ERVs à l'échelle du génome dans les cellules souches, nous avons d'abord réanalysé de nombreuses données épigénomiques (MeDIP-seq, ChIP-seq) déposées dans les banques publiques obtenues à partir de ESCs. Nos analyses mettent en évidence un profil épigénétique spécifique au niveau de la famille GLN caractérisé par la présence de marques actives (5hmC et H3K27ac) au niveau de leurs LTRs (qui colocalisent avec p53), ainsi qu'un enrichissement en marques répressives (5mC et H3K9me3) le long de la partie centrale de la séquence rétrovirale. Pour confirmer ces résultats et mesurer les conséquences d'une perte de p53 sur le paysage chromatinien des ERVs-GLN, nous avons ensuite analysé la distribution des 5mC et 5hmC par DIP-seq, ainsi que des marques H3K9me3 et H3K27ac par Cut&Tag dans des ESCs WT et KO pour p53. Nos données mettent en évidence un défaut de déposition de H3K27ac au niveau des LTRs de la famille GLN ainsi qu'une baisse globale de la quantité de H3K9me3 le long des séquences codantes de la séquence rétrovirale. L'expression des ERVs étant régulée par H3.3 dans les ESCs, nous postulons que ce variant est la principale cible des modifications post-traductionnelles de la famille H3 retrouvées au niveau des ERVs. Ainsi, la perte concomitante de facteurs épigénétiques activateurs (H3K27ac) et répresseurs (H3K9me3) au niveau des ERVs-GLN en absence de p53 pourrait être est la conséquence d'un défaut de déposition de H3.3 spécifiquement sur cette famille. Pour confirmer cette hypothèse, la distribution génomique de H3.3 et TRIM28 par ChIP-seq doit être analysée dans les ESCs WT et KO pour p53.

Dans leur très grande majorité, les rétrovirus endogènes, qui ne sont pas soumis à une pression de sélection, ont été progressivement rendus inactifs par simple accumulation de mutations ou de délétions au cours de l'évolution. De ce fait, parmi l'ensemble des copies d'une famille d'ERV, seuls quelques éléments ont conservé leur capacité à générer des particules virales infectieuses (Ribet et al. 2008). Certains membres de le famille ERVs-GLN sont des rétrovirus endogènes de classe I qui sont restés infectieux. En outre, bien que l'infection par un rétrovirus ait généralement un effet délétère sur l'individu, la découverte de gènes de rétrovirus intégrés au génome depuis des millions d'années, et remplissant des fonctions

physiologiques essentielles, révèle que ces éléments peuvent également jouer un rôle fondamental dans la biologie de leur hôte, comme par exemple les glycoprotéines de l'enveloppe (Env) codées par les rétrovirus endogènes. Un exemple particulièrement remarquable est celui de la syncitine, qui est capable d'induire la fusion cellulaire et est nécessaire à la formation de l'architecture placentaire des mammifères. Nous sommes demandés si les protéines de l'enveloppe codées par la famille GLN (Env-GLN) pouvaient jouer un rôle important chez la souris, et en particulier si cette fonction pouvait être impliqué dans l'activité suppresseur de tumeur médié par p53.

Nous avons d'abord examiné de plus près les différents isoformes de Env-GLN exprimées dans les MEF primaires. Bien que la plupart des membres de la famille ERVs-GLN exprimés dans les MEFs code pour une protéine Env-GLN pleine taille (Env WT), il est important de noter que le membre de la famille GLN le plus exprimé code pour une protéine de l'enveloppe délétée au niveau de son extrémité N-terminale (Env Δ1-320). Nous avons montré que p53 est essentiel à l'expression de l'ensemble des membres de la famille ERVs-GLN exprimés dans les MEFs, ceux codant à la fois pour Env WT et Env Δ1-320. Afin d'aborder le rôle potentiel des ERVs-GLN sur l'activité suppresseur de tumeur médiée par p53, nous avons induits des tumeurs par allogreffe chez la souris après injection sous-cutanée de MEFs KO pour p53 qui n'expriment plus les ERVs-GLN. Après une première expérience pilote qui a confirmé l'activité tumorigène des MEFs p53 KO lorsqu'elles sont injectées dans des souris sauvages, nous avons suivi pendant plusieurs semaines la croissance des tumeurs induites par la lignée tumorigène dans laquelle a été restaurée ou pas l'expression de la protéine Env WT ou Δ1-320. Nos expériences *in vivo* montrent que la restauration de l'expression de la protéine Env pleine taille ralentit la progression de la tumeur induite par les MEFs KO pour p53, alors que la restauration de l'expression par le mutant de délétion Δ1-320 accélère de manière drastique la croissance tumorale. Nous concluons que la protéine Env WT codée par les ERVs-GLN contribue par sa fonction à l'activité suppresseur de tumeur de p53.

Pour mieux comprendre comment la protéine Env-GLN peut exercer une telle fonction, nous avons purifié par double-immunoaffinité les complexes protéiques associées aux deux isoformes Env WT et Δ1-320. L'analyse par spectrométrie de masse a identifié de nombreuses protéines de la famille SLC, des transporteurs transmembranaires responsables principalement de l'influx et secondairement de l'efflux d'une très grande variété de substrats comme les ions, les acides aminés, les métaux, les sucres, les nucléotides, les vitamines, les hormones ou les neurotransmetteurs. Ces résultats suggèrent que les transporteurs SLC peuvent être des récepteurs potentiels des protéines Env GLN. Nous suggérons que l'interaction Env-SLC favorise le système immunitaire à distinguer les cellules tumorigènes des cellules saines par un mécanisme qui reste à découvrir.

Conclusion

Les séquences d'ADN répétées représentent près de la moitié du génome humain. Notre étude souligne l'importance d'analyser ces séquences dans les études fonctionnelles à

grande échelle, malgré les problèmes d'alignements innérents à leur nature répétitive et la plus faible couverture de ces régions dans les données génomiques. Parmi elles, les rétrovirus endogènes contiennent de nombreux sites de liaison de facteurs de transcription suggérant un rôle critique dans l'intégrité et la fonctionnalité du génome. Notre étude élucide le mécanisme moléculaire qui sous-tend le lien fonctionnel entre p53 et H3.3 dans la régulation des ERVs. Nous montrons que p53 se lie directement aux LTRs des ERVs-GLN, induit une modification du paysage chromatinien le long de la séquence rétrovirale qui favorise leur expression. Nos données suggèrent que p53 est responsable du recrutement de H3.3 au niveau des ERVs-GLN, et que, par conséquent, la régulation épigénétique de ces séquences médiée par les modifications post-traductionnelles de H3.3 (K27ac et K9me3) est perdue en absence de p53. De plus, cette étude démontre que le mécanisme de régulation des ERVs-GLN par p53 est préservé tout au long du développement murin, des cellules embryonnaires jusqu'aux tissus adultes. Le résultat le plus surprenant de ce travail est la mise en évidence d'un ralentissement du développement tumoral, induit par injection d'une lignée tumorigène KO pour p53 chez la souris, lorsque l'expression de Env-GLN est restaurée dans ces cellules. Ces résultats suggèrent que la fonction suppresseur de tumeur de p53 est médiée en partie par la glycoprotéine de l'enveloppe codée par les ERVs-GLN. Ce travail apporte une nouvelle preuve que les protéines rétrovirales endogènes n'ont pas que des rôles pathologiques, mais qu'elles peuvent être « domestiquées » et acquérir une fonction physiologique bénéfique pour l'hôte. L'identification des transporteurs SLC dans les complexes protéiques associées à Env-GLN, indique que les protéines de la famille SLC peuvent agir comme des récepteurs potentiels des glycoprotéines de l'enveloppe codée par les ERVs. Dans le futur, il sera important d'élucider si l'activité suppresseur de tumeur des protéines de l'enveloppe est médiée par les protéines SLC, et si elle mobilise le système immunitaire. Le cas échéant, adresser les mécanismes moléculaires par lesquels une interaction Env-SLC peut favoriser le système immunitaire à distinguer les cellules tumorigènes des cellules saines, et étudier si une telle fonction est retrouvée chez l'homme, seront des objectifs essentiels qui permettront de mieux comprendre les mécanismes de tumorigénèse.

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5caC: 5-carboxylcytosine, 22
5fC: 5-formylcytosine, 22
5hmC: 5-hydroxymethylcytosine, 22
5mC: 5-methylcytosine, 22
ATRX: Alpha-Thalassemia/mental Retardation syndrome protein, 31
CTCs: Circulating Tumor Cells, 102
DAXX: Death domain-associated protein, 31
DBD: DNA-Dinding Domain, 39
EMT: Epithelial Mesenchymal Transition, 49
ERVs: endogenous retrovirus elements, 33
ESCs: Embryonic Stem Cells, 24
HDAC: Histone Deacetylase, 26
HP1: heterochromatin protein 1, 56
iPSC: pluripotent stem cells, 24
KAP1: KRAB-associated protein 1, 56
KRAB-ZFPs: Krüpell-associated box-containing zinc finger proteins, 56
LTRs: Long Terminal Repeats, 33
MEFs: Mouse Embryonic Fibroblasts, 32
PBS: primer binding site, 60
pHGG: pediatric High-Grade Glioma, 34
PRC2: Polycomb-repressive complex 2, 32
PTMs: post-translational modifications, 20
RE: Response Elements, 39
RT: reverse transcriptase, 60
TAAs: Tumor-Associated Antigens, 68
TADs: Topological Associating Domains, 17
TDG: thymine DNA glycosylase, 22
TE: Transposable elements, 53

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Introduction

I. Introduction

A. Chapter 1: Chromatin organization and epigenetic

1. Overview of chromatin organization

DNA is the molecule that contains the genetic information. In eukaryotes, 2 m of genomic DNA are compacted in an organized manner in the nucleus, with a diameter of 10 µm. There are several levels of chromatin compaction. The first level of compaction is achieved by wrapping the negatively charged DNA around the basic core histone octamer, the nucleosome, which consists of the histones H2A, H2B, H3, and H4 (Maeshima, Hihara, and Eltsov 2010). This first level of compaction, known as beads on string, is a nucleofilament of 11 nm and was imaged for the first time in 1973 by Olins & Woodcock (Olins and Olins 1974, 2003; Oudet, Gross-Bellard, and Chambon 1975). The 11 nm filament is then compacted into a 30 nm fiber through interaction with the linker histone, thus constituting the second level of chromatin organization. Following the longstanding compaction model, the 30 nm fibers would further fold into 120 nm chromonema, 300 to 700 nm chromatids, and finally mitotic chromosomes (Figure 1A). Nevertheless, the compaction model is only based on *in vitro* observations, and no *in vivo* experiment has validated it over the 30 nm fibers. Recent studies using cutting-edge microscopy technics (Maslova and Krasikova 2021; Ou et al. 2017) have demonstrated that there is much less regularity in chromatin folding within the cell nucleus and propose that topological associating domains (TADs) (Figure 1B) are the fundamental units of the three-dimensional (3D) nuclear organization. More specifically, Hi-C analyses have revealed two distinct multi-TAD compartments, A and B. Compartment A is enriched in actively transcribed genes, open chromatin and activating epigenetic marks, whereas compartment B correlates with heterochromatic marks (Jerkovic and Cavalli 2021; Mohanta, Mishra, and Al-Harrasi 2021; Razin and Ulianov 2017; Woodcock and Ghosh 2010). The dynamic packaging of the genome is regulated by many effectors, such as histone chaperones, covalent DNA or histones modifications, ATP-dependent chromatin remodeling factors, histone variants, and many other factors, which control genome activity during processes such as replication, DNA-repair, or transcription, and define cell identity in complex organisms (Dong et al. 2020).

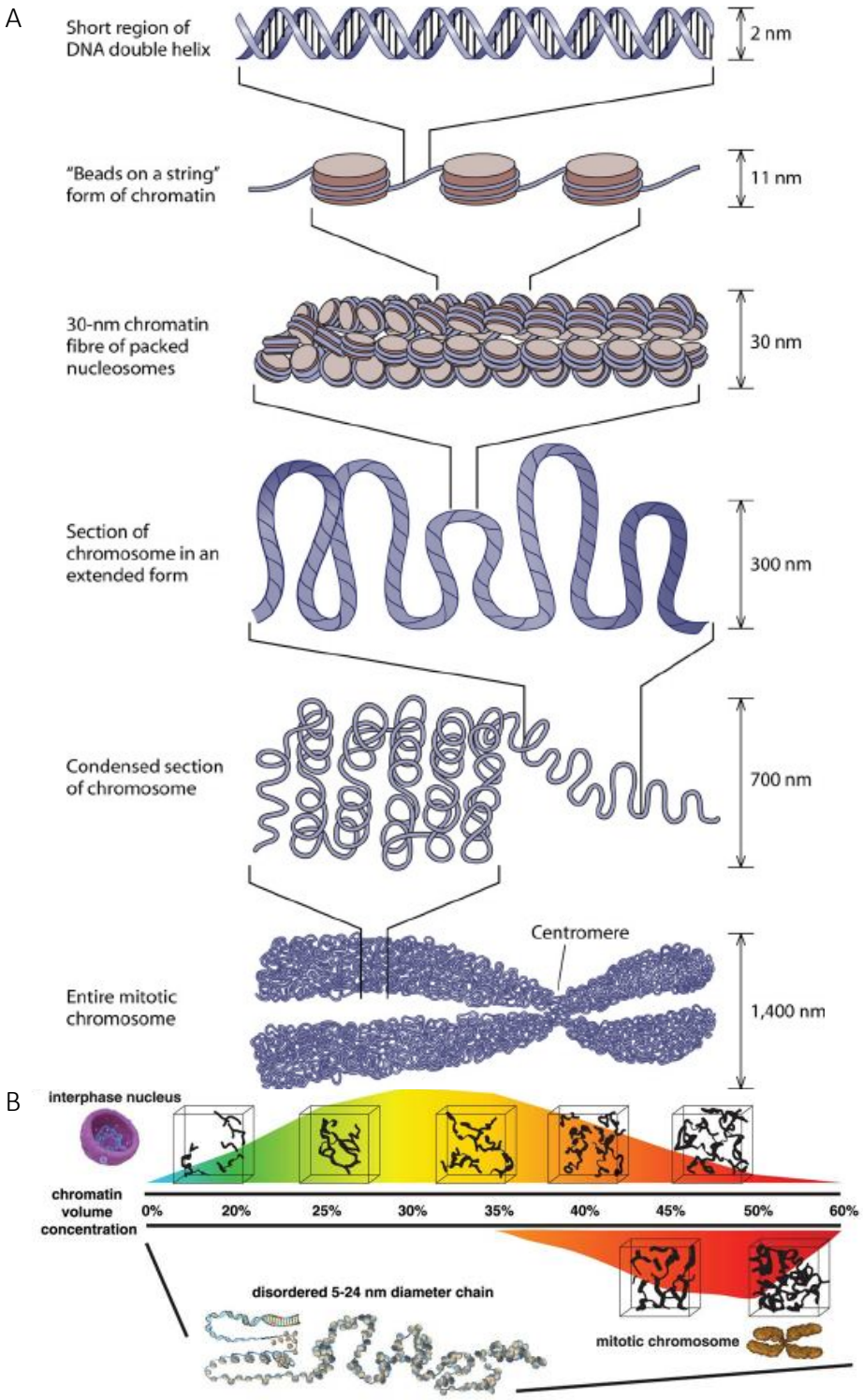
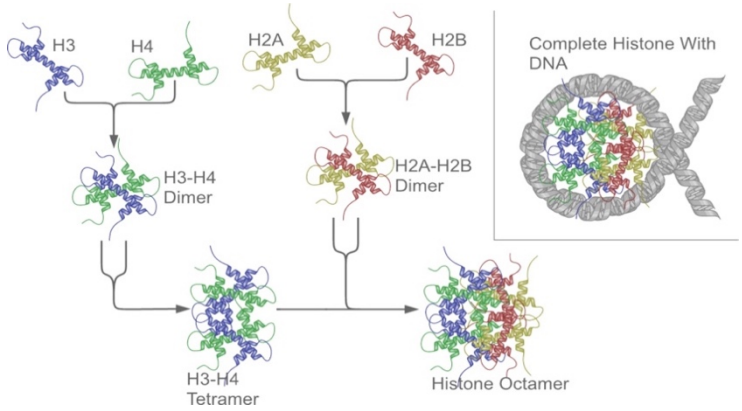


Figure 1: Models of chromatin higher levels of organization, adapted by (Felsenfeld and Groudine 2003; Ou et al. 2017). (A) Hierarchical chromatin-folding model. (B) Higher-disorder 3D chromatin packing. Chromatin is a flexible disordered 5- to 24-nm-diameter granular chain that is packed together at different 3D volume concentration density distributions in interphase nuclei and mitotic chromosomes.

2. Nucleosome

The nucleosome is the basic fundamental unit of chromatin. The nucleosome consists of a core histone octamer wrapped around 146 base pairs of DNA in about 1.7 turns. The histone octamer comprises two copies of the four core histones H2A, H2B, H3, and H4. More precisely, the nucleosome is assembled by two heterodimers H2A-H2B and a tetramer H3-H4 (Figure 2A) with the help of histone chaperones via a stepwise process (Das, Tyler, and Churchill 2010) and finally forms a cylinder with a diameter of 11 nm and 5.5 nm in height (Figure 2B) (Luger et al. 1997). The nucleosome core is highly conserved between species; however, the length of the linker DNA separating two nucleosomes varies between species and cell types. Thus, the total length of the nucleosome DNA can vary from 160 to 241 base pairs depending on the species.

A



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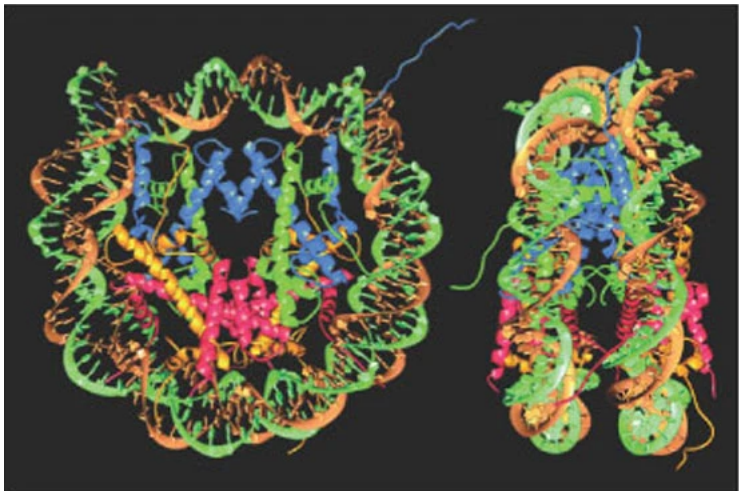


Figure 2: Nucleosome structure. (A) Schematic representation of the assembly of the core histones into the nucleosome (adapted by Richard Wheeler (Zephyris – Wikipedia). Histone H2A (yellow) dimerize with histone H2B (red) to form the H2A/H2B dimer. Histone H3 (purple) dimerize with histone H4 (green) to form the (H3-H4)2 tetramer which then assemble into an octamer by incorporating the two H2A-H2B dimers. The histone octamer wraps 146 bp of DNA to form the nucleosome. (B) Crystallographic structure of the core nucleosome at 2.8 Å (adapted by Luger et al. 1997). Eight histone proteins (blue: H3; green: H4; yellow: H2A; red: H2B) and 146 bp DNA (brown and turquoise). View down the DNA superhelix axis, on the left, and perpendicular to it, on the right.

3. Histones

There are five families of histones that can be grouped into two categories: the core/canonical histone families (H2A, H2B, H3, and H4) and the linker histone family (H1). The histone sequences are rich in positively charged lysine and arginine residues allowing DNA packaging and recruitment of transcriptional or chromatin remodeling machineries.
Canonical histones H2A, H2B, H3, and H4 are small proteins with molecular weights between 11 and 20 kDa. Each canonical histone family has similar structural characteristics that are conserved during evolution, despite the low sequence conservation. Histone fold domain proteins (HFDs) form "handshake" arrangements to give rise to the heterodimer H2A-H2B and the heterodimer H3-H4 (Arents et al. 1991; Khorasanizadeh 2004). The histone N- and C-terminal tails are disordered and float outside the nucleosome. Thus, they are highly accessible, and their amino acid residues are targets of post-translational modifications (PTMs), essential for many critical biological processes (discussed below).
The genes encoding the canonical histones are organized into a cluster containing several copies of histone genes with special mRNAs that lack introns and polyadenylated (polyA) tails, but instead have a 3′ stem-loop structure which allows the regulation of their transcription, translation and degradation (Sullivan, Steiniger, and Marzluff 2009). Thus, a high level of expression is ensured in S phase, when a large quantity of histones is required for replication (Duc and Thiriet 2021; Ejlassi-Lassallette and Thiriet 2012).

H1 'linker' histone interacts with the DNA that links two adjacent nucleosomes and further stabilizes the nucleosome structure. Unlike core histones, linker histones have a lower conservation rate between species. H1 has three structural domains: a short N-terminal domain (NTD), a central globular domain (GD), and a long C-terminal domain (CTD). The unstructured N- and C-terminal domains have high variability in sequence and length, a positive net charge due to the abundance of lysine residues (Bradbury et al. 1975) and are targets of post-translational modifications (Andrés et al. 2020). The globular domain contains a winged-helix motif and is responsible for the binding of H1 to nucleosomal DNA (Bednar et al. 2017), while the CTD is considered as the main determinant of H1-driven chromatin compaction (Th'ng et al. 2005) (Figure 3). Thus, H1 linker histone has important biological functions apart from structural component of chromatin such as a dynamic modulator of the transcription (Vicent, Wright, and Beato 2016) or silencer of repetitive elements with H3K9 methylation (Healton et al. 2020).

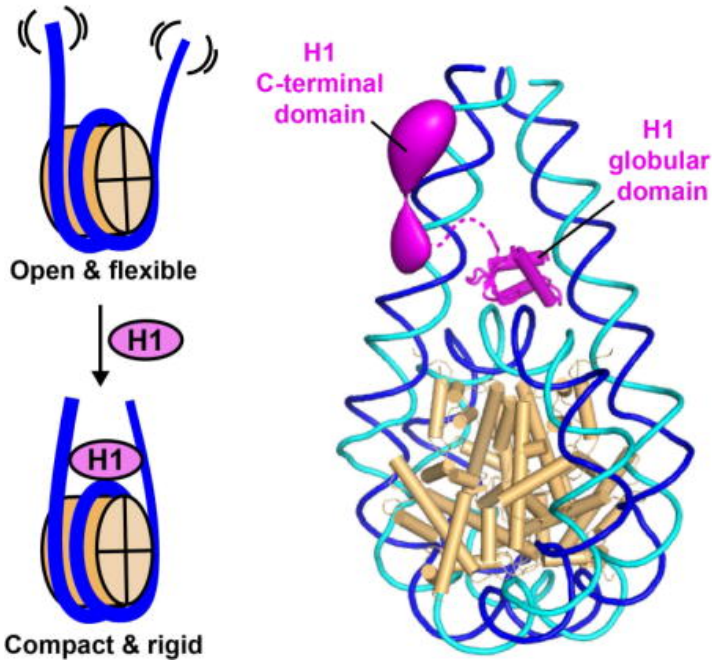


Figure 3: Nucleosome structure bound to linker histone H1. Schematic structural representation of H1 binding induces the nucleosome to adopt a more compact and rigid conformation from (Bednar et al. 2017).

4. Chromatin modifications: Compaction and remodeling

The dynamic packaging of the genome (Figure 1) is regulated by several actors, such as covalent modifications of DNA and histones, ATP-dependent chromatin remodeling complexes, the replacement of canonical histones by histone variants, which will be discussed below in detail. Chromatin remodeling is performed by specific chromatin remodeling protein complexes. Histones and DNA chemical modifications are deposited by specific proteins commonly named 'writers', and removed by their 'erasers' counterparts. The accessible regions and chromatin modifications are further recognized by the 'readers' (Figure 4) (Biswas and Rao 2018; Cao and Yan 2020).

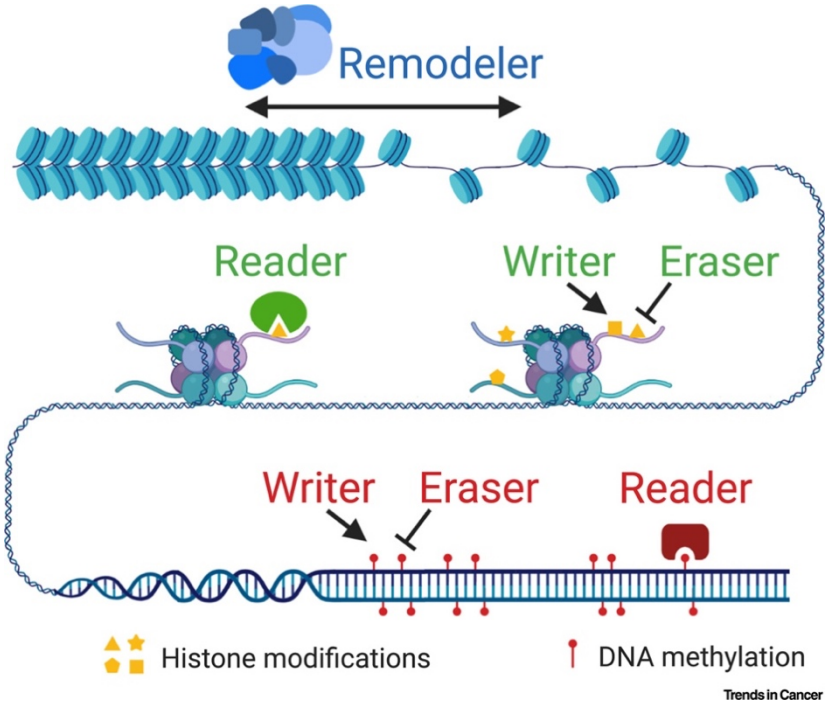


Figure 4: Molecular mechanisms of chromatin-based packaging and their regulators by (Cao and Yan 2020). Genomic DNA is wrapped around histone octamers to form nucleosomes, which are further packaged into the human nucleus in a highly organized manner. The packaging states of chromatin are dynamically regulated by chromatin-remodeling complexes ('remodelers') to allow or deny access of selected *cis*-elements by their *trans*-factors. DNA can be methylated and histones can be modified at multiple residues through covalent bonds by methylation, acetylation, phosphorylation, ubiquitination, and many other modifications. Histone post-translational modifications and DNA methylation are added or removed by specific enzymes ('writers' and 'erasers', respectively) and recognized by their binding proteins ('readers').

The level of chromatin packaging has a direct influence on genome accessibility and thus expression. Two distinct chromatin states exist, euchromatin, also called 'open' chromatin ; and heterochromatin, also called 'closed' chromatin. Euchromatin is associated with active transcription and is marked with active post-translational modifications (PTMs) as H3K9ac, H3K14ac, H3K4me3 and H3K79me3 (Morrison and Thakur 2021). Heterochromatin is further subdivided into constitutive and facultative heterochromatin. Constitutive heterochromatin is composed of the strongly silenced and packaged chromatin, rich in DNA repetitive elements, and located at telomeres, centromeres and peri-centromeric regions (Figure 27) (Bouzinba-Segard, Guais, and Francastel 2006; Saksouk, Simboeck, and Déjardin 2015). The differential compaction state of chromatin can be obtained thanks to the coordination of several actors

such as histone H1, architectural proteins and RNA (e.g. HP1, pericentric RNA), PTMs (e.g. H3K9me3, H3K27me3), and ATP-dependent chromatin remodeling complexes (Grewal and Jia 2007; Nishibuchi and Nakayama 2014).

In conclusion, the level of chromatin packaging has fundamental roles in chromatin structure and is linked to crucial biological processes such as gene expression, cell cycle regulation, DNA repair and chromosome condensation. Defects in the pathways related to chromatin packaging have been associated with the occurrence and progression of various diseases, such as cancer, heart failure, autoimmune diseases, and neurodegenerative disorders (Cao and Yan 2020; Ramazi, Allahverdi, and Zahiri 2020).

a) DNA Methylation

DNA methylation is the addition of a methyl group from the S-adenosyl methionine cofactor (SAM) to the 5th carbon of cytosine residues to form 5-methylcytosine (5mC) in eukaryotes. On the contrary, in prokaryotes, DNA methylation happens on various bases, giving rise to 5-methylcytosine, N6-methyladenine, and N4-methylcytosine, contributing to the host restriction system and protecting the prokaryote cells from foreign genetic material, such as viral DNA and destruction by proper restriction enzymes (Anton and Roberts 2021; Mohapatra and Biondi 2017).

In mammals, the cytosine bases at position 5 in CpG dinucleotides are modified genome-wide by dedicated DNA methyl-transferases (DNMT) to produce 5mC, which can be further oxidized by the Ten eleven translocation (TET1, 2, and 3) enzymes (He et al. 2011; Ito et al. 2010, 2011; Tahiliani et al. 2009) to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). Moreover, 5fC and 5caC can be excised to regenerate unmodified cytosines by the action of thymine DNA glycosylase (TDG) together with the base excision repair (BER) enzymes (Cortellino et al. 2011; He et al. 2011; Maiti and Drohat 2011) (Figure 5).

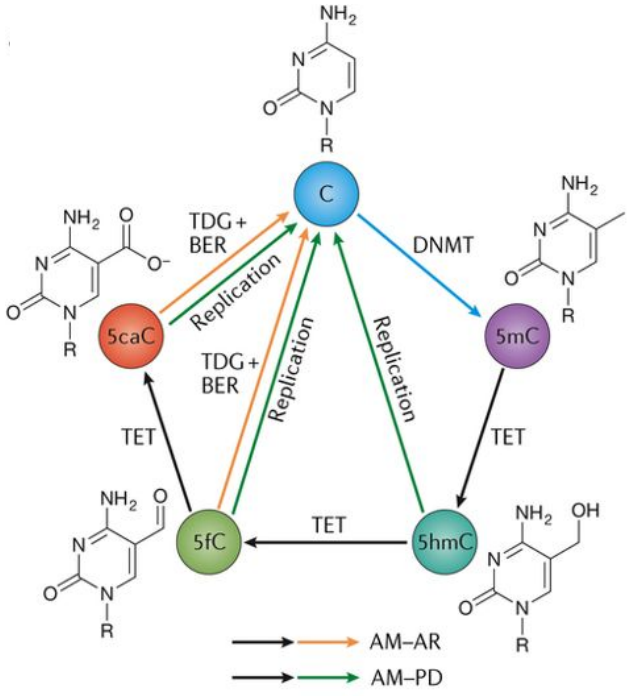


Figure 5: The cycle of active DNA demethylation (Wu and Zhang 2017). DNA methyltransferases convert unmodified cytosine to 5mC (5-methylcytosine). 5mC can further be oxidized by TET proteins to 5hmC (5-hydroxymethylcytosine), 5fC (5-formylcytosine) and 5caC (5-carboxylcytosine). 5fC or 5caC are further excised by thymidine DNA glycosylase (TDG) coupled with base excision repair (BER). AM–AR, active modification–active removal; AM–PD, active modification–passive dilution.

In mammalian genomes, CpG dinucleotides are the primary site for DNA methylation. The distribution of methylated CpGs across the genome is divided among SINEs (Short INterspersed Elements), LINEs (Long INterspersed Elements), LTRs (Long Terminal Repeats), and coding regions of functional genes (Edwards et al. 2017; Jones 2012) (Figure 6). At the same time,

unmethylated CpGs are often found at dense clusters of CpG dinucleotides, CpG islands (CGIs), encompass the promoter regions of >70% of the genes (Deaton and Bird 2011; Illingworth et al. 2010; Papin et al. 2021; Suzuki and Bird 2008). Thus, DNA methylation is classically associated with inactive transcription. However, the link between DNA methylation and transcription is far more complicated. Furthermore, DNA methylation plays a critical role in several biological processes apart from transcription, such as transposon silencing, X chromosome inactivation, imprinting, cell differentiation, and genome integrity (Meng et al. 2015).

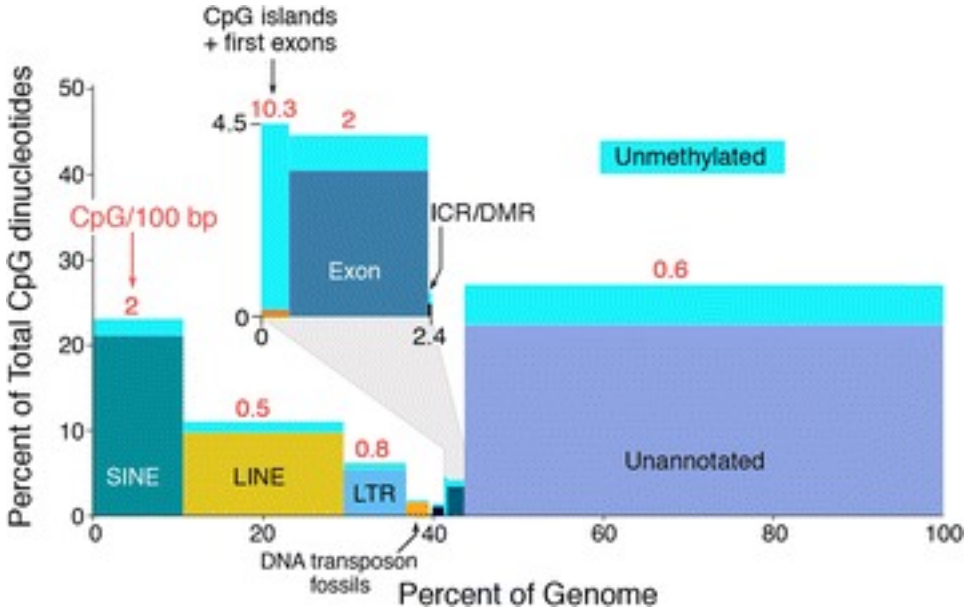


Figure 6: Distribution of DNA methylation across sequence compartments in the human genome (Edwards et al. 2017). The vertical *axis* indicates the percentage of total CpG dinucleotides in each indicated compartment; the horizontal *axis* indicates the percentage of the total genome in each compartment; *light blue* at the *top* of each compartment indicates the unmethylated fraction. Numerals in *red* denote CpG dinucleotides per 100 bp. The genome-wide CpG density expected on the basis of G + C content is 4.2 per 100 bp. Note that the only sequence compartment that exists in the largely unmethylated state is the CpG island/first exon compartment; this compartment occupies <0.5% of the genome. The ICR/DMR compartment (differentially methylated regions of imprinting control regions) represents ~0.001% of the genome and ~0.01% of total CpG dinucleotides. Introns are included in the unannotated compartment, as are putative enhancers.

DNA methylation patterns are dynamic during mammalian development in a time and tissue-specific manner. More specifically, the genome undergoes two waves of global demethylation and re-methylation in the life cycle. The first wave occurs in the germline, initiated with the erasure of global methylation in primordial germ cells (PGCs) and completed with a *de novo* methylation profile, specific to the maternal and paternal genome. The second wave occurs after fertilization, including the erasure of most methylation marks inherited from the gametes and the subsequent establishment of the embryonic methylation pattern. The DNA methyltransferases, Dnmt3a and Dnmt3b, are responsible for *de novo* methylation during early development and mammal gametogenesis (Okano et al. 1999; Tomizawa et al. 2011; Xie et al. 2012; Zeng and Chen 2019)(Figure 7). In somatic cells, DNA methylation is then transmitted after each cycle of DNA replication by the DNMT1 enzyme, allowing the methylation profile to be maintained during differentiation (Laurent et al. 2010). Nevertheless, non-CpG methylation is observed in several mammalian cells and tissues, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSC), and the brain (Lister et al. 2013), while changes in the methylation profile are linked to carcenogenesis and aging (Mangelinck and Mann 2021;

Nishiyama and Nakanishi 2021). Genome-wide hypomethylation of retroelements, as well as hypermethylation of CpG islands associated with tumor suppressor genes and developmental regulators, are characteristics of cancer cells.

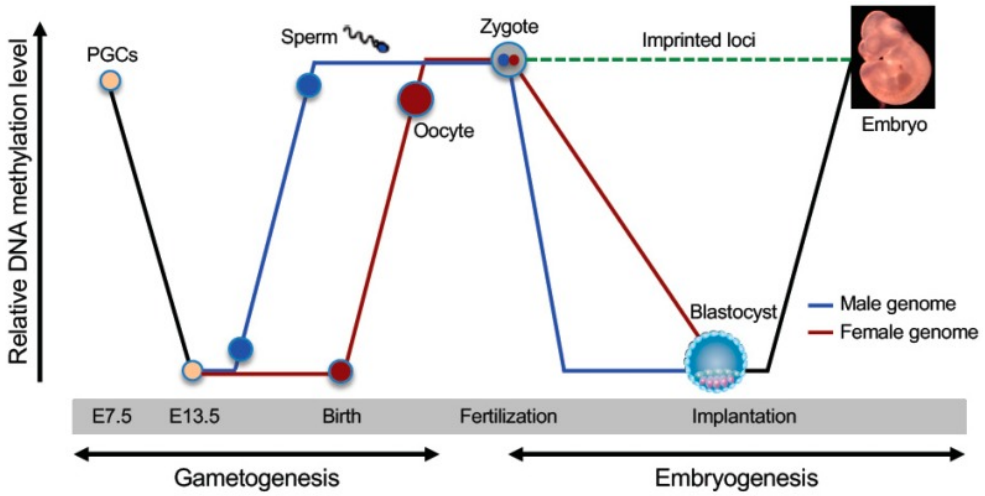


Figure 7: Dynamic changes in DNA methylation during mammalian development by (Zeng and Chen 2019). Schematic representation of the two waves of global DNA demethylation and remethylation during gametogenesis and early mammalian development. Primordial germ cells (PGCs) with initial high levels of DNA methylation are globally demethylated during PGC expansion and migration. At later stages of germ cell development (before birth in male and after birth in female), *de novo* methylation results in the establishment of sex-specific germ cell methylation patterns, including methylation marks at imprinted loci. Shortly after fertilization, the methylation marks inherited from the gametes are erased again (except those at imprinted loci and some retrotransposons), with the paternal genome undergoing active demethylation and the maternal genome undergoing passive demethylation. Upon implantation, a wave of *de novo* methylation establishes the initial embryonic methylation pattern.

b) Histone post-translational modifications

DNA is not the only chromatin component to be chemically modified. Canonical and linker histones are also covalently modified by post-translational modifications (PTMs), known as histone marks (Andrés et al. 2020; Huang et al. 2014). Histone tails protrude out from the nucleosome and are substrates of PTMS. The first discovered and most studied histone PTMs are located in N-terminal tails, while PTMs are also found in globular domains and C-terminal tails. PTMs mainly consist of acetylation, sumoylation, and ubiquitination of lysine, methylation of arginine and lysine, phosphorylation of serine, threonine, and tyrosine, glycosylation, and ADP-ribosylation (Figure 8).

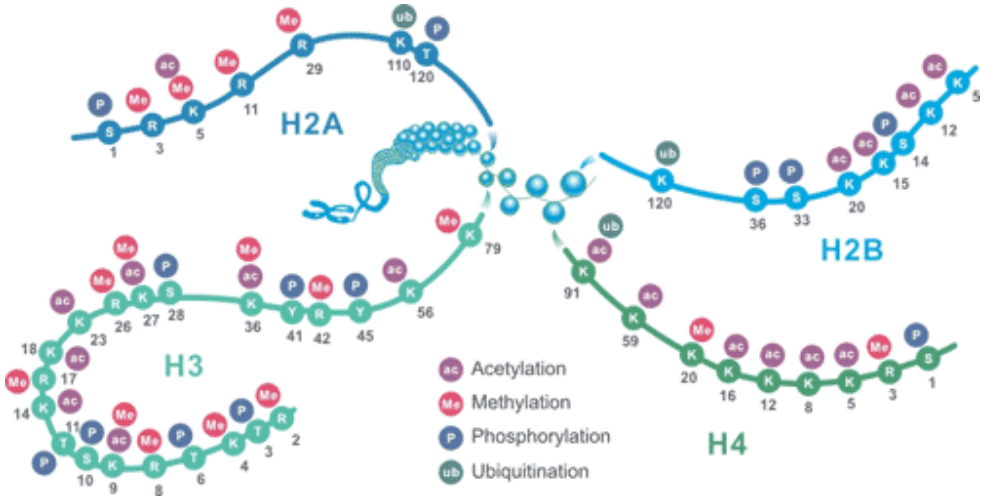


Figure 8: Schematic representation of the most important sites of histone post-translational modifications (PTMs) by Cusabio.com.

These PTMs are really dynamic and specific factors add (writers), remove (erasers) or recognize (readers) these modifications (Kouzarides 2007). The readers have specific protein domains, such as chromodomain and bromodomain that recognize a particular modification within a defined amino acid sequence (Figure 9).

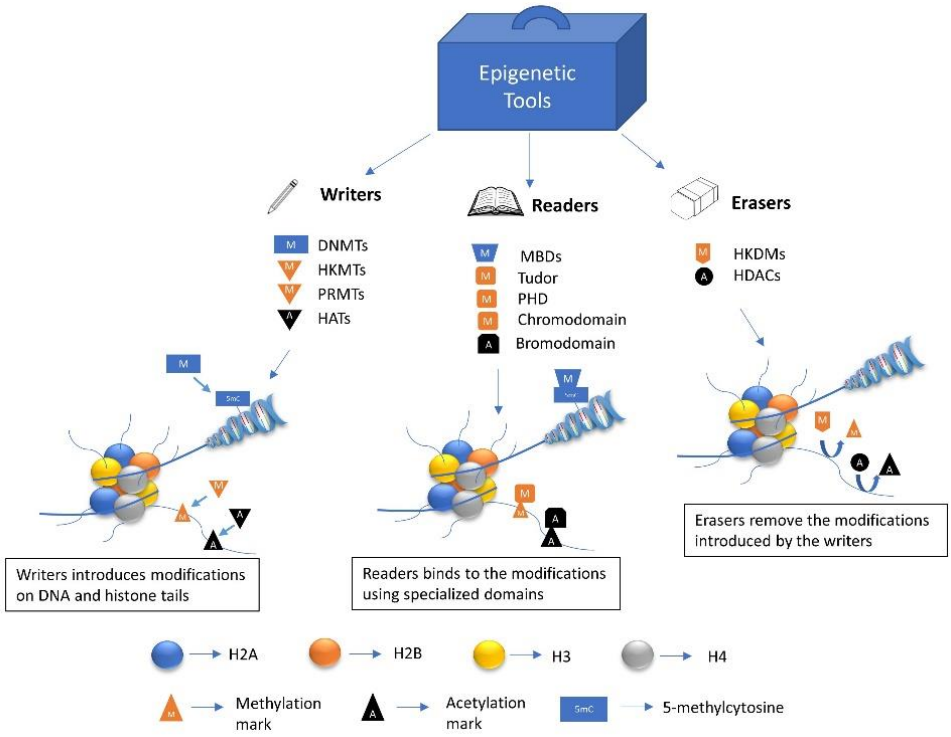


Figure 9: Representation of epigenetic writers, readers and erasers of histone post-translational modifications (PTMs) and DNA methylation by (Biswas and Rao 2018). DNMTs, DNA methyltransferases; HATs, Histone acetyltransferases; HDACs, Histone deacetylases; HKDMs, Histone lysine demethylases; HKMTs, Histone lysine methyltransferases; MBDs, Methyl-CpG-binding domains; PHD, Plant homeodomain; PRMTs, Protein arginine methyltransferases.

Acetylation consists of adding one or more acetyl groups to the amino groups α or ε of the lysine residues of histones and other proteins. This addition of acetyl groups is catalyzed by

histone Acetyltransferases (HATs), enzymes which are classified into five families: GNAT (GCN5-related N-terminal AcetylTransferases), MYST, p300/CBP, the general transcription factor HATs and the nuclear hormone-related HATs. The removal of the acetyl groups is catalyzed by histone deacetylases (HDAC) which are classified into three families: SIR2 (Silent Information Regulator 2), HD2 and RPD3/HDA1. Histone acetylation seems to contribute to form an open chromatin environment conducive to active transcription by allowing transcription factors to access the promoter regions of genes (Galvani and Thiriet 2015; Ramazi et al. 2020).

Methylation is a post-translational modification of the lysine and arginine residues particularly well characterized for histones H3 and H4. The lysine residue side chain is the target for mono, di or trimethylation while the arginine residue side chain can be mono or dimethylated. The addition of methyl group on histone residues is catalyzed by histone methyltransferases (HMT) enzymes, which use the S-adenosyl methionine cofactor. Methylation of arginine residues is traditionally associated with transcription activation, while methylation of lysine residues is more variable. For example, the trimethylation of H3 lysine residue 27 (H3K27me3) has a repressive effect on transcription, while the trimethylation of H3 lysine residue 4 (H3K4me3) has an activating effect on transcription and both of these histone marks coexist at gene promoter involved in cell differentiation in embryonic stem cells (ESCs) (Vastenhouw and Schier 2012).

Phosphorylation consists of the addition of a phosphate group, derived from triphosphate nucleotides (ATP, GTP, AMPc), to the hydroxyl group in the side chain of serine, threonine and sometimes tyrosine residues by kinase and its removal by phosphatase enzymes. Histone phosphorylation plays an essential role in many cellular processes such as transcription regulation, apoptosis, cell cycle, DNA repair, chromosome condensation, protein degradation and enzyme activity regulation (Johansen and Johansen 2006; Loomis et al. 2009:20; Thiriet and Hayes 2005). For example, phosphorylation at serine and threonine residues of histone tails is involved in chromatin condensation during mitosis and meiosis (Banerjee and Chakravarti 2011).

Histone ubiquitination differs from the other PTMs because it entails the covalent binding of a 76-amino acid protein (8 kDa), ubiquitin (Ub). Ubiquitination results from sequential actions of E1 activating, E2 conjugating, and E3 ligase enzymes, yielding the covalent conjugation of Ub to the amine group ε of the lysine residues on proteins, or on Ub itself to form different flavors of polyUb chains. Responsible for Ub removal are deubiquitinating enzymes (DUBs) (Mattiroli and Penengo 2021). In general, ubiquitination targets proteins for proteasomal degradation, but interestingly histone ubiquitination is associated with DNA damage response. In the same category of PTMs as ubiquitination, we also find sumoylation. Sumoylation is the addition of one or more SUMO (Small Ubiquitin-like MOdifier) proteins to lysine residues. Histone sumoylation has been linked to transcriptional repression and genome stability.

c) Chromatin remodeling complexes

The ATP-dependent chromatin remodeling factors use the energy provided by the ATP hydrolysis and modify the structure or position of the nucleosome resulting in chromatin compaction changes. These complexes are highly conserved from yeast to humans and belong to the super-family of helicases 2 (SF2 helicase). The chromatin remodeling complexes are divided into four families based on similarities and differences between domains harbored within their ATPase subunits: the SWI/SNF family, the ISWI family, the CHD1 family and the INO80 family (Clapier 2021; Clapier et al. 2017; Paul and Bartholomew 2018; Torchy, Hamiche, and Klaholz 2015)(Figure 10). The chromatin remodeling complexes ISWI and CHD are responsible for the assembly and proper spacing process of nucleosomes during replication and transcription (Fei et al. 2015; Torigoe et al. 2011). The SWI/SNF complexes enable chromatin opening by ejecting part- or full nucleosomes in order to make the chromatin more accessible to proteins such as transcription factors (TFs) (Clapier et al. 2016, 2020; Shukla et al. 2019; Szerlong et al. 2008). The INO80 complexes, known as nucleosome editing remodelers, replace canonical histones by replication-independent histone variants (Brahma et al. 2017; Papamichos-Chronakis et al. 2011; Pradhan et al. 2016; Udugama, Sabri, and Bartholomew 2011; Yen, Vinayachandran, and Pugh 2013). Eukaryotes contain at least one remodeler from each of the four subfamilies, supporting the notion that their functions are largely non-redundant. Furthermore, eukaryotes have evolved a broader set of remodelers within each subfamily by increasing compositional diversity through a modular and combinatorial architecture involving subunit paralogs, leading to functionally tailored remodelers, such as cell-type specific or developmentally specific remodelers.

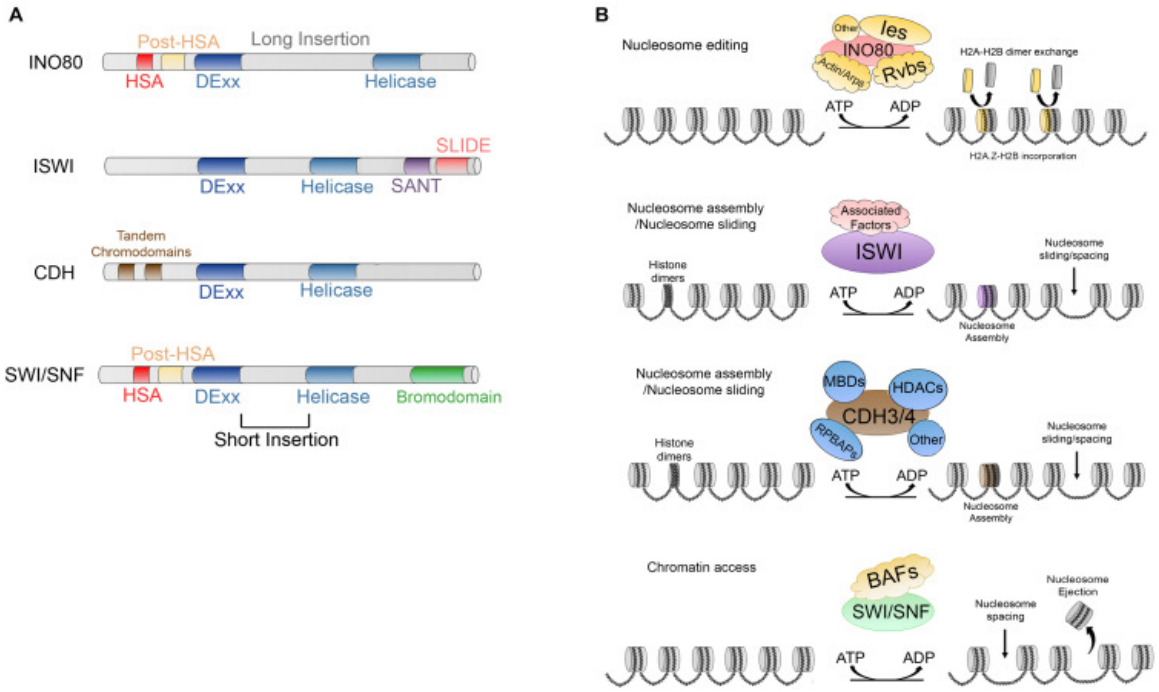


Figure 10: ATP-dependent chromatin remodeling complexes adapted by (Magaña-Acosta and Valadez-Graham 2020). (A) Main characteristics and domains of INO80, IWSI, CHD, and SWI/SNF families. The DExx and helicase domains compose the ATPase subunits. (B) ATP-dependent activities are carried out by INO80, which is responsible for nucleosome editing by exchanging H2A-H2B and H2A.Z-H2B dimers. ISWI and CDH are involved in nucleosome assembly as well as in nucleosome spacing and sliding. SWI/SNF members enable chromatin access through nucleosome spacing, nucleosome ejection as well as dimer eviction.

d) Histone Variants

Histone variants are non-allelic isoforms of canonical histones. Unlike the canonical histone, histone variants are encoded by single genes with introns and polyA tails and are deposited to chromatin throughout the cell cycle (replication-independent). They are sharing an overall similar structure, while having a relatively different primary structure (33-97% homology in sequence). In some cases, the variants differ in only a few amino acids from the canonical histones (e.g. H3.3 and H3.1) whereas other variants can show larger sequence dissimilarities (e.g. H2A variants). In higher eukaryotes, non-allelic isoforms exist for all histones, except for H4 (Figure 11). Overall, eight variants of H2A (H2A.X, H2A.Z.1, H2A.Z.2.1, H2A.Z.2.2, H2A.Bbd: H2A Barr body deficient; also known as H2A.B, macroH2A1.1, macroH2A1.2, and macroH2A2), six variants of H3 (H3.3, histone H3-like centromeric protein A: CENP-A, H3.1T, H3.5, H3.X; also known as H3.Y.2, and H3.Y; also known as H3.Y.1), and two testis-specific variants of H2B (H2BFWT; also known as H2B.W, and TSH2B: testis-specific histone H2B; also known as histone H2B type 1A) (Figure 11) (Buschbeck and Hake 2017; Talbert and Henikoff 2021) have been identified in vertebrates.

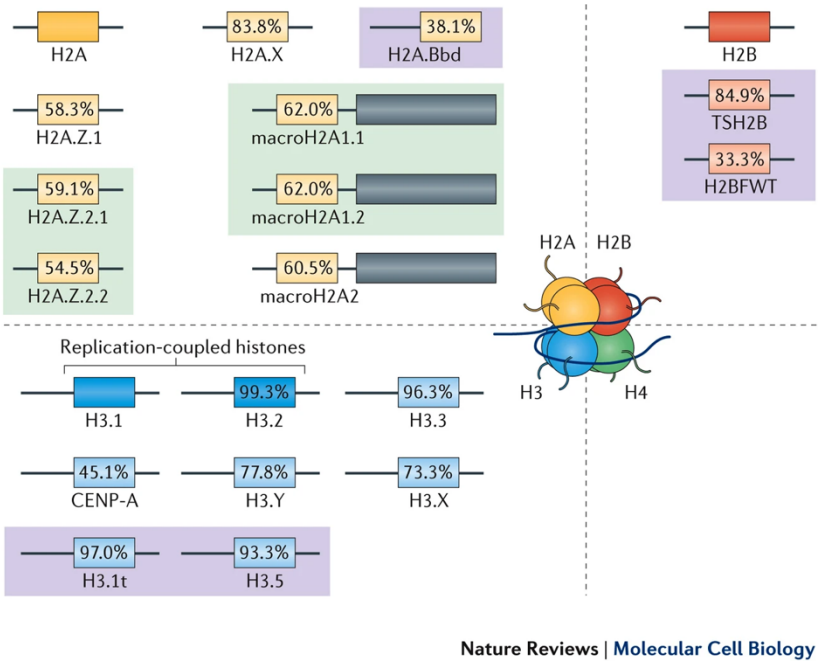


Figure 11: Canonical histones and their variants adapted by (Buschbeck and Hake 2017). Canonical histones H2A (yellow), H2B (red) and H3 (blue) and their associated variants in pale yellow, pale red and pale blue respectively. Rectangles represent core regions, and lines represent flexible histone tails. No variant of H4 (green) has yet been discovered in human. Tissue-specific (testis) histone variants are highlighted by light purple boxes and alternative splice isoforms by light green boxes. Percentages indicate total amino acid sequence conservation (% sequence identity) of the variants relative to their replication-coupled counterparts. CENP-A, histone H3-like centromeric protein A; H2BFWT, histone H2B type WT; TSH2B, testis-specific histone H2B.

Histone variants have spatio-temporal regulation linked to their distinct functions in cell division, transcription, DNA repair, differentiation, and chromatin remodeling. There are distinct chaperones and remodeling complexes for different histone variants and for different stages and pathways of the histone assembly (Gurard-Levin, Quivy, and Almouzni 2014; Talbert and Henikoff 2017). For instance, the canonical histones H3.1/2 are deposited genome-wide by the CAF-1 complex (Latreille et al. 2014; Tagami et al. 2004), while the variant CENP-A is

specifically deposited at centromeres by the chaperone HJURP (Athwal et al. 2015; Lacoste et al. 2014). Furthermore, H3.3 is recognized by two different complexes (HIRA and DAXX/ATRX) (Delbarre et al. 2013; Drané et al. 2010:3; Elsässer et al. 2012), leading to its deposition at different genomic locations (Figure 12). Another example is the assembly and disassembly of H2A-H2B dimers by mainly the histone chaperones NAP1 (nucleosome assembly protein 1) and FACT (facilitates chromatin transcription) (Huang, Dai, and Zhou 2020), while H2A.Z incorporation is facilitated by the SRCAP (Snf2-Related CREBBP Activator Protein) and p400/TIP60 (E1A-binding protein p400/Tat-Interactive Protein 60) remodeling complexes (Gévry et al. 2007; Mizuguchi et al. 2004; Ruhl et al. 2006) with the chaperones YL1 (Latrick et al. 2016:1) and ANP32 (Obri et al. 2014) in higher eukaryotes. In addition, It is worth to be mentioned that H2A.X is linked to DNA damage and H2A.Z has a complex role in the transcriptional regulation (Belotti et al. 2020; Giaimo et al. 2019), while we will discuss below the role of H3.3 (see section: Biological functions).

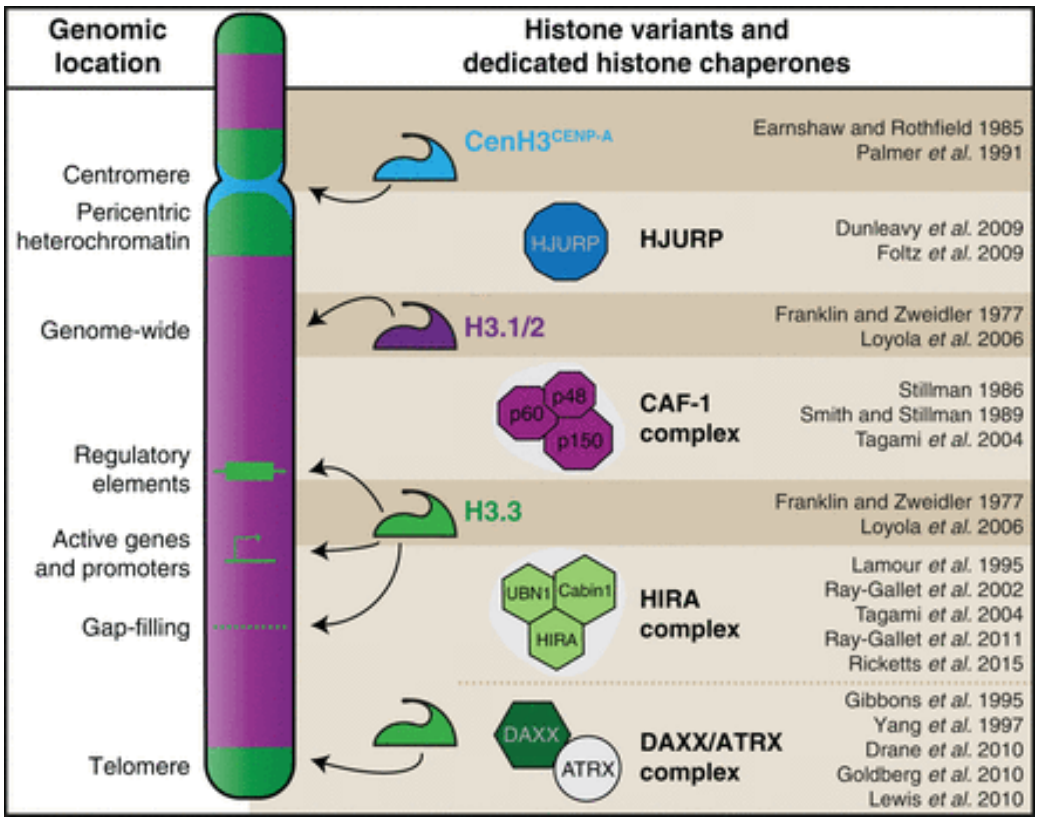


Figure 12: Spatio-temporal regulation of histone variants by their associated histone chaperones that mark specific chromosomal regions, adapted by (Sitbon et al. 2017). CENP-A is incorporated at centromeres by HJURP. Canonical H3.1/2 is incorporated genome-wide by the CAF-1 complex. H3.3 is incorporated at regulatory elements and gene bodies by the HIRA complex and at telomeres and pericentric heterochromatin by the DAXX/ATRX complex.

B. Chapter 2: Histone H3.3 and p53 functions in tumorigenesis

1. Histone H3.3

a) Structure

In human, mouse, and *Drosophila,* the histone variant H3.3 is encoded by the genes *H3f3a* and *H3f3b*. In human, *H3f3a* and *H3f3b* are located on chromosomes 1 and 17, respectively (Frank, Doenecke, and Albig 2003). At the protein level, H3.3 differs only in five or four residues when compared to the canonical H3.1 or H3.2, respectively. Four of them (i.e., A87, I89, G90, and S96) are placed in the histone fold domain, while the serine 31 is located in the N-terminal tail (Figure 13) (Szenker, Ray-Gallet, and Almouzni 2011:3). These variations in sequence don't affect the nucleosome structure *in vitro* (Tachiwana et al. 2011).

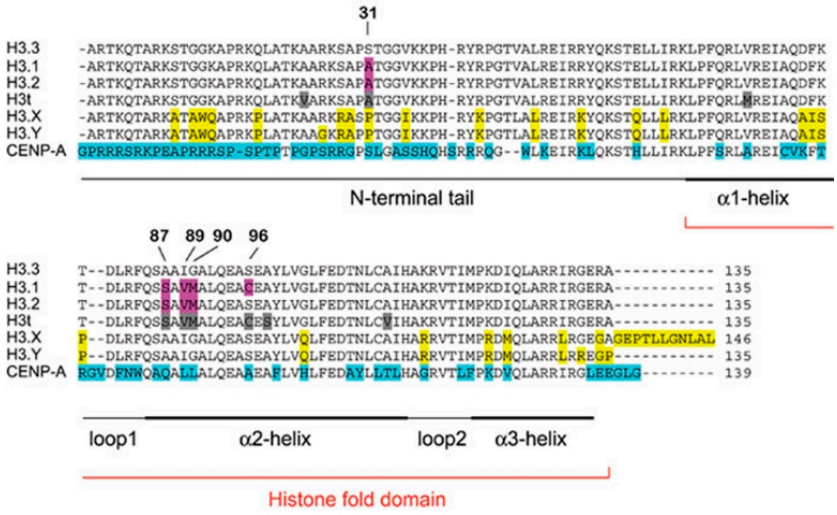


Figure 13: Amino acid sequence alignment of human H3 variants, adapted by (Szenker et al. 2011). Sequences are compared with the H3.3 variant and the amino acid differences are highlighted. H3.1 and H3.2 differences are highlighted in purple, H3t in gray, H3.X and H3.Y in yellow, and CENP-A in light blue. The position numbers of amino acids that are different between H3.3 and H3.1/2 are indicated. The positions of the N-terminal tail and of the α-helixes of the histone-fold motif are depicted according to the secondary protein structure.

b) Chaperones

Canonical histones H3.1 and H3.2 are deposited into chromatin in a replication-dependent manner by the CAF-1 complex. Contrary, H3.3 histone variant is incorporated at specific regions of the genome throughout the cell cycle, suggesting distinct mechanisms and deposition factors. Indeed, it has been shown that the incorporation of H3.3 into chromatin is ensured by two specific chaperones HIRA and DAXX.

The histone cell cycle regulator 1 complex (HIRA complex) takes part in the assembly of H3.3-H4 dimers into nucleosomes. The HIRA complex is composed of HIRA, calcineurin-binding protein 1 (Cabin1), ubinuclein 1 (UBN1) or ubinuclein 2 (UBN2) (Ricketts and Marmorstein 2017) and proceeds to H3.3 deposition in genomic regions characterized by an open chromatin state, such as the regulatory elements and gene bodies of actively transcribed genes (Goldberg et al. 2010; Pchelintsev et al. 2013; Xiong et al. 2018) (Figure 12). The mechanism by which

HIRA specifically recognizes and deposits H3.3 remains elusive, but two pathways have been proposed recently (Torné et al. 2020). However, the interaction of HIRA with H3.3 was questioned by recent studies showing that it is Ubinuclein that confers H3.3 recognition and selectivity to the HIRA complex (Ricketts et al. 2015, 2019; Xiong et al. 2018). These data suggest that Ubinuclein, not HIRA, is the H3.3 histone chaperone.

The death domain-associated protein DAXX and the chromatin remodeling factor ATRX (alpha-thalassemia/mental retardation syndrome protein) form the complex DAXX/ATRX which is responsible for the deposition of H3.3 at telomeres, pericentromeric heterochromatin, and endogenous retroviral elements (Drané et al. 2010; Elsaesser, Goldberg, and Allis 2010:3; Elsässer et al. 2015; Fromental-Ramain, Ramain, and Hamiche 2017; Goldberg et al. 2010; Lewis et al. 2010; Udugama et al. 2015) (Figure 12). In the absence of DAXX, H3.3 has been found to be associated with the CAF-1 complex, suggesting an alternative mechanism of deposition (Drané et al. 2010).

c) Biological functions

Despite the high homology between histone variant H3.3 and its canonical counterparts, H3.1/2 cannot rescue H3.3 loss without deleterious defects in mammals (Bush et al. 2013; Couldrey et al. 1999; Tang et al. 2013; M. C. W. Tang et al. 2015). The available data suggest that H3.3 genes are required for mouse development, viability, and fertility and are implicated in several essential cellular processes such as transcription, DNA repair, mitosis, and chromosome segregation. However, its functional roles remain mainly unknown, therefore below we will summarize the current knowledge about H3.3 biological functions.

H3.3 role in transcription

The histone variant H3.3 is found at transcriptionally active chromatin enriched in active post-translational modifications (Ahmad and Henikoff 2002; McKittrick et al. 2004). H3.3 plays an active role in the maintenance of accessible chromatin structure at enhancer and actively transcribed genes. In addition, nucleosomes harboring the hybrid H3.3/H2A.Z histones have been shown to be intrinsically unstable and to promote gene activation (Chen et al. 2013; Jin et al. 2009; Jin and Felsenfeld 2007). In addition, H3.3 is enriched at transcription start sites (TSS) of both active and repressed CG-rich promoters, and in the gene bodies and transcriptional end site of active sequences (Goldberg et al. 2010). H3.3 has also been shown to promote transcriptional recovery after genotoxic stress in a HIRA-dependent manner (Adam, Polo, and Almouzni 2013). The deposition of H3.3 by HIRA has been shown to be stimulated by interferon to regulate interferon-stimulated genes (Bachu et al. 2019). Furthermore, phosphorylation of H3.3 at serine 31 positively correlates with open chromatin and active transcription (Armache et al. 2020; Martire et al. 2019). Despite the numerous studies correlating H3.3 with active chromatin its role in transcription activation is debated. The absence of H3.3 has a mild effect on transcription (Bush et al. 2013; Jang et al. 2015; Ors et al. 2017). This leads to the conclusion that H3.3 might not play a pivotal role in transcriptional activation despite its importance during development.

H3.3 role during development

To decipher its biological role, H3.3 had been deleted or inactivated in several organisms. In flies, loss or inactivation of the two H3.3 genes did not perturbate embryonic and postnatal development but led to infertility, which can be rescued by ectopic expression of H3.2 (Hödl and Basler 2009:3, 2012; Sakai et al. 2009). Contrary, overexpression of H3.3 in the S-phase can also rescue growth defects in the H3.2-null flies (Hödl and Basler 2012). Therefore, it seems that it is the overall level of H3 histones which is important for fly development and that H3.3 variants are interchangeable with canonical histones. In *Caenorhabditis elegans,* loss of H3.3 has no effect on viability or fertility (Piazzesi et al. 2016), while in *Xenopus,* its loss leads to gastrulation defects that cannot be rescued by H3.2 overexpression (Szenker, Lacoste, and Almouzni 2012). In zebrafish, H3.3 seems to be important for the proper cranial neural crest cell differentiation (Cox et al. 2012).

In mice, H3.3 has significant function in gametogenesis, in zygote and in early development. In particular, partial or complete loss of both *H3f3a* alleles led to reduced viability and subfertility or infertility of males (Couldrey et al. 1999; Tang et al. 2013). Mice lacking *H3f3b* also had reduced viability and both homozygous and heterozygous were infertile (Bush et al. 2013; Fontaine et al. 2022; Tang et al. 2013; M. C. W. Tang et al. 2015; Yuen et al. 2014). Another study showed that mouse models with a single knock-out of *H3f3a* or *H3f3b* are normal and fertile in both sexes (Jang et al. 2015), while depletion of both *H3f3a* and *H3f3b* causes developmental retardation and embryonic lethality. The discrepancy between the aforementioned studies is likely due to differences in genetic background. Nevertheless, all of the studies concluded to a partial redundancy of H3.3 genes, and that one of the two H3.3 genes is required for mouse development, viability and fertility. From an evolutionary perspective, *H3f3b* resembles to an ancestral form of H3.3, while *H3f3a* could have arisen later in evolution, possibly thought a duplication event. The existence of two independent H3.3-coding genes seems to enable the fine-tuned expression of H3.3 genes in different cellular programs (Muhire, Booker, and Tolstorukov 2019).
Furthermore, H3.3 seems to have a dual role in spermatogenesis (Fontaine et al. 2022). Indeed, H3.3 is exclusively incorporated during mammalian meiotic sex chromosome inactivation and is required for gene silencing in the male germ line (van der Heijden et al. 2007). After oocyte activation, the canonical histone H3.1 is replaced by maternal-derived H3.3 in the donor nucleus through the HIRA-dependent pathway. Immediately after fertilization, maternal H3.3 invades paternal chromatin by replacing protamines (Loppin et al. 2005; Torres-Padilla et al. 2006). Additionally, HIRA-mediated H3.3 deposition is also required for rRNA transcription in addition to its role in the parental genome reprogramming (Lin et al. 2014). Moreover, H3.3R26 and H3.3K27 residues and their PTMs are essential for proper oogenesis and good partitioning of the cells to the inner cell mass of the early embryo (L. Zhou et al. 2017).
In mouse zygotes, knockdown of H3.3 causes defect in nuclear envelope formation and change in chromosome condensation (Inoue and Zhang 2014). In mESC, developmentally-regulated gene promoters are decorated by both the H3K27me3 repressive mark and the H3K4me3 activation mark (Bernstein et al. 2006). Those 'bivalent' domains are marked by H3.3 which facilitates the recruitment of the Polycomb-repressive complex 2 (PRC2) (Banaszynski et al. 2013). Last but not least, H3.3 also plays a critical role in cell fate transition, initially by

maintaining the parental cell identities during reprogramming and secondly by its deposition on genes implicated in lineage reprogramming (Fang et al. 2018).

H3.3 and mitosis

H3.3 has an important role in mitotic progression. Depletion of H3.3 in mESC and mouse embryonic fibroblasts caused mitotic defects such as anaphase bridges and lagging chromosomes (Jang et al. 2015; Ors et al. 2017). Furthermore, deletion of H3.3 tail at residue 21 was shown to lock the cells in the senescent process (Duarte et al. 2014). H3.3 is also involved in the maintenance of the replication fork and in the transcription restart after UV damage (Adam et al. 2013; Frey et al. 2014).

H3.3 role in heterochromatin

Apart from the H3.3 role in active chromatin loci, H3.3 is deposited by DAXX/ATRX complex at heterochromatin, more precisely, telomeres, pericentromeric heterochromatin, and endogenous retroviral element (Drané et al. 2010; Goldberg et al. 2010). It has been observed decondensed chromatin at telomeres, centromeres, and pericentromeres in MEFs that lacks H3.3 (Jang et al. 2015).

H3.3 is enriched at the telomeric (TTAGGG)n repeat in a ATRX-dependent manner, specifically at interphase telomeres in mESC (Goldberg et al. 2010; Ivanauskiene et al. 2014; Wong et al. 2010). Furthermore, the specific interaction among ATRX, CBX5 (HP1) and H3.3 is essential in the maintenance of the telomere structural integrity (Wong et al. 2010). H3K9me3 serves as an ATRX docking site and is essential for the telomere transcriptional repression (Udugama et al. 2015).

H3.3 plays a key role in pericentric heterochromatin where it is deposited by the ATRX/DAXX complex in a PML-dependent manner (Delbarre et al. 2017; Drané et al. 2010). Moreover, H3.3 recruitment by DAXX into the PML nuclear bodies has been shown to be essential for the transcriptional regulation of pericentromeric satellite repeats in both mouse and human (Morozov et al. 2012).

Last but not least, H3.3 was found enriched at the long terminal repeats (LTRs) of endogenous retrovirus elements (ERVs). In mESC, ERVs expression is repressed by H3K9me3 and a co-repressor complex containing KAP1, also known as TRIM28 (Rowe et al. 2010; Rowe, Kapopoulou, et al. 2013). Depleting H3.3 in mESCs results in ERV-associated H3K9me3 decrease and ERV de-repression (Elsässer et al. 2015). Thus, H3.3 has a critical role in establishment and maintenance of ERVs silencing.

d) Post translational modifications of H3.3

Covalent modifications of histones can occur in all H3 variants at the amino acid residues of the N-terminal tails and the internal histone fold domain (Figure 14 & Figure 8). Whether H3.3 can show specific PTM patterns that differ from the canonical H3 variants and if this helps define unique chromosomal domains is still of great interest (Trovato et al. 2020). Previous studies ranging from *Drosophila* to *Arabidopsis* and mammals showed that H3.3 is enriched in PTMs that correlate with an active chromatin state (Hake et al. 2006; Johnson et al. 2004;

McKittrick et al. 2004). For instance, H3K4me, H3K9ac, and H3K36me1/2, which mark active transcription, are more enriched for H3.3 compared to H3.1 and H3.2. Some PTM enrichment on H3.3 showed co-occurrence, including K9ac-K14ac and K18ac-K23ac (Hake et al. 2006). On the other hand, some marks have a dual role depending on the place and time of enrichment. H3K9me3 is known as a repressive transcription mark at heterochromatin but is also associated with transcriptionally active genes (Carrozza et al. 2005; Vakoc et al. 2005). For instance, H3.3K9me3 was located at actively-transcribing repeats, for example, telomeres in stem cells (Udugama et al. 2015).

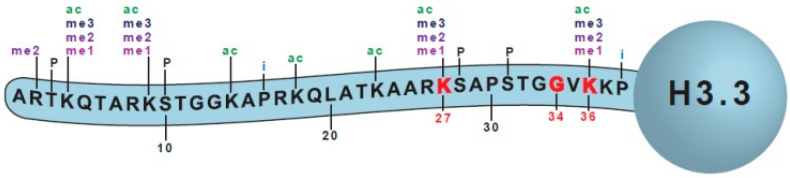
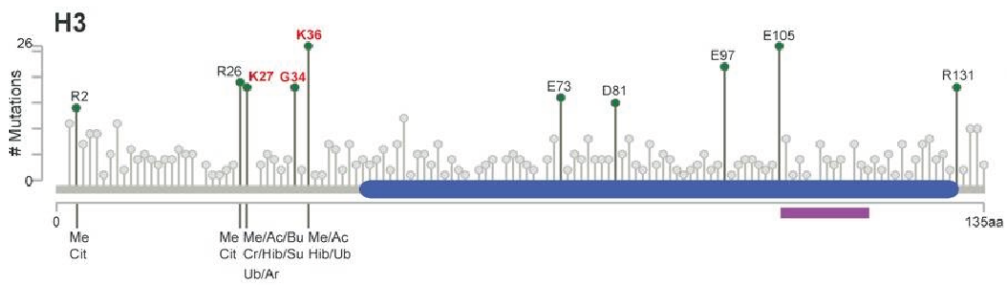


Figure 14: Main post-translational modifications on the N-terminal tail of histone H3.3, adapted by (Lowe et al. 2019). Commonly mutated sites are highlighted in red (K27, G34, K36) and the sites of modification are depicted as me: methylation, ac: acetylation, P: phosphorylation, and i; isomerization.

e) H3.3 mutations in cancer

Histone H3 was the first histone found to be frequently mutated in cancer. In 2012, two studies showed the association between histone H3 K27M mutation and pediatric high-grade glioma (pHGG), an observation which was confirmed by other studies (Buczkowicz et al. 2014; Fontebasso et al. 2014; Morgan and Shilatifard 2013; Schwartzentruber et al. 2012; G. Wu et al. 2012; Wu et al. 2014). Later, further cancerogenic malignancies linked to H3.3 mutations have been identified, including chondroblastoma, chondrosarcoma, osteosarcoma, head, and neck squamous cell carcinoma, pediatric soft tissue sarcoma, bladder cancer, melanoma and acute myeloid leukemia (Behjati et al. 2013; Bennett et al. 2019; Gessi et al. 2016; Kleinschmidt-DeMasters et al. 2017; Lehnertz et al. 2017; Lu et al. 2016; Nacev et al. 2019; Papillon-Cavanagh et al. 2017). In general, the role of H3K27M is critical for the brain (El-Hashash 2021) and is responsible for neurodegenerative disorders (Bryant et al. 2020).
The most studied H3 mutations, K27M, K36M and G34 mutations (Bender et al. 2013; Castel et al. 2018; Chan et al. 2013; Chan, Mao, and Ng 2016; Fang et al. 2016; Jiao and Liu 2015:2; Justin et al. 2016:2; Larson et al. 2019; Lewis et al. 2013; Lutsik et al. 2020; Mohammad et al. 2017; Silveira et al. 2019; Stafford et al. 2018; Sturm et al. 2012; Tatavosian et al. 2018; Venneti et al. 2013; Yi et al. 2019), are localized in the N-terminal domain and play their oncogenic role by perturbing the pattern of histone PTMs (Chaouch et al. 2021). There are also several residues in the globular domain such as E97, E105, and R131 that have been found mutated at similar or even higher rates (Figure 15) (Arimura et al. 2018; Bennett et al. 2019; Nacev et al. 2019). These H3.3 mutations, as well as mutations in its dedicated deposition machinery (ATRX/DAXX) (Buschbeck and Hake 2017:3), are likely to be responsible in changes in nucleosome structure, function or higher order chromatin organization. Interestingly, recent studies reported that H3.3 mutations (K27M and K36M) de-repress transposable elements through perturbation of antagonistic chromatin marks (Chaouch et al. 2021; Elsässer et al. 2015; Stekelenburg et al. 2022).

A



B

Histone	Mutations	Preferential genes	Histone domain	Proposed mechanism	Biological evidence	Primary sites of cancer
H3	K27M	H3F3A (H3.3)	N-TT	Interference with residue methylation (dominant-negative)	Tumor growth and differentiation (in vitro and in vivo); associated with more aggressive tumor phenotype (in patients)	Diffuse intrinsic pontine glioma (pediatric)
	K36M	H3F3B (H3.3)	N-TT	Interference with residue methylation (dominant-negative)	Tumor development (in vivo); enhanced colony formation and less sensitivity to apoptotic stimuli (in vitro)	Chondroblastoma (children and young adults)
	G34R/V	H3F3A (H3.3)	N-TT	Interference with K36 and K27 methylation (dominant-negative)	None	Cortical pediatric high-grade glioma (young adults)
	G34W/L	H3F3A (H3.3)	N-TT	Interference with K36 and K27 methylation (dominant-negative); interference with DNA methylation and histone modifications at heterochromatic and bivalent regions	Enhanced colony formation, infiltration and proliferation (G34W—in vitro and in vivo); reduced differentiation of GCTB stromal cells (primary cells)	Giant cell tumors of bone
	E97K	H3.1 genes	HFD	Interference with nucleosome structure	Enhanced colony formation (in vitro)	None
	E105K/V	H3B (slight preference)	HFD	Interference with nucleosome structure	None	None
	R131C	None	HFD	Interference with nucleosome structure	None	None

Figure 15: Cancer-associated histone mutations occur at sites of PTMs in H3 tail and globular domain, adapted by (Amatori et al. 2021; Nacev et al. 2019). (A) Schematic representation and (B) table summarizing the current knowledge. The ten most frequently mutated positions are shown in green circles. Established oncohistone mutations are indicated in red. The blue bar represents the globular domain (HDF).

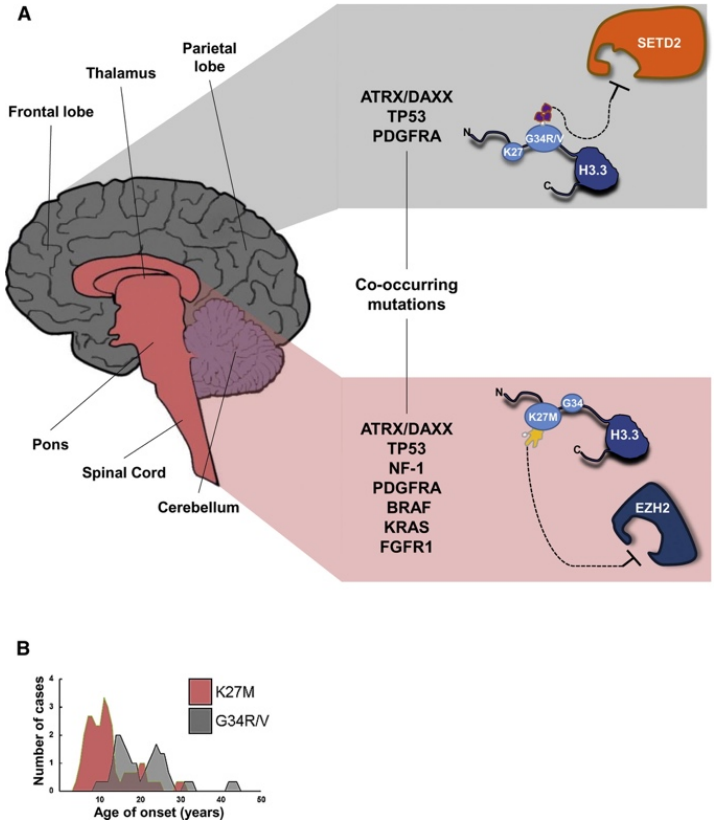
f) H3.3 mutations in pHGG

Sequencing of tumor cells revealed that histone H3 is frequently mutated in pediatric high-grade glioma (pHGG), with up to 78 % of diffuse intrinsic pontine gliomas (DIPGs) carrying K27M and 36 % of non-brainstem gliomas carrying either K27M or G34R/V mutations (Schwartzentruber et al. 2012; G. Wu et al. 2012). Both groups reported recurrent somatic heterozygous mutations in the gene encoding the histone variant H3.3A (i.e., *H3f3a)*. This was the first demonstration that histone mutations may be drivers of disease and especially in pHGG, but the mechanistic aspects of H3.3 mutation functions are still unknown.

It is worth to be mentioned that despite the fact that both K27 and G34 are in close vicinity on H3.3 N-terminal tail, an important antagonism is observed between those two mutation sites in pHGG. In particular, K27M and G34R/V mutations differ on their age of diagnosis, location, prognosis, DNA methylation pattern and transcriptional profile. The age incidence profile of K27M pHGG peaks at 7 years and ranges from 5 to 29 years patients, while G34R/V pHGG occurs in slightly older patients (range, 9–42 years) and peaks at 14 years (Bjerke et al. 2013; Yuen and Knoepfler 2013) (Figure 16B). It is important to note that H3.3K27M and H3.3G34R/V mutations have different anatomical localizations. H3.3K27M tumors are localized throughout the midline structures, such as the brainstem, cerebellum, thalamus, and spine, while H3.3G34R/V tumors are exclusively occurring in cerebral hemispheres (nonmidline supratentorial areas)(Diaz and Baker 2014)(Figure 16A). In terms of prognosis, the H3.3K27M pHGG has been depicted as more aggressive tumors with a median of survival of 11 months

and less than 5% of 2-year overall survival. On the other hand, H3.3G34R/V pHGG has been associated with longer overall survival compared to K27M pHGG (median at 18 months and 27% of 2-year overall survival)(Mackay et al. 2017). Finally, K27M and G34R/V tumors can be discriminated based on their DNA methylation profile (Castel et al. 2018) and transcriptional profile that follows the tumor localization (K27M-midline vs. G34R/V-hemispheres) (Bjerke et al. 2013; Castel et al. 2018; Paugh et al. 2010; Schwartzentruber et al. 2012).

Intriguingly, H3.3 mutations are also found to simultaneously overlap with other mutations within the same tumor. Approximately 30% of K27M mutations are associated with mutations in ATRX/DAXX and 60% with mutations in the tumor suppressor gene *TP53* (coding for p53)(Schwartzentruber et al. 2012). K27M mutations have also been found at much lower frequency alongside mutations in NF-1, PDGFRA, BRAF, KRAS, and FGFR1 in gliomas (Khuong-Quang et al. 2012; Schwartzentruber et al. 2012)(Khuong-Quang et al. 2012; Schwartzentruber et al. 2012). Meanwhile, G34R/V mutations completely overlap with tumors containing mutations in p53 and ATRX/DAXX, but have also been found together with mutations in PDGFRA (Figure 16A) (Schwartzentruber et al. 2012). Tumors with mutations in H3.3, p53, and ATRX/DAXX contain a higher abundance of copy number alterations (Schwartzentruber et al. 2012), although there was no clear association between increased copy number alterations and *H3f3a* mutations. Given that *H3f3a* mutations are found in heterozygous form and that these gliomas exhibit similar rates of p53 mutation with other glioma types, H3.3K27M and H3.3G34R/V may act as driver mutations with p53 mutations occurring as a second hit (Khuong-Quang et al. 2012; Schwartzentruber et al. 2012), although it is important to note that G34R/V mutations display much higher association with mutations in ATRX/DAXX and p53.



In conclusion, it is tempting to speculate that H3.3 mutations affect heterochromatin formation and transposable elements silencing, in line with recent observations (Chaouch et al. 2021; Elsässer et al. 2015). Moreover, the pHGG tumor-specific overlap of H3.3 mutations with the p53 and ATRX/DAXX mutations indicates an additional functional link between these proteins, probably at the level of transposable elements regulation.

Figure 16: Histone H3.3 mutations in cancer (Yuen and Knoepfler 2013). K27M and G34R/V mutations of H3.3 (*H3F3A)* display distinct characteristics from one another. (A) G34R/V mutations (gray, top) in *H3f3a* localize primarily to cerebral/cortical hemispheres. K27M mutations (pink, bottom) in *H3f3a* localize primarily to midline locations, including the spinal cord, thalamus, pons, and brainstem. G34R/V mutations overlap with mutations in p53 and ATRX/DAXX and PDGFRA. K27M mutations overlap with mutations in p53 and ATRX/DAXX, NF-1, PDGFRA, BRAF, KRAS, and FGFR1. (B) Age of onset of H3.3-mutated tumors. K27M mutations are more prevalent in younger patients (median age, 12 years) while G34R/V mutations are more prevalent in older patients (median age, 20 years).

2. Tumor suppressor p53

The tumor suppressor protein p53 is the guardian of the genome and was first discovered in the 1970s. Initially, it was thought to be an oncogene given that the mutated protein was found in abundance in many cancer tissues. p53 initial discovery was found to be associated with the large T-antigen simian virus 40 (SV40), where the introduction of this virus resulted in transformed malignant cells (Lane and Crawford 1979; Linzer and Levine 1979). A decade later, it was uncovered that p53 is a tumor suppressor (Baker et al. 1989; Buchman et al. 1988; Harris et al. 1986; Lamb and Crawford 1986; Zakut-Houri et al. 1985).

The wild-type p53 protein is a transcription factor that selectively regulates the transcriptional activation of several genes and transposable elements in diverse cells (Boutelle and Attardi 2021). p53 activation by various stress environmental signals results in the activation of numerous pathways related to cell cycle arrest, senescence (Itahana, Dimri, and Campisi 2001:5; Rufini et al. 2013:53), apoptosis, autophagy, cell death, metabolic alterations, DNA repair (Kastan et al. 1991; Kocyłowski et al. 2015:168), immune response (Blagih, Buck, and Vousden 2020), and tumor suppression (Figure 17). Contrary to most stress response genes that are transcribed when there is a stress signal, p53 is constantly expressed in normal cells. Reduced levels or insufficient p53 activity are major risk factors for cancer development, and more than half of all human cancers exhibit diminished p53 expression or function (Kastenhuber and Lowe 2017). In contrast, hyperactive p53 has been linked to impaired wound healing, obesity, and accelerated aging (Rufini et al. 2013:53). It is remarkable that depletion of the *TP53* gene does not lead directly to lethal phenotypes, considering the nature of this pleiotropic transcription factor. There is an active debate about which p53-related pathways are responsible for the tumor suppressor phenotype and what type of stress activates p53. Thus, the p53 protein ensures a quality of life either by repair or by death (Vousden and Prives 2009:53).

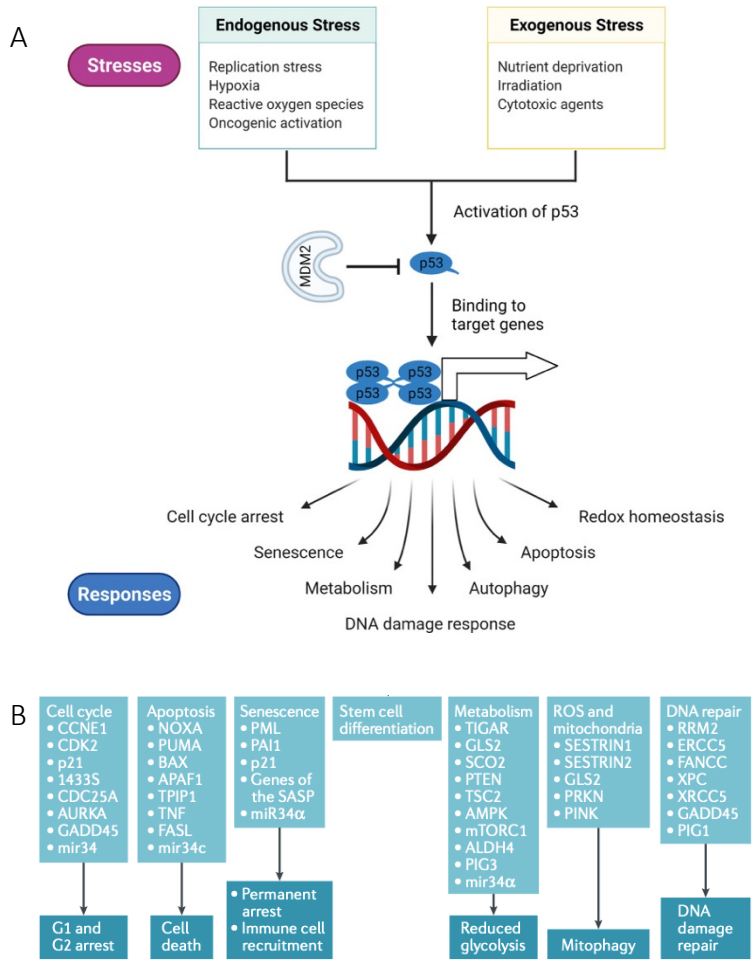


Figure 17: Overview of p53 pathway and its functions in normal cells by (Hu et al. 2021). (A) p53 is an important tumor suppressor in normal cells to maintain homeostasis. Throughout their lifespan, cells are faced with continuing stresses including endogenous and exogenous stresses. To overcome these stresses, p53 is activated to mediate a series of cellular responses via its transcription-dependent functions or direct protein-to-protein interactions. p53-mediated responses also rely on the type and degree of insults, as well as the cell types and the context in which the insult occurs. (B) List of protein products expressed due to activation of p53-mediated transcription and are involved in a range of downstream cellular processes.

a) The p53 family

The universal stress sensor, p53, belongs to a group of highly conserved transcription factors of p53/p63/p73 gene family (Belyi et al. 2010). An ancestral gene of p53/p63/p73 family first appeared in the early metazoans, specifically in the sea anemone, 600-800 million years ago. Interestingly, the primary function of this ancestral gene is to protect the germ line from genomic instability in response to cellular stress (Chakravarti et al. 2022). From insects, worms, clams, vertebrates, to humans, this function has been maintained for over one billion years of evolution. During evolution, the somatic stem cell with the ability to regenerate adult tissues emerged, and p53 took on the function of protecting these somatic stem cells and progenitor cells from genotoxic stress. Thus, p53 has retained during evolution its structural features and functional activities for which it is now well-known in higher vertebrates. p63, similar to p53, responds to DNA damage by inducing cell death, but in female and male germ lines (Beyer et al. 2011; Deutsch et al. 2011; Suh et al. 2006; Tomasini et al. 2008). p63 is also a critical transcription factor in the generation and regeneration of squamous cell epithelium throughout the body and contributes to craniofacial structures, limbs, and the central nervous system. p73 is required for the production of ciliated epithelial cells and so plays a role in many tissues that contain these cells (male germ line, immune system, hearing, trachea, lung, central nervous system, and so forth)(Marshall et al. 2016).

The protein domain structures of p53 (DeLeo et al. 1979; Kress et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979), p63 (Yang et al. 1998), and p73 (Jost, Marin, and Kaelin 1997; Kaghad et al. 1997) are similar, with the three DNA binding domains being almost structurally identical, binding to similar DNA specific sequences and regulating the transcription of some identical genes and some distinct genes. The C-terminal domains differ from each other in size, sequence, and functionality, regulate DNA binding and transcription and mediate both intra-protein and interprotein functional interactions. The N-terminal sequences of all three proteins encode at least two different transcriptional activation domains (Belyi et al. 2010; Belyi and Levine 2009).

b) p53 structure and isoforms

p53 is a DNA binding protein (Kern et al. 1991) consisting of 393 amino acids which are divided into three functional regions (Figure 18): an N-terminal domain (NTD) (1–93), containing two acidic transcriptional activation domain (TADI & II) and a proline-rich domain; a core DNA-binding domain (DBD) (102–292); and a C-terminal domain (CTD), consisting of a homo-tetramerization (TD) (320 – 356) and a regulatory domain (RD) (363 – 393). These functional regions are flanked by three flexible, unstructured regions: an N-terminal transactivation region, a linker region between the DNA binding and tetramerization domains, and a C-terminal basic region (Bell et al. 2002; Joerger and Fersht 2008:53). A p53 consensus DNA response element (RE) is composed of a tandem of two decameric palindromic sequences 5′-RRRCWWGYYY-3′, where R = purine, Y = pyrimidine and W is either A or T, separated by a spacer of 0 to 13 base-pairs. In general, p53 binds to its specific DNA consensus sequence with the highest affinity as a tetramer to activate target responsive elements (RE) with each monomer interacting with five base-pairs of the consensus sequence via its DBD (Bell et al. 2002; Ma and Levine 2007) (Figure 18 & Figure 19). The DBD structure is conserved from

Drosophila, *Caenorhabditis elegans* to human, as determined by X-ray crystallography, and is a hotspot for mutations in cancer.

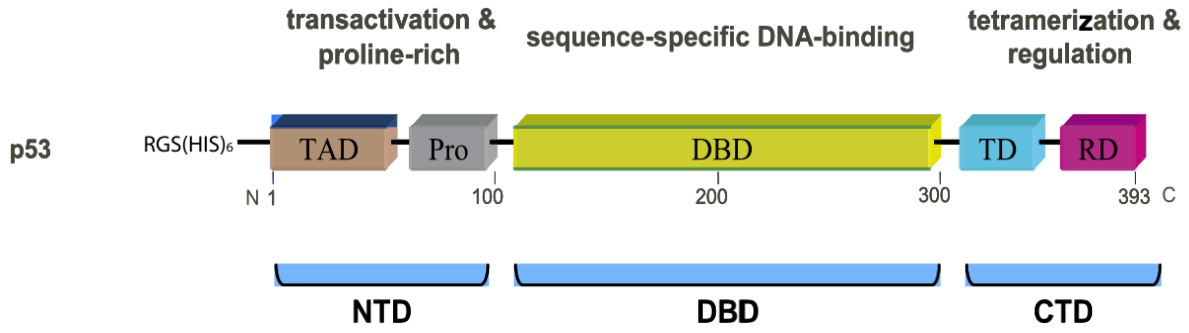


Figure 18: Schematic representation of p53 adapted by (Bell et al. 2002). Functional regions are depicted in different shades: N-terminal transcriptional activation domain (TAD), brown; proline-rich domain (Pro), grey; DNA-binding domain (DBD), ocher; tetramerization domain (TD), cyan; C-terminal regulatory domain (RD), purple.

In particular, there are several three-dimensional structures of p53. Most of these encompass only the core DNA-binding domain, either by itself or in complex with DNA (Chen, Dey, and Chen 2010; Cho et al. 1994; Ho, Fitzgerald, and Marmorstein 2006; Kitayner et al. 2006, 2010; Ma and Levine 2007; Malecka, Ho, and Marmorstein 2009). Later studies managed to solve the structure of p53 polypeptide containing both the DNA-binding and homo-tetramerization domains in its free form and bound to specific DNA sites (Emamzadah, Tropia, and Halazonetis 2011; Petty et al. 2011). Moreover, the same group showed that loop L1 of the p53 DNA-binding domain adopts an extended conformation in the absence of DNA, while two p53 subunits switch to a recessed loop L1 conformation when bound to DNA as a tetramer (Emamzadah et al. 2014).

Interestingly, the p53 tetramer has been shown to bind to bent DNA (Brázda and Coufal 2017; Brázda and Fojta 2019; Demir, Ieong, and Amaro 2017; Jett et al. 2000; Nagaich et al. 1999, 1999; Nagaich, Appella, and Harrington 1997:53; Niederweis and Hillen 1993; Petty et al. 2011; Tubbs and Tainer 2011), an observation in line with *in vivo* results showing a preferential binding of p53 to 3D DNA loops (Coufal et al. 2013; Jett et al. 2000; Stros et al. 2004; Vaughan et al. 2014).

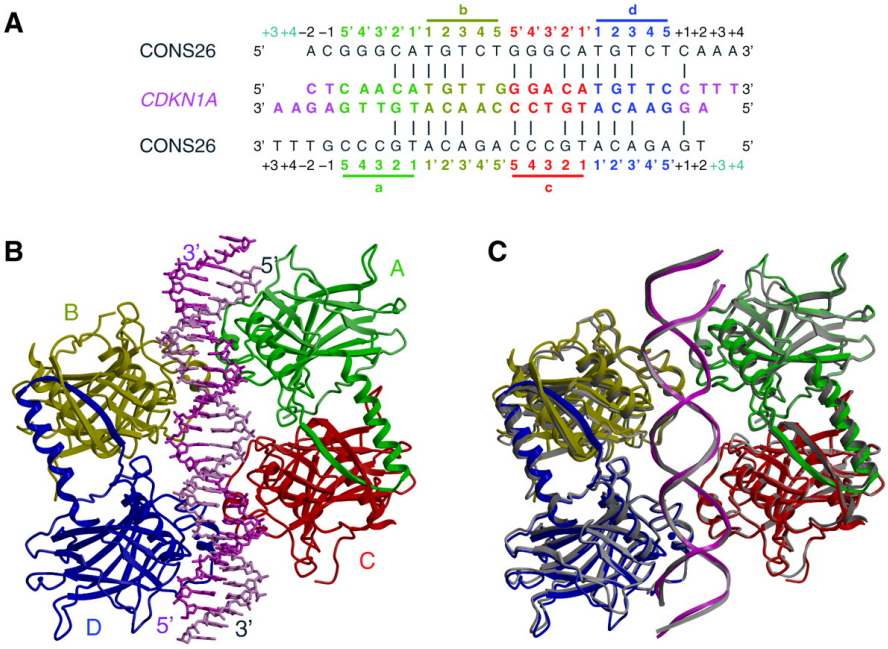


Figure 19: 3D structure of a multidomain p53 oligomer bound to the *CDKN1A*–p53-response element by (Emamzadah et al. 2011). (A) sequence of the oligonucleotides containing the *CDKN1A*–p53-response element (*CDKN1A*), and comparison to the sequence of oligonucleotides (CONS26) containing an artificial consensus p53-binding site.

The p53-binding site consists of 4 contiguous pentamer repeats (a–d). (B) overall 3-dimensional structure of 2 p53CR2 (modified p53 protein) dimers bound to the *CDKN1A*–p53-response element. The p53 monomers are labeled A–D, corresponding to the DNA pentamer repeats a–d, respectively. (C) superimposition of the p53CR2–*CDKN1A* (colored) and p53CR2–CONS26 (gray) structures.

In addition to the DNA-binding and homotetramerization domains, the transactivation domains (TADs; TAD I in residues 20-40 and TAD II in residues 40-60), located at the N-terminus of the protein, are of great interest. They are responsible for the interaction with MDM2, the negative regulator of p53, which binds to the TAD to block transcription and promote ubiquitination of the C-terminal domain of p53 and its degradation (Kussie et al. 1996; Lin et al. 1994). Loss of TAD I function, most frequently achieved by simultaneous mutation of leucine 22 (L22) and tryptophan 23 (W23) (p53L22Q/W23S), produces a p53 mutant that was initially thought to be virtually devoided of all transcriptional activity. Contrary, it has been shown that this mutant (p53L22Q/W23S) can activate a subset of p53 proapoptotic targets (Baptiste-Okoh, Barsotti, and Prives 2008; Johnson et al. 2005; Jung et al. 2006). These findings reveal that p53 regions, such as TAD II and the proline-rich domain, possess the capacity to function autonomously in transcriptional activation. Although TADs may be able to act independently, they also work in concert to recruit specific components of the multisubunit transcriptional activator STAGA complex, namely GCN5, Taf9, and ADA2b, to activate target genes such as p21, Puma, and GADD45 (Gamper and Roeder 2008; Vousden and Prives 2009:53).
It would be an oversight, not to mention that the p53 protein exists in multiple isoforms as a consequence of two promoters at the *TP53* gene, post-transcriptional events like alternative mRNA splicing and internal ribosome entry site. A total of twelve isoforms are known namely p53, p53 (β, γ), Δ40p53 (α β, γ), Δ133p53 (α β, γ), and Δ160p53 (α β, γ) (Khoury and Bourdon 2011). p53 isoforms are expressed in normal and cancer cells and can be simultaneously expressed with full-length p53 protein (Bourdon et al. 2005). It has been mentioned that Δ isoforms have a dominant-negative effect on wild type p53 and consequently prevent the p53-mediated apoptosis (Bourdon et al. 2005), while β isoforms enhance p53 target gene expression. Nevertheless, the exact role of p53 isoforms is not fully characterized.

c) Cell cycle

Cell cycle regulation is mostly controlled by Rb-E2F and MDM2-p53 pathways, which crosstalk between them (Hernández Borrero and El-Deiry 2021; Lim and Kaldis 2013; Polager and Ginsberg 2009; Timmers et al. 2007). In unstressed cells, p53 is targeted by the E3 ubiquitin ligase MDM2 for degradation, keeping p53 at low level. A variety of stress signals recognized by sensor proteins (ATM, ATR, Chk1, Chk2, HIPK2, DNA-PK, and p14ARF) relieve p53 from MDM2 inhibition. p53 binds DNA in a sequence-specific manner and recruits transcriptional machinery components to activate the expression of a network of target genes with various functions. Proteins encoded by p53 target genes function in multiple processes that include, but are not limited to, cell cycle arrest (p21, GADD25 and 14-3-3 σ) and apoptosis (PUMA, BAX, NOXA, APAF1, DR5, PIGs). Cell cycle arrest genes control G1/S (Harper et al. 1995:21; Nakanishi et al. 1999; Stewart, Leach, and Pietenpol 1999) and/or G2/M checkpoints (Bates et al. 1998:53; Engeland 2022; Rainey et al. 2008; Salvador, Brown-Clay, and Fornace 2013) through cyclin-mediated pathway promoting DNA repair. Apoptotic genes promote controllable cell death, in order to eliminate damaged cells (Figure 20).

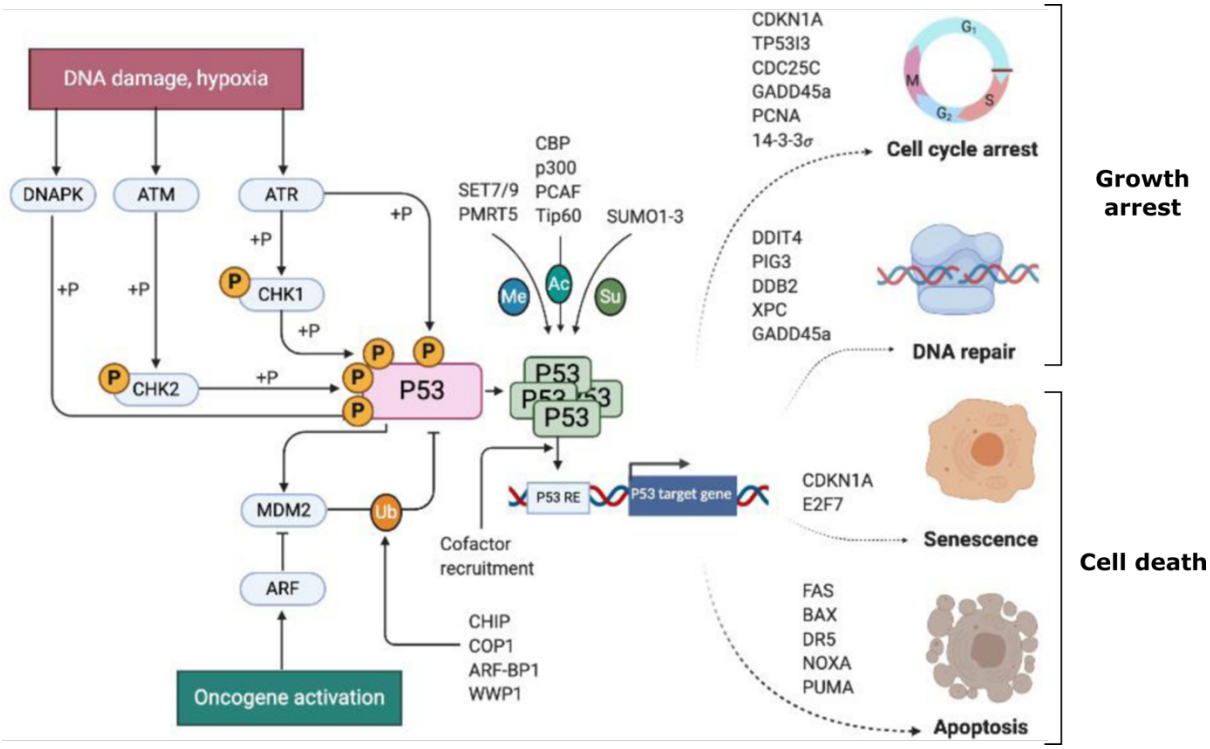


Figure 20: Cell cycle regulation by p53, adapted by (Polager and Ginsberg 2009) and (Hernández Borrero and El-Deiry 2021). Numerous cellular stresses activate stress sensor proteins such as ATM, ATR, Chk1, Chk2, DNA-PK, and p14ARF. These kinases phosphorylate p53 leading to its stabilization, oligomerization, and binding to the p53RE. P53 stability is mainly regulated by the E3 ubiquitin ligase, MDM2, which is also a p53-target, thus forming a negative feedback loop. Further protein modifiers and cofactors that bind to the p53 protein regulate the transcriptional activity of its target genes. When p53 is activated, it induces several biological responses, including cell cycle arrest, DNA repair, apoptosis, and senescence. p53-induced growth arrest is mediated by transactivated genes that encode inhibitors of cell cycle progression, such as the cyclin-dependent kinase (CDK) inhibitor p21. p53-induced cell death involves transactivation of numerous pro-apoptotic genes, as well as transcription-independent mechanisms, the latter typically involving the mitochondria.

In cell cycle arrest, the most well-documented link between p53 and E2Fs is the CDK inhibitor p21, a classic transcriptional target of p53 that impinges on the CDK–Rb–E2F pathway leading to the repression of E2f activity and cell cycle arrest (Engeland 2022) (Figure 21). In particular, the *Cdkn1A* gene codes for the p21 protein, which inhibit the phosphorylation of Rb mediated by CDKs, thus Rb remains bound to E2Fs preventing the transcription necessary for cell-cycle progression. Specifically, p21 interaction with cyclin E/CDK2 and cyclin D/CDK4 promotes Rb binding to E2F resulting in G1 arrest. On the other hand, p21 association with cyclin B/CDK1 results in G2/M cell cycle arrest. Furthermore, studies have provided evidence for additional p53–E2F crosstalk in cell cycle arrest, whereby activator E2Fs repress p53 activity and repressor E2Fs function downstream of p53 (Polager and Ginsberg 2009). Recent studies highlight the importance of p53 in cell cycle regulation in mESCs (Ter Huurne et al. 2020), reminding its ancestral role in germ lines.

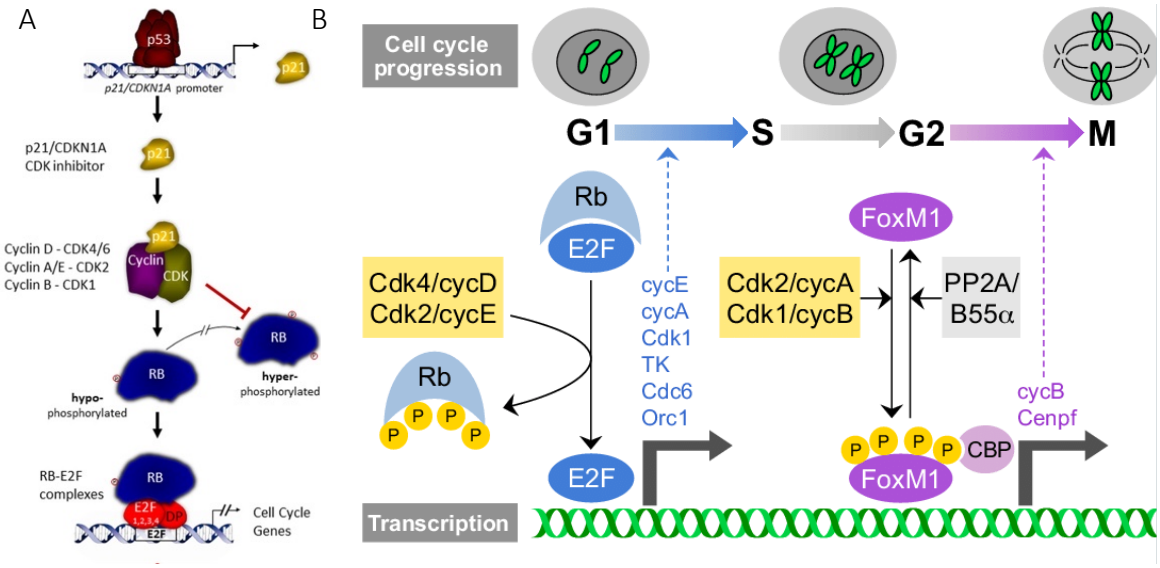


Figure 21: p53 pathway indirectly controls G1/S and G2/M checkpoints, adapted by (Engeland 2022; Lim and Kaldis 2013). (A) Following p53 activation, transcription of p21/CDKN1A is strongly induced as a direct target of p53. The cyclin-dependent kinase inhibitor p21 then blocks activity of several cyclin-CDK complexes, leading to hypophosphorylation of Rb, Rb-E2F complex formation and transcriptional repression of many cell cycle regulators, which result in cell cycle arrest. (B) In unstressed cells, CDK/cyclin complexes regulate Rb/E2F- and FoxM1-mediated transcription and thus control G1/S and G2/M checkpoints. During the G1 phase of the cell cycle, CDK4/cyclin D (cycD) and CDK2/cyclin E (cycE) complexes sequentially phosphorylate (P) Rb, leading to the activation of E2F proteins and the expression of E2F-responsive genes. This cluster of genes encodes cell cycle regulators required for G1/S transition [cyclin E, cyclin A (CycA) and CDK1], enzymes involved in nucleotide biosynthesis [thymidine kinase (TK)] and components of the DNA replication machinery [Cdc6 and origin recognition complex subunit 1 (Orc1)]. During the G2 phase of the cell cycle, CDK2/cyclin A and CDK1/cyclin B (CycB) complexes sequentially phosphorylate FoxM1, leading to the relief of its self-inhibition and the recruitment of a histone deacetylase p300/CREB binding protein (CBP) that activates the expression of FoxM1 target genes. This cluster of genes encodes cell cycle regulators required for the execution of mitosis (cyclin B) and interactors of the kinetochore complex crucial for proper chromosome segregation [centromere protein F (Cenpf)]. The effects of CDK phosphorylation on FoxM1 can be counteracted by the phosphatase PP2A/B55α.

d) Interactors and post-translational modifications

P53 interactors are proteins promoting p53 covalent modifications (PTMs), controlling its stability and subcellular localization, determining its specificity for selected promoters, or modulating its transactivation potential. At public protein–protein interaction databases, there are more than 300 interactors of p53 (Figure 22) and had been reviewed at the following publications (Chen, Liu, and Tao 2020; Green and Kroemer 2009:53; Hernández Borrero and El-Deiry 2021; Kruse and Gu 2009; Y. Liu, Tavana, and Gu 2019; Vousden and Prives 2009:53). Therefore, we will briefly mention the most important and well-studied.

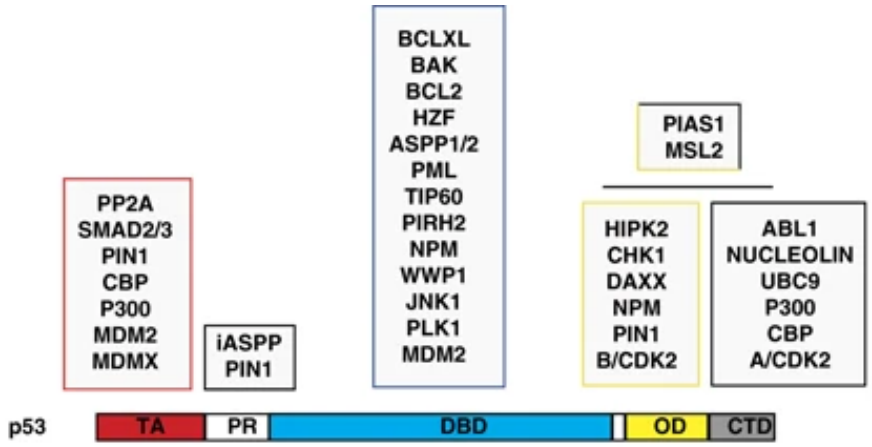
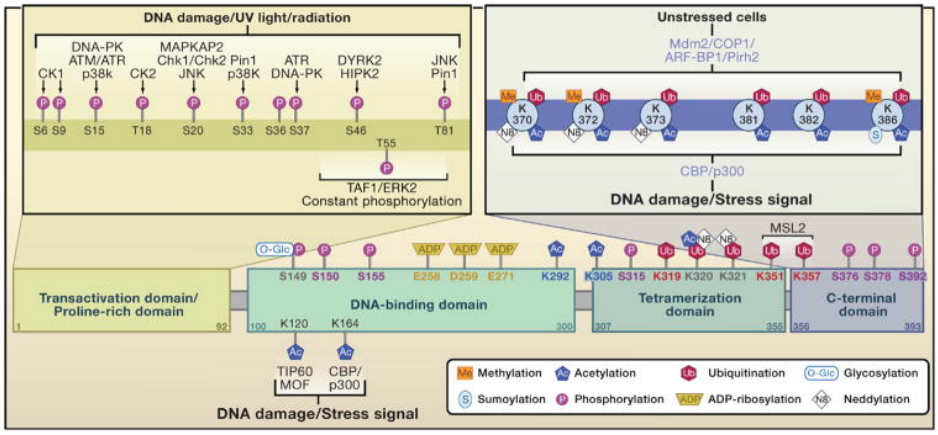


Figure 22: Graphic representation of p53 protein-protein interactions according to their region of binding, adapted by (Collavin, Lunardi, and Del Sal 2010).

p53 is regulated by an array of posttranslational modifications (PTMs, including phosphorylation, ubiquitination, acetylation, methylation, sumoylation, neddylation, O-GlcNAcylation, ADP-ribosylation, hydroxylation, and β-hydroxybutyrylation) both during normal homeostasis and in stress-induced responses (Kruse and Gu 2008, 2009; Y. Liu et al. 2019; Vousden and Prives 2009:53). Proteins implicated in p53 modification and p53-PTM recognition such as MDM2, TIP60 and CBP/p300 have important roles for p53 function and regulation (Figure 23A). For example, Serine 46 (S46) phosphorylation correlates with activation of p53 apoptotic activity (D'Orazi et al. 2002; Hofmann et al. 2002:2; Olsson et al. 2007; Taira et al. 2007), while of serine 106 (S106) phosphorylation by Aurora-A inhibits the interaction between p53 and MDM2 (Hsueh et al. 2013:2).

A



B

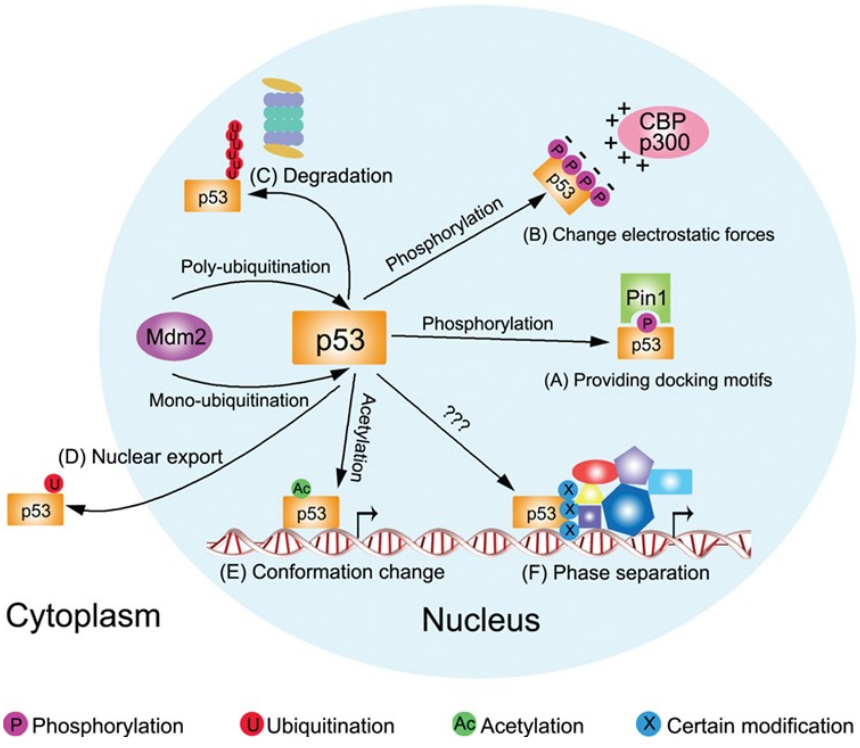


Figure 23: Post-translational modifications of p53 adapted by *(Kruse and Gu 2009)* and *(Y. Liu et al. 2019)*. (A) The graphical representation depicts the major sites of p53 phosphorylation (P), ubiquitination (Ub), and acetylation (Ac) with the corresponding major modifying enzymes and signals. Aditionally, major sites of methylation (Me), sumoylation (S), neddylation (N8), glycosylation (O-Glc), and ribosylation (ADP), are indicated. (B) PTMs can regulate p53 in different modes. (a) Phosphorylation at S33, T81, and S315 of p53 provides docking motif for Pin1. (b) Phosphorylations at p53 N-terminal mask it with negative electrostatic forces to facilitate the binding of CBP/p300 with positive electrostatic forces. (c-d) Mdm2 polyubiquitinates p53 for proteasomal degradation whereas monoubiquitinates it for nuclear export. (e) Acetylation at K120 by Tip60 may cause p53 conformational change, making it bind to specific target gene promoter. (f) Certain p53 modifications may help establish phase separation with other regulators.

Another important p53 interactor is the complex CBP/p300 (CBP:co-activators CREB-binding protein, p300: paralog E1A-binding protein) which acetylate p53 at several C-terminal lysines (K370, K372, K373, K381, K382, and K386) (Gu and Roeder 1997) and boost p53 binding at REs and activate downstream pathways. Moreover, K120 residue on the DBD is another extensively studied p53 acetylation site, as it is frequently mutated in cancers, and its acetylation is catalyzed by three members of the MYST HAT family (Tip60, MOF, and MOZ). Tip60 acetylates p53 at K120 to selectively induce the expression of proapoptotic genes (like PUMA and Bax), but not cell cycle arrest genes (like p21). Probably, K120 acetylation by Tip60 changes the conformation of the DBD, causing target promoter selectivity (Figure 23B_e)
The most well studied p53 interactors are its negative regulators Mdm2 and MdmX, which are structural homologs (Klein et al. 2021:53; Michael and Oren 2003, 2003). Mdm2 is an E3 ubiquitin ligase responsible for the proteasomal degradation of p53 by ubiquitinilating p53 at six different lysine residues within the CTD (K370, K372, K373, K381, K382, and K386) through an autoregulatory feedback loop (Rodriguez et al. 2000). The phosphorylation of Ser166 stabilizes Mdm2 to regulate p53 activity. A high level of Mdm2 promotes p53 polyubiquitination and degradation (Li et al. 2003:2) (Figure 23B_c), whereas a low level induces monoubiquitination and nuclear export of p53 for transcription-independent p53 functions (Green and Kroemer 2009:53) (Figure 23B_d). Interestingly, loss of p53 rescues the early mice lethality caused by an Mdm2 mutant lacking E3 ligase activity, which indicate that the primary role of Mdm2 is the degradation p53 (Brooks and Gu 2006; Itahana et al. 2007; Moyer, Larsson, and Lozano 2017). Furthermore, Mdm2 itself is a transcriptional target of p53. Thus, p53 and Mdm2 can form a double-negative regulatory loop (Lu 2017; X. Zhou, Cao, and Lu 2017). Mdm2 has also been shown to reduce p53 acetylation, by displacing p300 from p53 (Ito et al. 2001:2; Kobet et al. 2000; Teufel et al. 2007:53), and repress acetylation of either p53 or histones in the vicinity of p53-binding sites by recruiting the histone deacetylases HDAC1 (Ito et al. 2002) and KAP1 (Wang et al. 2005). Apart from Mdm2's ability to displace acetyltransferases, Mdm2 can directly ubiquitinate histone H2B (Minsky and Oren 2004) to negatively regulate p53 transactivation at target p53 response elements. In addition, MDM2 promotes DNA compaction through stabilization of histone deacetylase (Choi et al. 2019) and association with the polycomb repressive complex 2 (PRC), resulting in both trimethylation and monoubiquitination of histones (Wienken et al. 2016:53), while increasing DNA accessibility via degradation of the major methyltransferase suppressor SUV39H1 (Mungamuri et al. 2016). Interestingly, MDMX also interacts with members of the PRC complex and thereby supports histone ubiquitination (Wohlberedt et al. 2020).

e) p53 & DNA methylation

CpG island methylation is a common feature of many human cancers and is thought to play an important role in cancer initiation and progression. Aberrant promoter hypermethylation of

CpG islands associated with tumor suppressor gene can lead to transcriptional silencing resulting in tumorigenesis. (Diala and Hoffman 1982, 1982; Hoffman 2017; Pérez et al. 2018). This phenotype could be a result of negative regulation of the *Dnmt1* promoter by p53. More precisely, p53 forms a repressor complex with the specificity protein 1 (Sp1) and chromatin modifiers on the *Dnmt1* promoter leading to a reduced level of DNMT1 expression (Lin et al. 2010) (Figure 24). In line with this observation, deletion or mutation of p53 results in overexpression of DNMT1 (Guo et al. 2008; Peterson, Bögler, and Taylor 2003). Additionally, in brain tumors and lung cancers, it had been observed an association of promoter hypermethylation and p53 mutations (Shamsara et al. 2009; Wirsching, Galanis, and Weller 2016; Wolf et al. 2001). Furthermore, other studies have shown that DNMT1 physically interacts and binds to p53 into the nucleus inducing CpG methylation (Estève, Chin, and Pradhan 2005) (Figure 24) and establishing heterochromatin through HDAC recruitment (Fuks et al. 2000; Hoh et al. 2002).

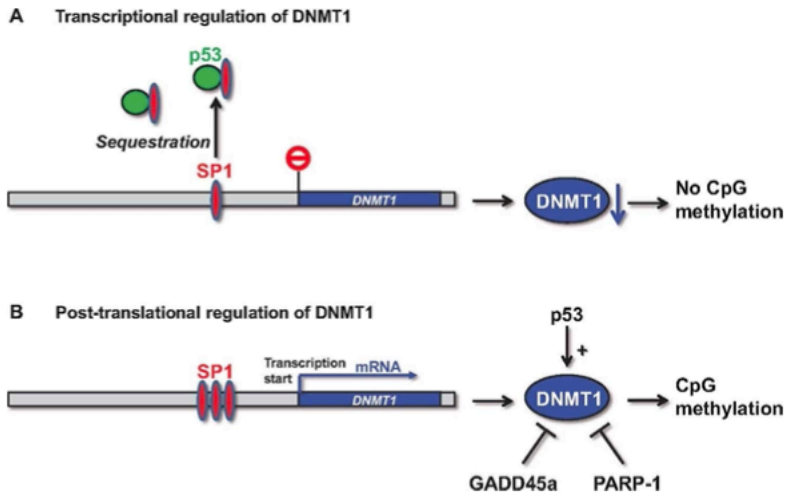


Figure 24: p53 mediated regulation of DNMT1 at (A) transcriptional and (B) post-translational level adapted by (Christmann and Kaina 2019).

Interestingly, a recent study provides evidence that p53 binds via its DBD domain with higher affinity to methylated CpGs *in vitro* and provides evidence for increased *in vivo* p53 occupancy at methylated binding sites, correlating with primed enhancer histone marks (Kribelbauer et al. 2017). These findings raise the question of whether changes in the methylome could trigger differential TF binding and, thus, contribute to the onset of disease and its treatment using epigenetic drugs that alter the DNA methylation profile (Levine 2017).

f) Tumor suppressor activity of P53

The gene encoding the tumor suppressor protein p53 (Boutelle and Attardi 2021) is the most frequently mutated gene in human cancer, about half of all tumors carry mutations or deletions of this gene, suggesting a strong selection against p53 function during tumorigenesis (Bouaoun et al. 2016; Kandoth et al. 2013). Loss of p53 pathway function gives cancer cells a survival advantage to bypass the resolution of oncogenic signals and DNA damage to continue abnormal proliferation. Compared to other tumor suppressors, p53 is unique as mutations can influence its function into different outcomes. p53 mutations are known first and foremost to inactivate the oncosuppressive properties of the wild-type p53 protein as a transcription factor (loss-of-function – LOF). p53 mutant can exert dominant-negative (DN) effects over WT p53, especially if the mutant protein is excessively expressed over the WT one, by forming mixed tetramers that are incapable of DNA binding and transactivation. Mutant p53 proteins can also acquire novel pro-oncogenic properties, an effect known as gain of function (GOF) (Zhang and Lozano 2017). Furthermore, p53 mutants can be classified as class I structural mutants and class II contact mutants. The structural mutants have lower thermodynamic stability and result in misfolded protein compared to the wild-type p53, while contact mutants retain a folded

protein structure but the mutation at the core DBD prevents its binding to the DNA p53RE. Most of the *p53* mutations in human cancer target the DBD (Hainaut et al. 1997; Hollstein et al. 1991). Approximately 95% of p53 observed mutations are located at the DBD.
Li-Fraumeni syndrome (LFS) (Gargallo et al. 2020; Guha and Malkin 2017; Li et al. 2020) is a rare inherited disorder, with frequency of 0.005 – 0.02% worldwide and is characterized by mutations in the *TP53* gene (GOF) predisposing patients to early onset cancer development. The inheritance of a mutant *TP53* allele observed in Li-Fraumeni syndrome further underscore the role of p53 in tumor suppression. The importance of p53 as a tumor suppressor is cemented by experimental evidence from $p53^{-/-}$ mice, which are viable and have normal embryonic development, but die of cancer at young age, mostly thymic lymphomas, with 100% penetrance (Donehower et al. 1992; Kaiser and Attardi 2018).
Moreover, it is known that p53 is a regulator of transposable elements, in addition of being a transcriptional activator of genes in response to cellular stress. A large number of p53 DNA binding sites were detected in transposable elements. Numerous studies in recent years indicate that these genomic elements contribute to p53's tumor suppression role preserving genome integrity (Guidez 2014; Harris et al. 2009; Levine, Ting, and Greenbaum 2016; McDonald et al. 2021; Wylie et al. 2016; Yang et al. 1996). Interestingly, the regulation of transposable elements by p53 is closely associated with the immune response in cancer, particularly the interferon response, a conserved mechanism, known as viral mimicry (Figure 35) (Jansz and Faulkner 2021; Leonova et al. 2013, 2018; Xiaolei Zhou et al. 2021).

3. H3.3 and P53, key players in pHGG

a) Basis of Tumorigenesis

Cancer is defined as the uncontrollable proliferation of cells which results in the formation of tumors and is a medical condition with a molecular basis. The conversion of a healthy cell into a cancer cell results from a series of genetic and epigenetic alterations that give the cell new characteristics. Activation of oncogenes and repression of tumor suppressor genes are the initial cause of cancer due to stress response (Gaillard, García-Muse, and Aguilera 2015; Kontomanolis et al. 2020). The mis-regulation of these genes cause a cascade of transmitting signals allowing cancer cell to survive, proliferate and disseminate (Hanahan and Weinberg 2011). In particular, oncogenes activate autocrine proliferative stimulation or stimulate normal cells to produce growth factors and induce chromosomal translocation, point mutation, and gene amplification resulting in genome instability, while tumor suppressor genes, such as p53, operate as gatekeeper of cell-cycle progression and control the decision between cell division and cell death (Figure 20). Furthermore, these genes activate other signaling pathways related to mitochondrial metabolism, angiogenesis, and inflammation (Figure 17).
It is well known that tumors promote angiogenesis through vascular endothelial (VEGF) or fibroblast (FGF) growth factor, as they have high need for nutrients, oxygen and to evacuate metabolic wastes (Carmona-Fontaine et al. 2017; Jain and Carmeliet 2012; Lugano, Ramachandran, and Dimberg 2020; Rojas-Puentes et al. 2016). It is not surprising that angiogenesis is observed early during cancer invasion (Raica, Cimpean, and Ribatti 2009). In addition, tumors have high energetic and anabolic demands due to the enhanced cell proliferation, therefore they use metabolic alterations. Normally, cells catabolize glucose to pyruvate through glycolysis in the cytoplasm and sequentially, pyruvate to carbon dioxide in the mitochondria. Contrary, cancer cells produce energy not through the 'usual' citric acid

cycle and oxidative phosphorylation in the mitochondria as observed in normal cells, but through a less efficient process of 'aerobic glycolysis', known as Warburg effect. To compensate the lower efficiency of energy production from the transformation of glucose to lactate in cytosol, cancer cells increase glucose uptake by upregulating glucose transporters, such as GLUT1 (Bose and Le 2018; Jones and Thompson 2009; Park, Pyun, and Park 2020; Vazquez et al. 2016).

In addition, it is worth pointing out the significant role of telomerase reverse transcriptase (TERT) in the replication immortality of cancer cells. Telomeres protect the ends of the chromosomes and can be compared as a clocking device determining the limited replicative potential of normal cells, a limit that cancer cells need to overcome. Telomerase, the DNA polymerase responsible for the telomere addition at the ends of telomeric DNA, is over-expressed in most cancer cells. In contrast, its expression levels gradually reduce with the increasing number of cell divisions in normal cells. It is not a surprise that the DAXX/ATRX complex, responsible for the H3.3 deposition at telomeres (see section: Chaperones), has been found to interact with TERT and their mutations to coincide with gliomas (Ak Aksoy et al. 2021; Appin and Brat 2015; M. Tang et al. 2015).

(1) Metastasis & Invasion

Cancer metastasis and invasion are the processes involving dissemination of cancer cells from a primary lesion to distal organs and are the principal causes of cancer lethality (Figure 25). Dissemination of cells from a primary tumor involves many cellular mechanisms. These include invading through, or colluding with, stroma, escaping immune surveillance by inhibiting or co-opting their anti-tumorigenic processes, and evading and adapting the tissue microenvironment (Fischer et al. 2015; Friedl and Gilmour 2009; Kalluri 2016; Li et al. 2016; Massagué and Obenauf 2016; Schaller and Agudo 2020).

E-cadherin is the most studied invasion potentiator which facilitates the cell to cell adhesion. Normally, E-cadherin helps to assemble epithelial cell sheets and maintain the quiescence of the cells within these sheets. Overexpression of E-cadherin negatively correlates with invasion and metastasis. Contrary, loss of its expression in association with the epithelial-to-mesenchymal transition (EMT) occurs frequently during tumor metastasis. However, many metastases continue to express E-cadherin, and a full EMT is not always necessary for metastasis (Na et al. 2020; Rojas-Puentes et al. 2016; Zheng et al. 2015). EMT defines a biological process in which adherent epithelial cells acquire mesenchymal characteristics and become more migratory/invasive. EMT/MET is closely associated with cancer. EMT is thought to be activated in cancer cells, linked to their dissociation from the primary tumor and their intravasation into blood vessels. EMT/MET is a complex network, which involves a variety of factors such as transcription factors (TFs), coding genes and microRNAs (miRs). However, as whole the epigenetics of EMT/MET remains largely elusive. An interesting study showed that the histone variant H2A.Z is implicated in epithelial-to-mesenchymal transition (EMT). The loss of H2A.Z in Xenopus laevis impaired the cell movement required for the formation of the mesoderm and neural crest (Ridgway et al. 2004). Other histone variants, such as H3.3, may also be involved in epithelial-to-mesenchymal transition (EMT).

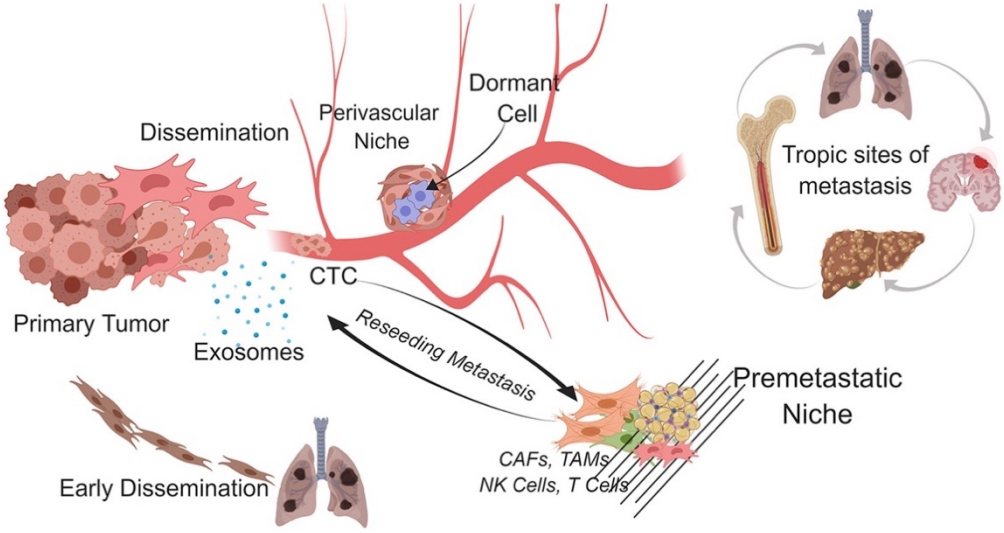


Figure 25: Overview of metastasis by (Suhail et al. 2019). Metastasis is a complex, multiscale process that involves multiple sub-processes occurring in parallel through partially overlapping routes. Premalignant lesions are capable of giving rise to distant, latent metastasis and are not associated with only late-stage primary tumors. However, metastasis occurs mainly through dissemination from malignant lesions when microenvironmental stressors induce cellular reprogramming events that facilitate cellular migration and invasion toward more nutrient-rich niches. These stressors, associated with metabolic reprogramming, can trigger phenotypic changes in cancer cells to adopt more mesenchymal-like states that are not binary, but plastic, with the cells capable of sampling these dynamic states throughout the metastatic process. While this may advance a cell's ability to metastasize, it is now appreciated that it Is likely not the only mechanism by which metastasis occurs. Indeed, there are multiple parallel mechanism co-opted by cancer cells. Lymphatics and blood vasculature are the primary route of cell seeding into the common metastatic organs across cancer types (lymph nodes, liver, lung, bone marrow, and brain), though the tropism cancer exhibit for specific organs is still poorly understood. The combination of genetic and epigenetic changes, and interactions with the diverse milieu of cells in the host microenvironment, determines cancer cell survival and outgrowth.

(2) Immunology of cancer

Pathologists have known for a long time that certain cancers have an infiltration of immune cells from both the innate and adaptive arms (Dvorak 1986; Pagès et al. 2010). Initially, this immune response assumed to be an attempt of the immune system to eradicate tumors. Later, evidences prove that the tumor-associated immune-response is not only associated with eradication signals, but paradoxical tumor-promoting signals. Inflammation can supply the tumor micro-environment with many molecules, including growth factors that sustain proliferation, survival factors that limit cell death, proangiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and factors that lead to activation of EMT (Colotta et al. 2009; DeNardo, Andreu, and Coussens 2010; Grivennikov, Greten, and Karin 2010; Karnoub and Weinberg 2006; Qian and Pollard 2010). Additionally, inflammatory cells can release chemicals, such as reactive oxygen species, which promote mutagenesis and thus tumorigenesis (Grivennikov et al. 2010). Moreover, some cancer cells seem to evade immune destruction by impairing components of the immune system (e.g. disablement of cytotoxic T lymphocytes and natural killer by TGF- β secretion (Yang, Pang, and Moses 2010) or by escapping immune surveillance (Ge et al. 2020; Schaller and Agudo 2020).

b) pHGG: pediatric High-Grade Gliomas

(1) Definition & location

Gliomas are brain tumors arising from glial cells, particularly astrocytes, oligodendrocytes or their precursors. Gliomas are ranked from low-grade gliomas (LGG, grades I and II) to high-grade gliomas (HGG, grades III and IV). Adult HGG arise predominantly in the cerebral cortex, while pediatric HGG (pHGG) have a broader spectrum of locations (Jones, Perryman, and Hargrave 2012; Wu et al. 2014). pHGG can further be divided according to their brain location in diffusive intrinsic pontine gliomas (DIPGs) and in pediatric non-brainstem HGGs (NBS-HGGs). As indicated by its name, DIPGs occur in the brainstem at the pons, while NBS-HGGs can arise either in midline structures (thalamus or cerebellum) or in the cortex hemispheres (Figure 26).

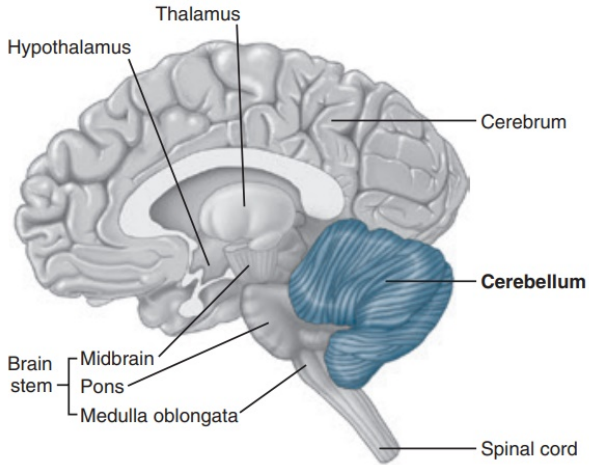


Figure 26: Anatomic representation of brain, adapted by pharmacy180.com

(2) Epidemiology & Diagnosis

High-grade gliomas (HGG) are the most common malignant brain tumors in the pediatric population with the highest incidence in children aged 5 to 9 years (Braunstein et al. 2017; Hoffman et al. 2018; Jones et al. 2012; Juratli et al. 2018). Five-year survival rates for pHGG as a whole are less than 20% despite treatment.

The signs and symptoms of pHGG greatly vary depending on patient age, tumor location and aggressiveness. Patients with pHGG present impairment of recent memory, persistent headache awakening children during night, nausea and vomiting, irritability and change in feeding pattern.

For diagnosis of pHGG, it is first essential to use Magnetic resonance imaging (MRI) which gives indications of the location, size, density, shape and borders of the tumor. After MRI, a biopsy of the tumor is performed in order to classify the tumor and to select the most suitable treatment. Tumor classification is performed through histopathology and molecular classification according to the 2016 WHO (World Health Organization) classification of tumors of the CNS. The histopathology analysis provides information regarding the nuclear atypia, the cellular polymorphism, the number of mitoses, the microvasculature and the presence of necrosis and helps to tumor pre-classification as WHO grade II-III or WHO grade IV. The

molecular analysis of the tumor concludes the final tumor classification based on its genetic alterations. pHGGs present mostly mutations in the histone variant H3.3 (see section: H3.3 mutations in pHGG), in the death DAXX/ATRX complex, in the tumor suppressor p53, in isocitrate dehydrogenase (IDH) and in genes that participate at the RTK-RAS-PI3K signaling pathway, such as NF-1, PDGFRA, BRAF, KRAS, and FGFR1. The rapid advances and cost reductions of high-throughput sequencing solutions lead to a progressive generalization of genome-wide identification of molecular alterations.

(3) Epigenetic background of pHGG

The genetic and epigenetic profiling of pHGG reveals distinct characteristics from adult glioma. A high rate of somatic missense mutations in genes encoding histone H3 isoforms, notably *H3f3a* and *Hist1h3b*, is detected in pHGGs (see section: H3.3 mutations in pHGG), which is accompanied by a reduction in global genomic H3K27 trimethylation (H3K27me3), a transcriptionally repressive mark rendered by the polycomb-repressivecomplex 2 (PRC2) (PRC2). The loss of H3K27me3 results in a global increase in H3K27 acetylation (H3K27ac), a mark that is recognized by bromodomain (BRD) and extraterminal (BET)/BRD proteins that recruit RNA polymerase II and activate transcription. Furthermore, recent studies revealed variant-specific enhancer architecture in DIPG (Nagaraja et al. 2019), and a global increase in the level of other activating histone marks such as H3K36 di- and trimethylation (H3K36me2/3)(Stafford et al. 2018). Therefore, studies are now investigating whether 3D genome alterations may play a critical role in the pHGG epigenetic landscape and thereby contribute to pediatric gliomagenesis (Wang et al. 2021).

(4) Treatment

Current treatments for pHGG include surgery (when possible), followed by radiation therapy combined or not with chemotherapy, but the result remains dismal. Surgery is especially challenging and in the case of DIPG impossible. Radiation therapy is the only palliative measure and can extend life by a few months. For several years, patients with pHGG were treated using aHGG regimens, however the results were inconclusive. Therefore, the need for the development of genetic or epigenetic drugs is vital for pHGG treatment. There are numerous clinical trials ongoing which target kinase that participate at the PI3K/EGFR/PDGFR/VEGF/AXL or BRAF/MET/NTRK/ALK signaling pathways, as well as epigenetic protein which determine the 3D chromatin structure, such as HDACs, demethylases LSD1/JMJD3, methyltransferase EZH2, chromatin reader bromodomains, and chromatin remodeler subunit BMI-1 (Bailey et al. 2018; Haase et al. 2020; Lapin, Tsoli, and Ziegler 2017; Sun et al. 2020). Nevertheless, no efficient treatment is yet available and pHGG prognosis remains really poor. Another limitation to overcome is the limited delivery of the drug to the brain when given systemically due to the blood-brain barrier.

C. Chapter 3: Endogenous retrovirus and their dual role in cancer

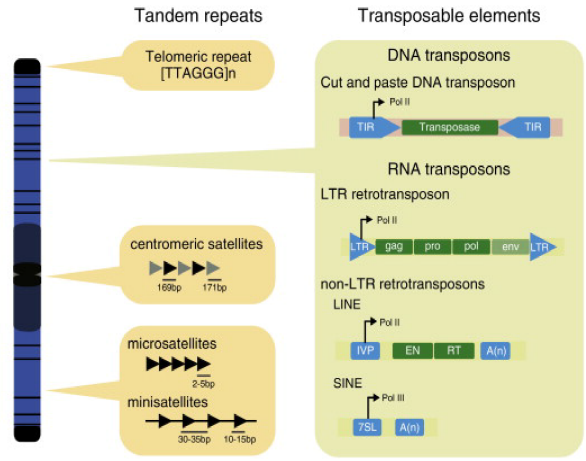
1. Transposable elements

With 70 years of research since the first transposable element was described (McClintock 1950), the non-coding part of the genome previously considered as 'junk' or 'selfish' DNA (Orgel and Crick 1980) has become a field of intense study. The human genome is composed of ~ 3 billion base pairs and less than 2% of its DNA encode genes. In contrast, more than 45% of the human genome is composed of transposable elements (Bannert and Kurth 2004) (Figure 27b). Transposable elements play a vital role in genome stability maintenance and contribute to genomic diversity and evolution (Catlin and Josephs 2022). They are considered as essential contributors to evolution thanks to their ability to modify genomic architecture, gene expression, etc. (Friedli and Trono 2015). Their activity is regulated by various mechanisms considering the deleterious effects of these mobile elements (Senft and Macfarlan 2021). This chapter will provide a brief overview of the classification of transposable elements and their role in evolution and will focus on endogenous retroviruses, their regulation and proposed role in cancer.

a) Classification of transposable elements

Transposable elements (TE) classification is based on parameters such as mechanism of mobilization or sequence homology. TE are divided in two main classes: retrotransposons (class I) and DNA transposons (class II) (Pace and Feschotte 2007; Wicker et al. 2007) (Figure 27). In addition to these two classes, the tandem repetitive sequences (known as satellite DNA) enlarge the DNA repetitive elements family (Figure 27A).

A



B

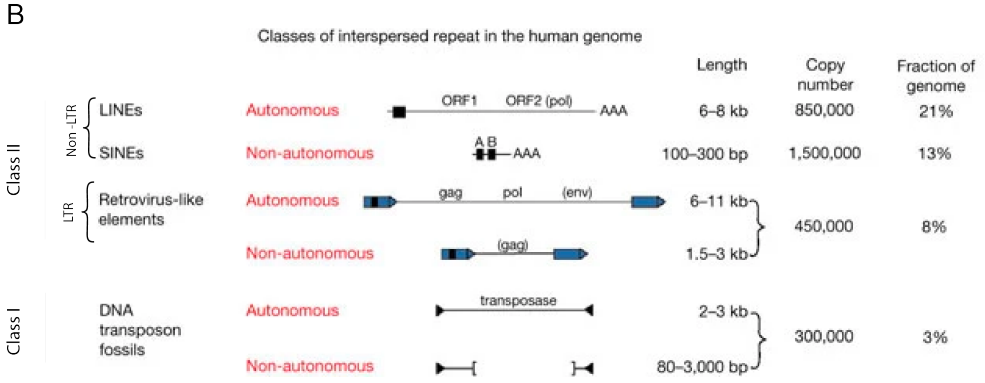


Figure 27: Classification of transposable elements, adapted by (Lander et al. 2001; Padeken, Zeller, and Gasser 2015) A) Chromosomal location of DNA repetitive elements. B) Overall classification of transposable elements with percentage of the overall genome, length and copy numbers are provided in district columns. LTR: long-terminal repeat; SINE: short-interspersed nucleotide elements; LINE: long-interspersed nucleotide elements.

DNA transposons accounts approximately 3% of the genome and performs transposition via a 'cut-and-paste' mechanism without RNA intermediate. Members of this class of TE are active in zebrafish and *Drosophila*, but not in humans, with the last transposition-competent element in primates dating back to 37 million years (Pace and Feschotte 2007; Padeken et al. 2015).

Contrary, retrotransposons amplify via a 'copy-and-paste' mechanism and rely on an RNA transcript which is retro-transcribed by a reverse transcriptase and further integrated into the genome. Active retrotransposons are present in all higher species, including humans. Retrotransposons can be further divided in 'LTR-' and 'Non-LTR'-containing retrotransposons (Beck et al. 2011:201; Castro-Diaz, Friedli, and Trono 2015; Friedli and Trono 2015) (Figure 27).

The LTR-retrotransposons are commonly called endogenous retroviruses (ERVs) as they are derived from ancient exogenous retrovirus infections and they are coding for the viral proteins Gag, Pol and Env which makes them autonomous for retrotransposition (Figure 27 & Figure 30). To note, only a small proportion of DNA sequences are coding for full length TEs and have thus the potential to transpose. More precisely, all humans ERVs are transposition-defective, while several hundred murine ERVs are active and capable of retrotransposition, accounting for up to 10% of spontaneous mutations observed in inbred mice (Maksakova et al. 2006).

The Non-LTR family is sub-composed of autonomous and non-autonomous retrotransposons. The autonomous Non-LTR retrotransposons include LINEs (long-interspersed nucleotide elements). LINEs encode a reverse transcriptase and a nuclease essential for transposition (ORF1 and 2, see Figure 27B). The Non-autonomous are composed of the SINEs (short-interspersed nucleotide elements) and the hominid specific SVAs (SINE-VNTR-Alus). As their name indicates, they rely on other retro-transcription machineries (e.g., coded by LINEs).

b) Transposable elements and their role in genome evolution

TEs promote genetic innovation but also threaten genome architecture and stability. Once a TE infects germ line cells of the host, it becomes a permanent element in the host germ line and is subject to natural selection (Dupressoir, Lavialle, and Heidmann 2012:664; Parrish and Tomonaga 2016). A minuscule proportion of exogenous virus would have invaded host germ lines.

TEs can have a plethora of impacts on the genome. They can directly and indirectly shape genomes. More precisely, they can directly induce deleterious mutations responsible for diseases, which can have disastrous outcomes. For example more than 100 insertions of LINE-1 cause human disease by disrupting host genes (Belancio, Hedges, and Deininger 2008; Goodier and Kazazian 2008), and LTRs can drive oncogenes (Babaian and Mager 2016; Payer and Burns 2019). TEs can disrupt genes (via alternative splicing, truncation or insertion of new exons) or modify their expression (via promoter, enhancer (Chuong et al. 2013) or repressor effects). Additionally, the repetitive nature of TE promotes ectopic recombination and chromosomal rearrangements, such as deletions, duplications or translocations. Also, they can

alter genome architecture via insulator sequences or by nucleating short- and long-range chromatin interactions, or provide entirely novel open reading frames (Figure 28) (Bourque et al. 2018; Ecco, Imbeault, and Trono 2017).

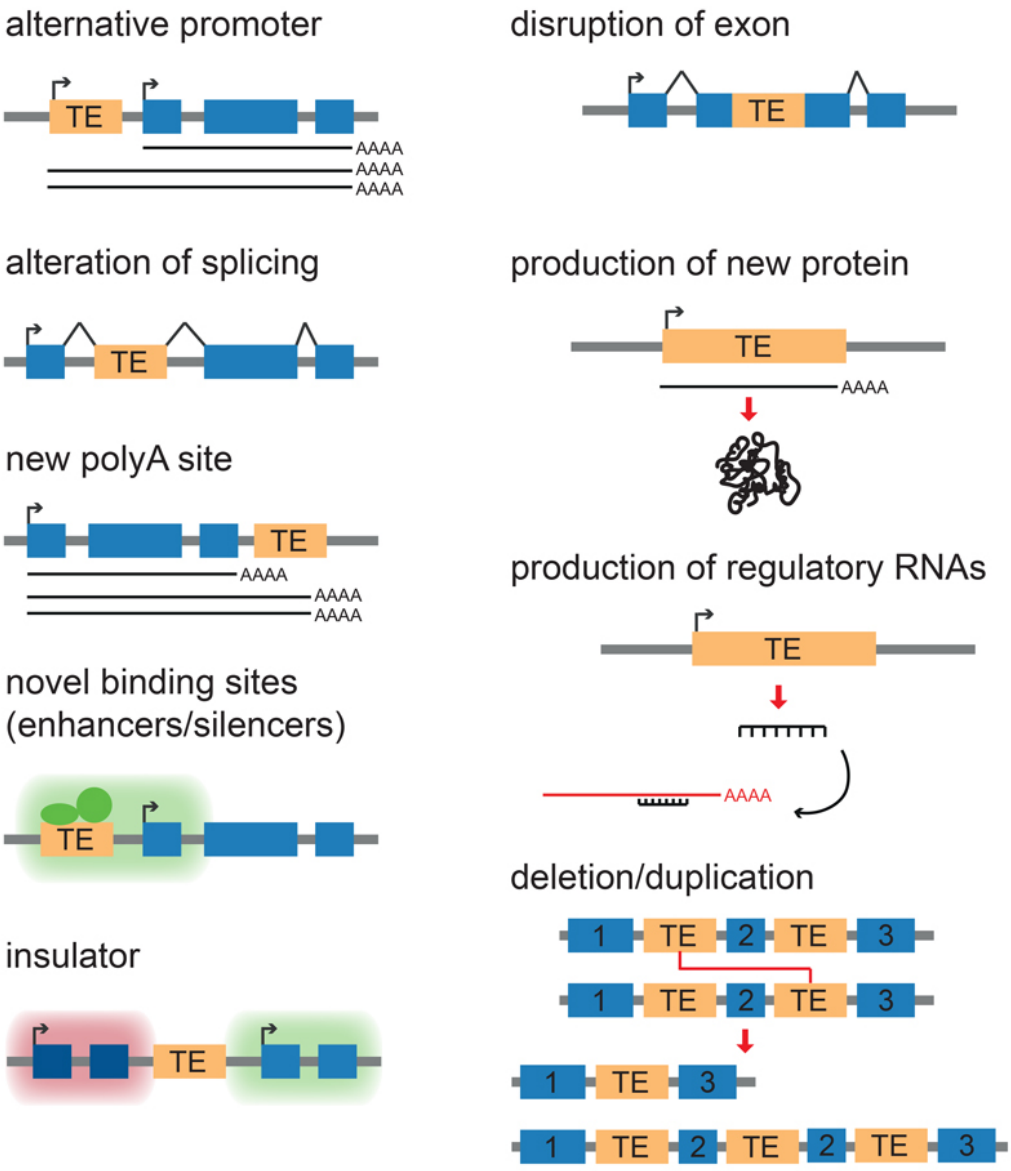


Figure 28: Transposable elements (TEs) and their genomic impact (Ecco et al. 2017). TEs often bear promoters, enhancers, suppressors, insulators, splice sites or transcriptional stop signals and can thus disrupt genes (via alternative splicing, truncation or insertion of new exons) or modify their expression (via promoter, enhancer or repressor effects). Due to their highly repetitive nature, TEs can also provoke recombination events that can lead to deletions, duplications, rearrangements or translocations. In addition, they can alter genome architecture *via* insulator sequences, long-range interaction modifications or they can provide entirely novel open reading frames.

TEs indirectly shape genomes via KRAB-ZFPs (Krüpell-associated box-containing zinc finger proteins). KRAB-ZFPs bound to DNA via their C2H2-type zinc finger arrays, the KRAB domain recruits chromatin modifiers. One cofactor crucial for embryonic development and transcriptional repression downstream of KRAB-ZFPs is the KRAB-associated protein 1 (KAP1) (encoded by the *Trim28* gene). KAP1 constitutes a scaffold for the recruitment of a heterochromatin-inducing machinery composed of the histone methyltransferase SETDB1 (also known as ESET), the histone deacetylase-containing complex NuRD, the heterochromatin

protein 1 (HP1) and the DNA methyltransferases (Figure 33). More details for KRAB-ZFP–KAP1 system will be discussed below (see section: KRAB-ZFPs/KAP1 system).

The repetitive zinc finger arrays of KRAB-ZFPs frequently recombine and acquire mutations in DNA-contacting zinc fingerprint amino acids to achieve diverse and highly specific DNA binding. TEs need to evolve to escape repression in order to remain active. This evolution, along with new colonizations of TEs, pressures KRAB-ZFPs to turn over in response, leading to an 'arms race ' (Figure 29A, B) (Bruno, Mahgoub, and Macfarlan 2019; Ecco et al. 2017). This quick turnover may explain why an astonishing amount of KRAB-ZFPs are species-specific.

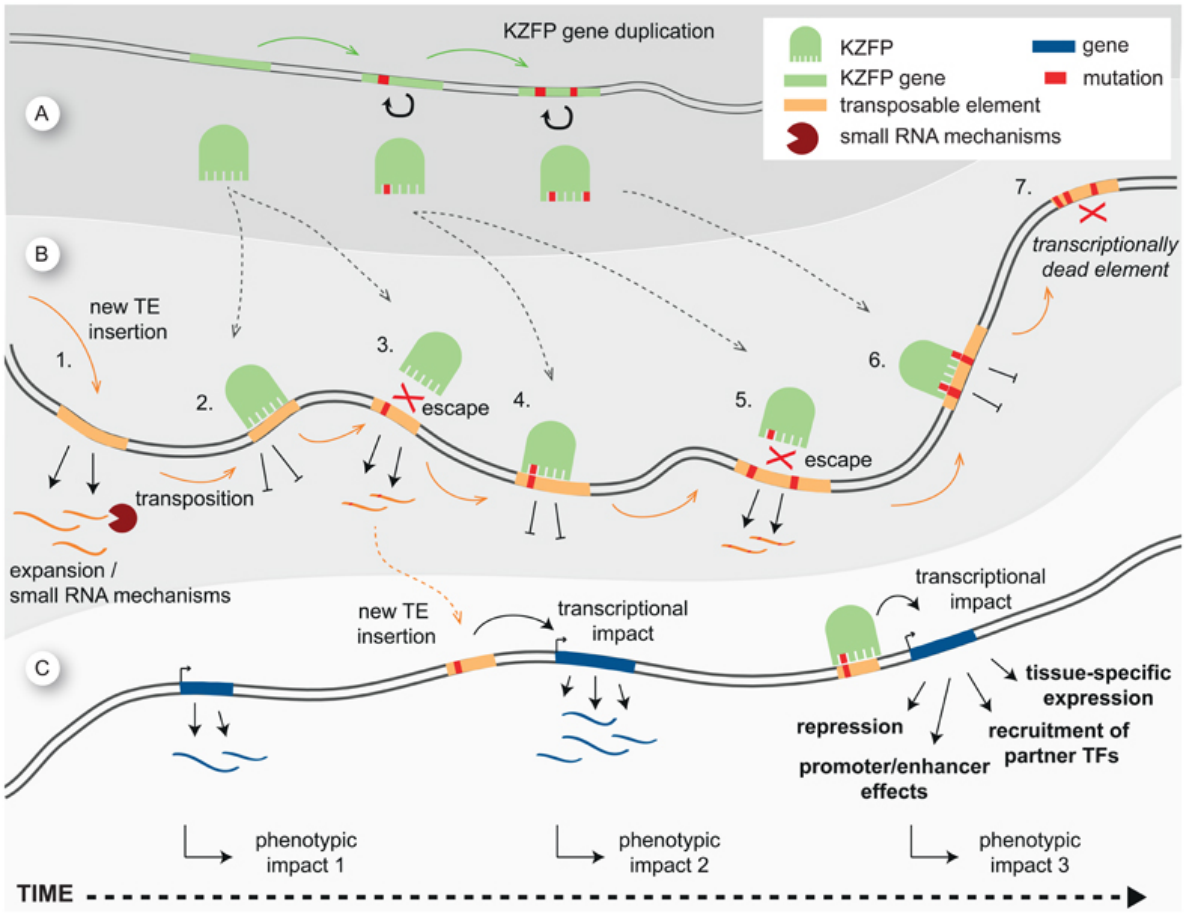


Figure 29: The dual evolutionary drive of KZFPs: arms race and TE domestication (Ecco et al. 2017). (A) KZFPs gene expansion over time. Duplication events and accumulation of mutations are depicted. (B) An evolutionary arms race between KZFPs and TEs. When a novel TE enters the host, it starts to be expressed and to transpose, albeit partly controlled by a first, small RNA-based, line of defense (1). Over time, KZFPs genes duplicate and paralogs emerge that can bind these TEs (2). In parallel, transposons accumulate mutations and escape repression (3 and 5), yet new KZFPs appear that can suppress the expression of these escapees (4 and 6). Eventually some of these TEs accumulate mutations and are rendered inactive (7) (C) From deleterious mutations to co-option. Left, a hypothetical gene would initially have a certain phenotypic impact (phenotypic impact 1). Middle, transposon insertions near genes can lead to transcriptional effects with phenotypic consequences (causing a phenotypic impact 2). Right, some of these could be beneficial for the host, notably if modulated by KZFP-mediated control, which can incur a variety of transcriptional and phenotypic effects. The TE/KZFP pair then can become fixed in evolution, completing the co-option process. KZFPs, KRAB-zinc finger proteins; TEs, transposable element s; TFs, transcription factors.

Collectively, KZFPs and their TE targets partner up to establish species-specific regulatory networks that provide novel host functions, a process called co-option, rather than

transposition blocking (Figure 29C). TE-KZFPs actively shape mammalian-specific developmental processes, particularly during pre-implantation and extra-embryonic development and at the maternal–fetal interface (Parrish and Tomonaga 2016; Senft and Macfarlan 2021). A well know examples of co-option during maternal-fetal interface is syncytin. Syncytin 1 and syncytin 2 in humans and syncytin A and syncytin B in mice are membrane proteins originating from *env* genes of ERVs. These ERVs are involved in the fusion of trophoblast cells, resulting in multinucleated syncytiotrophoblast formation, necessary for nutrient and gas exchange (Dupressoir et al. 2012; Imakawa, Nakagawa, and Miyazawa 2015). To note, even if the mouse and human syncytin have a similar function, they are not at orthologous positions, suggesting that the ERVs of origin were independently co-opted. The function of env-derived syncytin genes is not limited to cell fusion. Syncytins also repress the antiviral host immune response, which was presumably necessary for them to enter the host. The mammalian host, in turn, counteracts syncytin-mediated cell fusion.

In summary, TEs constitute a powerful and essential motor of evolution and hosts co-evolve with them through a fine balance between threat and benefit.

2. Endogenous retroviruses

Endogenous retroviruses (ERVs) are genetic elements that reside as proviruses (Figure 30) in their host's genome, presenting the only known "fossil" record of an infectious agent. 8% of the human and 10% of the mouse genome is of retroviral and retrotransposon origin. The first endogenous retroviruses were discovered in the late 1960s. Retroviruses usually infect somatic cells, but once a retrovirus infects a germline cell, the acquired provirus can be inherited and subjected to natural selection. The majority of proviruses have progressively become disabled by simple accumulation of mutations, deletions or silenced through epigenetic mechanisms (Boeke and Stoye 1997). As a result, among all the copies of a given ERV family, only a few ERVs may still be infectious in some host species. The so called recently "endogenized" ERVs are still able to replicate and integrate new proviral copies within the germline. Examples of relatively recent ERVs integration are the koala retrovirus (KoRV) (Greenwood et al. 2018), cervid ERV (CrERV) in populations of mule deer (Yang et al. 2021), the mouse leukemia viruses (MLVs) and the mouse mammary tumor virus (MMTV) (Stocking and Kozak 2008), or the endogenous jaagsiekte sheep retrovirus (enJSRV) (Varela et al. 2009), which are excellent ways to study the evolution of ERVs on a prospective basis. In other rare cases, only some of the retroviral genes were preserved and have remained functional over several millions of years following ERV integration, such as viral *env* genes.

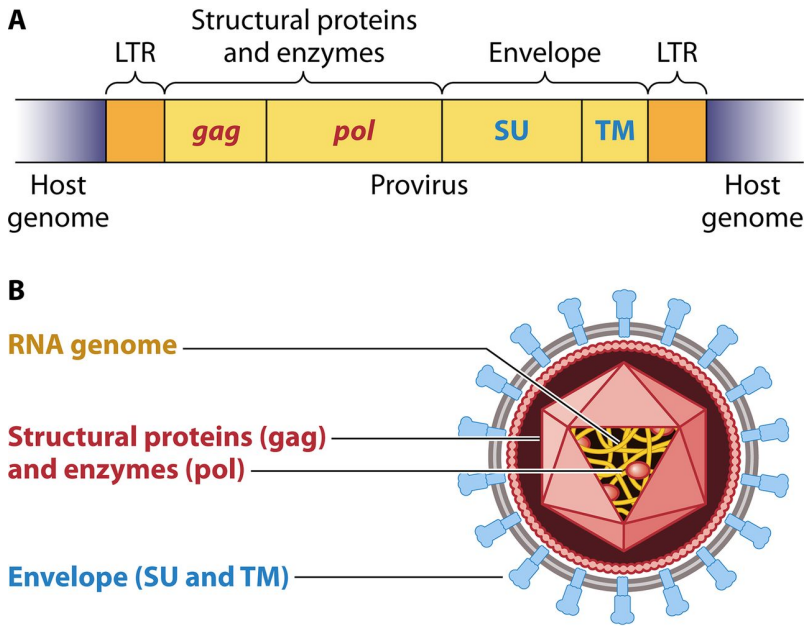


Figure 30: Schematic representation of orthoretrovirus (Greenwood et al. 2018). (A) An integrated double-stranded DNA provirus (yellow) of a simple orthoretrovirus within the host genome (gray) is shown. The long terminal repeats (LTRs) are at both the 5′ and 3′ ends of the provirus and flank the retroviral *gag*, *pol*, and *env* coding regions. Regions coding for enzymes and other proteins are shown with font colors corresponding to their depiction in panel B. (B) Schematic drawing of a simple orthoretrovirus. All orthoretroviruses have three component parts: (i) the RNA genome, shown in yellow; (ii) internal proteins, shown in red, including internal structural proteins (Gag) and the reverse transcriptase (Pol), which makes a DNA copy of the RNA genome that will be integrated into the host cell genome; and (iii) the envelope proteins, shown in blue. Env consists of two components: the TM moiety is embedded in the membrane (depicted in gray and white) of a host cell and is incorporated into the virion during the budding process, and the surface glycoprotein SU forms the knobs and is the part of the virus that binds to receptors on susceptible cells of the host.

a) ERV classification: Three main classes

Currently, there is no well-established standard for naming and classifying all ERVs, due to their repetitive nature, which makes the sequencing alignment challenging. Thus, accurate and locus specific analysis are difficult. Moreover, the lack of consensus in a precise classification led to different denominations for the same elements among different studies. Based on the most accepted method, ERVs are divided into three classes according to their similarities with modern exogenous retroviruses in mouse and human genome. Class I is comprising ERVs clustering with gamma- and epsilon-retrovirus, Class II with lentivirus, alpha-, beta-, and delta-retroviruses and Class III with spumaviruses (Figure 31). Shared characteristics used for ERVs classification are translational strategy, number of zinc finger proteins in the NC (nuclear capside) of gag, the presence and location of dUTPase, presence of a GPY/F motif in the C-terminal end of the Pol integrase (IN), accessory genes, and especially the reverse transcriptase gene. Even though mice and human share a common ancestor approximately 100 million years ago, as it is proved by their homology, a markedly dissimilar evolutionary history has been noted, both in the distribution and number of ERV families within the different classes but also in the fact that ERVs are nearly extinct in human, whereas in mouse there are many active members (Stocking and Kozak 2008).

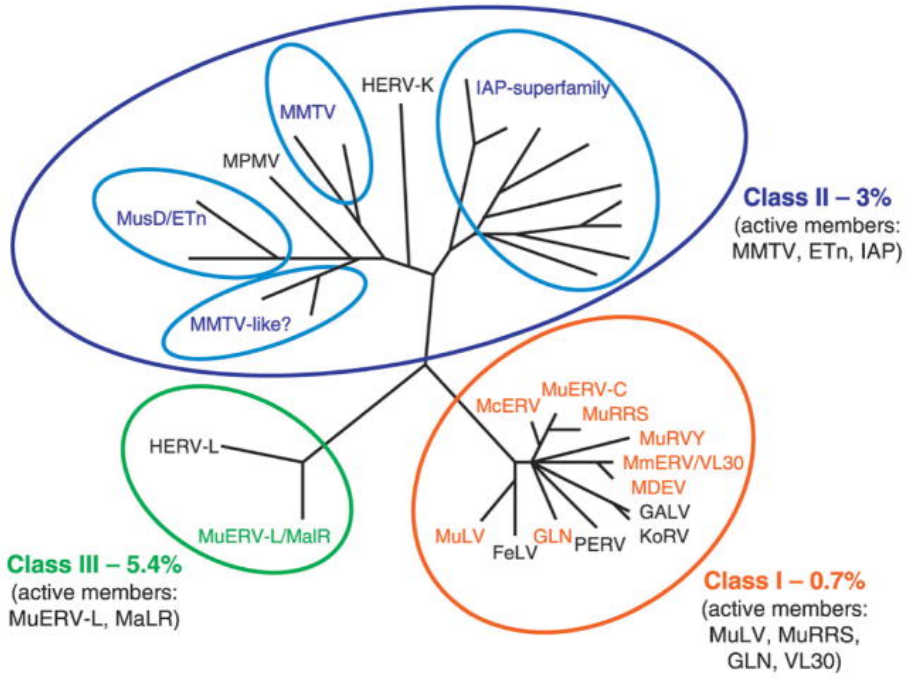


Figure 31: Phylogenetic analysis of ERV reverse transcriptase (RT) domains leading to three classes of mouse ERVs (Stocking and Kozak 2008). ERVs from host species other than mouse are included for comparison and are in black letters. Four distinct superfamilies are defined for the Class II ERVs, one of which (MMTV-like) is poorly characterized. Non-autonomous elements, such as the abundant VL30 s (Class I), eTns (Class II), and MaLRs (Class III) are listed with their presumed parental ERVs, as they do not contain RT domains.

b) ERV mechanism of reverse transcription and genome integration

The majority of LTR-retroelements use host tRNA to prime reverse transcription and copy their RNA into DNA for insertion into the genome (Figure 32)(Chapman, Byström, and Boeke 1992; Le Grice 2003; Levin 1995; Schorn and Martienssen 2018). In the case of HIV-1, $tRNA^{Lys}$ is the selected primer (Kleiman and Cen 2004:1). tRNAs are abundantly available at the host cytoplasm during viral proteins translation. Retroviral proteins bind specific tRNAs with high affinity and recruit them to the virus particle where their 3′-end initiates reverse transcription at the tRNA primer binding site (PBS). Then, the reverse transcriptase (RT) synthesizes a minus-strand DNA and produces an RNA-DNA hybrid. Continuing, the RNA-DNA duplex becomes the substrate of the RNaseH activity of RT, which cleaves the RNA strand at numerous points, leaving short RNA fragments hybridized to nascent DNA. Within these RNAs, two specific purine-rich sequences, known as the polypurine tracts (PPTs), serve as primers to initiate the synthesis of plus-strand DNA, thus creating the double-stranded DNA genome. Specific cleavages by RNaseH then remove the PPT primers and expose the integration sequence of the viral DNA into the host chromosome through recombination (Hughes and Coffin 2004; Jern and Coffin 2008; Katzourakis, Pereira, and Tristem 2007; Stoye 2001).

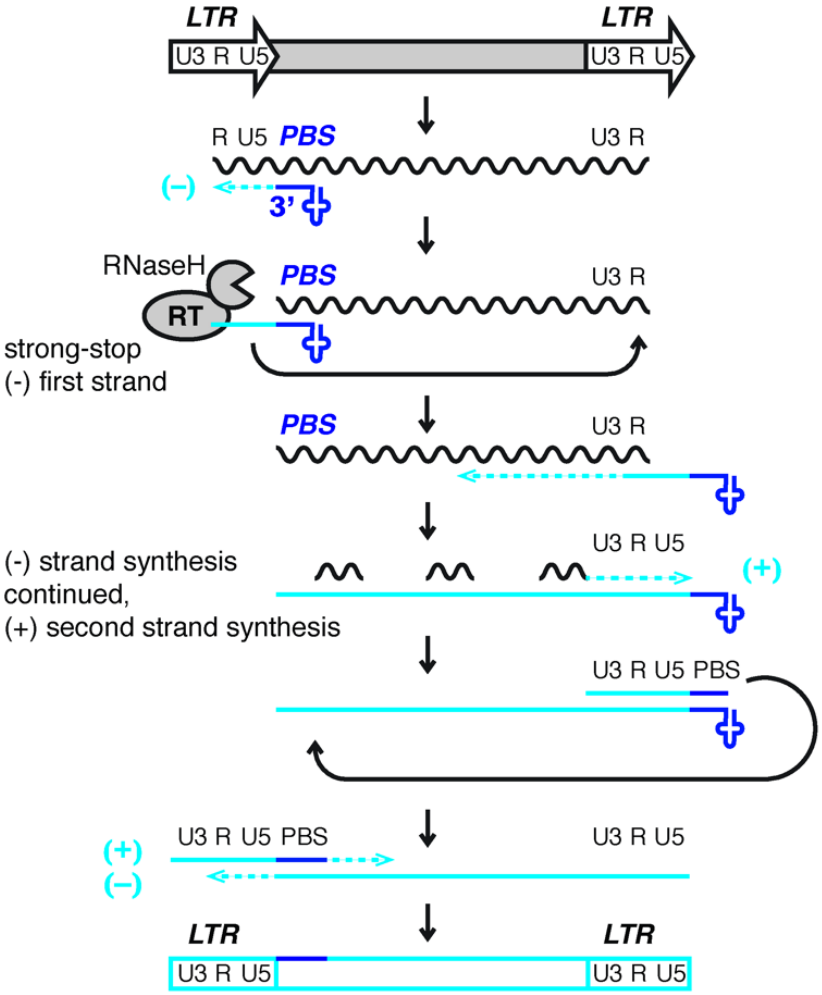


Figure 32: Model of reverse transcription of long terminal repeat (LTR)-retrotransposons and -viruses by (Cullen and Schorn 2020). LTRs encode promoter elements and termination signals. The RNA transcript contains a region repeated at either end (R), a 5′ unique segment (U5), and a segment only included at the 3′-end of the RNA (U3). The 3′-end of cellular tRNAs (blue cloverleaf) primes reverse transcription by hybridizing to the primer binding site (PBS). While this segment is being copied into first-strand cDNA (light blue line), also called minus (–) strong stop DNA, the RNaseH activity of reverse transcriptase (RT) degrades the template RNA. The elongating cDNA is transferred to the 3′-end of the retrotransposon transcript hybridizing to the R region. The remaining RNA is partially degraded by RNaseH leaving behind primers for second-strand, plus (+) cDNA synthesis. In *Retroviridae*, the plus strand PBS is a copy of the tRNA primer, while the minus strand is a copy of the original PBS sequence. After another transfer event, first (–) and second (+) strand synthesis are completed to result in a full-length, double-stranded retroviral DNA that will be integrated into the host genome.

c) ERV regulation

It became quite obvious that transcriptional expression and transposition of ERVs need to be tightly regulated from early development to provide genomic stability and avoid aberrant expression. Retrotransposition in germ cells would lead to germline mutagenesis and vertical transmission (Boeke and Stoye 1997; Friedli and Trono 2015; Maksakova et al. 2006), whereas mutagenesis in somatic cells could cause oncogenic transformation (Callahan and Smith 2000; Howard et al. 2008). Thus, the majority of TEs are epigenetically silenced *via* several mechanisms which induce dynamic heterochromatin formation (Figure 34), including DNA methylation, histone modifications, remodeling complexes, histone variants and RNA-mediated silencing.

(1) KRAB-ZFPs/KAP1 system and chromatin remodeling complexes

Krüpell-associated box-containing zinc finger proteins (KRAB-ZFPs) are the largest family of transcriptional repressors in higher vertebrates and are about 420 million years old (Imbeault, Helleboid, and Trono 2017; Yang, Wang, and Macfarlan 2017). KRAB-ZFPs contain an array of a variable number of tandem copies of C2H2 zinc fingers, which confer high DNA-binding specificity. KRAB-ZFPs bind TEs thanks to their C-terminal zinc fingers domain, whereas they recruit the KRAB-associated protein 1 (KAP1, also known as TRIM28) through their N-terminal KRAB domain (Friedman et al. 1996). TRIM28 further recruits corepressors and other chromatin modifiers to induce transcriptional suppression and heterochromatin formation. More precisely, Trim28 act as a scaffold protein for the recruitment of a heterochromatin-inducing machinery composed of the histone methyltransferase SETDB1 (also known as ESET), the histone deacetylase-containing complex NuRD, the heterochromatin protein 1 (HP1), and the DNA methyltransferases (Figure 33) (Giménez-Orenga and Oltra 2021; Imbeault et al. 2017; Yang et al. 2017). SETDB1, one of the H3-specific histone methyltransferases (rest HMTs of H3: Suv39h1, Suv39h2, Glp, and G9a) deposits the methylated H3K9 at ERV sites. H3K9me3 forms a binding site with high affinity for HP1 (Anon n.d.; Jacobs et al. 2001:3; Jacobs and Khorasanizadeh 2002; Lachner et al. 2001). This way KRAB-ZFPs/KAP1 complex is able to suppress activity of promoters or response elements (RE) several tens of kilobases distant from their DNA-binding sites. This long-rage heterochromatin spreading probably is achieved by DNA looping (Groner et al. 2010).

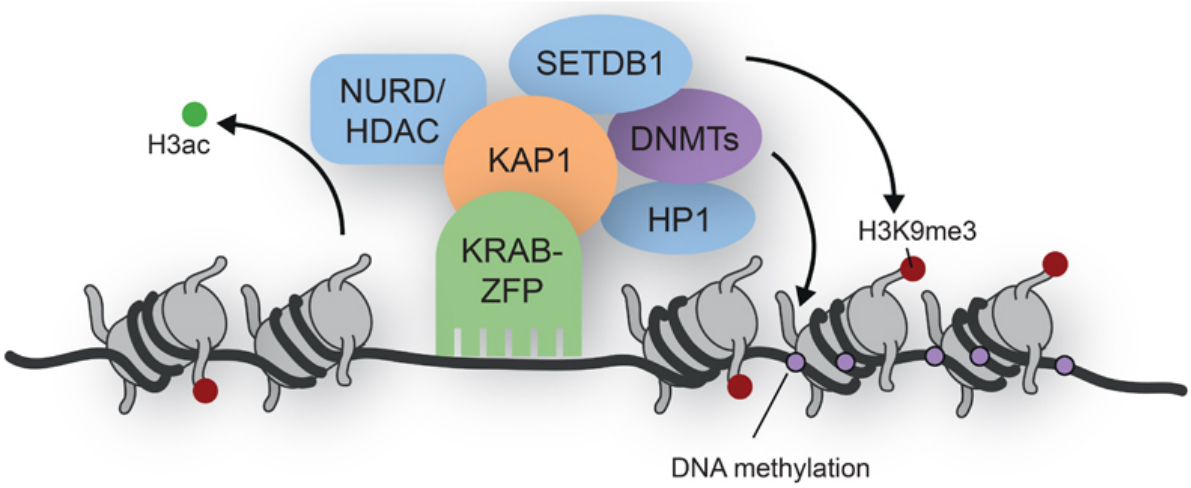


Figure 33: The KRAB-ZFP/KAP1 repressor complex by (Imbeault et al. 2017). KRAB-ZFPs (green) bind to DNA via their zinc fingers and recruit KAP1 (orange) via their KRAB domain. KAP1 then recruits a repressor complex, leading to heterochromatin formation through histone methylation (H3K9me3), DNA methylation, histone deacetylation (H3ac), and transcriptional silencing. DNMT, DNA methyltransferase; H3ac, acetylated histone H3; HDAC, histone deacetylase; HP1, heterochromatin protein 1; KAP1, Krüppel-associated box (KRAB)-associated protein 1; KRAB-ZFP, KRAB-zinc finger protein; NuRD, nucleosome remodeling deacetylase complex; SETDB1, SET domain bifurcated histone methyltransferase 1.

Loss of TRIM28 leads to overexpression of a wide range of ERVs, loss of H3K9me3, and, thus, absence of heterochromatin formation at ERVs (Rowe et al. 2010), which seems to directly impact euchromatin regions with reductions in chromatin accessibility, histone acetylation, and TF binding in mESC, because de-repressed ERVs effectively compete with euchromatic regions for TF binding (O'Hara and Banaszynski 2022). This observation indicates that heterochromatin at ERVs is a necessity for euchromatic TF binding and transcriptional homoeostasis. Furthermore, it proposes that ERVs de-repression during pre-implantation may provide plasticity required for cell fate decision (O'Hara and Banaszynski 2022). In line with this observation, loss of TRIM28 leads to transcriptional activation of adjacent genes, apart from ERVs de-repression and loss of H3K9me3 in neural progenitor cells (NPCs) (Fasching et al. 2015).
Furthermore, TRIM28 and SETDB1 together seem to regulate ERVs to a different extent than TRIM28 alone. Setdb1 knock-out results in derepression of additional ERV classes, mostly class I and II, beyond those seen with single knock-out of *Trim28* (Karimi et al. 2011b; Matsui et al. 2010; Rowe et al. 2010). SETDB1 acts through a KAP1-independent pathway, and directly bind to H3K9me1/K14ac or H3K9me2/K14ac histone tails through its N-terminal triple Tudor domain (TTD) to deacetylate K14 via SETDB1-associated histone deacetylases (HDACs) and further methylate K9 to achieve a trimethylated (H3K9me3) state (Jurkowska et al. 2017).
Recently, the chromatin remodeling factor SWI/SNF subunit SMARCAD1 has also been identified as a TRIM28 interactor and a key regulator of ERVs, in mESCs (Ding et al. 2018; Sachs et al. 2019), suggesting that many chromatin remodeling complexes may regulate the expression of ERVs by interacting with KRAB-ZFPs/KAP1 complex and promoting histone dynamic and exchange (Navarro et al. 2020).
Last but not least, the reinstalment of silenced ERVs is facilitated by TRIM28 after DNA replication, as this scaffold protein also interacts with several DNA replication factors (Jang et al. 2018; Talbert and Henikoff 2017).

(2) DNA methylation

Several evidences reported the importance of DNA methylation in ERVs silencing. From the very first steps of mammalian embryogenesis, ERVs undergo de novo DNA methylation, and DNMTs recruitment seems to need the KRAB-ZFPs/KAP1 and SETDB1 binding in advance (Rowe, Friedli, et al. 2013; Rowe and Trono 2011). Probably by using the mechanism of ERVs silencing described above (Figure 33) and by imprinting stable epigenetic marks at ERVs which are then maintained throughout development. In line with this observation, Papin et al. showed the existence of a highly dynamic and distinct DNA methylation patterns at ERVs during differentiation (Papin et al. 2017). They proposed a model in which IAP/ERVK methylation is highly dynamic and dependent on TET/TDG activities in mESC and become fully and stably methylated in differentiated cells. The role of DNA methylation on ERVs repression has been studied in knockout mouse models for DNA methyltransferases (Dnmt1, Dnmt3a and Dnmt3b) (see also section DNA Methylation). In testis, depletion of Dnmt3l and subsequent increased IAP expression caused infertility due to loss of germ cells (Bourc'his and Bestor 2004). During

mouse development, DNMT1 loss led to termination of embryogenesis, while in somatic cells it lead to transcriptional activation of ERVs, including CpG-rich IAP (Kurihara et al. 2008:1; Min et al. 2020; Sharif et al. 2016; Walsh, Chaillet, and Bestor 1998). Double knock-down of Dnmt3a and Dnmt3b led to severe embryonic phenotypes similar to Dnmt1 knock-down (Maksakova, Mager, and Reiss 2008), while loss of one of the two *de novo* methyltransferases didn't alter the ERV's DNA methylation pattern. Triple knock-out showed a complete loss of DNA methylation on ERV sequences, but only to a minor reactivation of transcriptional activity (Karimi et al. 2011b; Tsumura et al. 2006:3) Moreover, DNA methyltransferase inhibitors induce ERVs demethylation and upregulation in tumor cells (Chiappinelli et al. 2015).

Overall, it seems that ERVs silencing in early development might be controlled by SETDB1 and TRIM28-mediated silencing via H3K9me3 (Deniz et al. 2018; Rowe, Kapopoulou, et al. 2013), while during differentiation by DNA methylation. Interestingly, a recent study proposed a correlation between the mechanism of silencing and the evolutionary age of ERVs, with expression of the youngest LTRs being suppressed by DNA methylation and that of intermediate-aged LTRs by histone modifications, such as H3K9me3 and H3K9me2 (Ohtani et al. 2018).

(3) Histone variant H3.3, its chaperones and PTMs

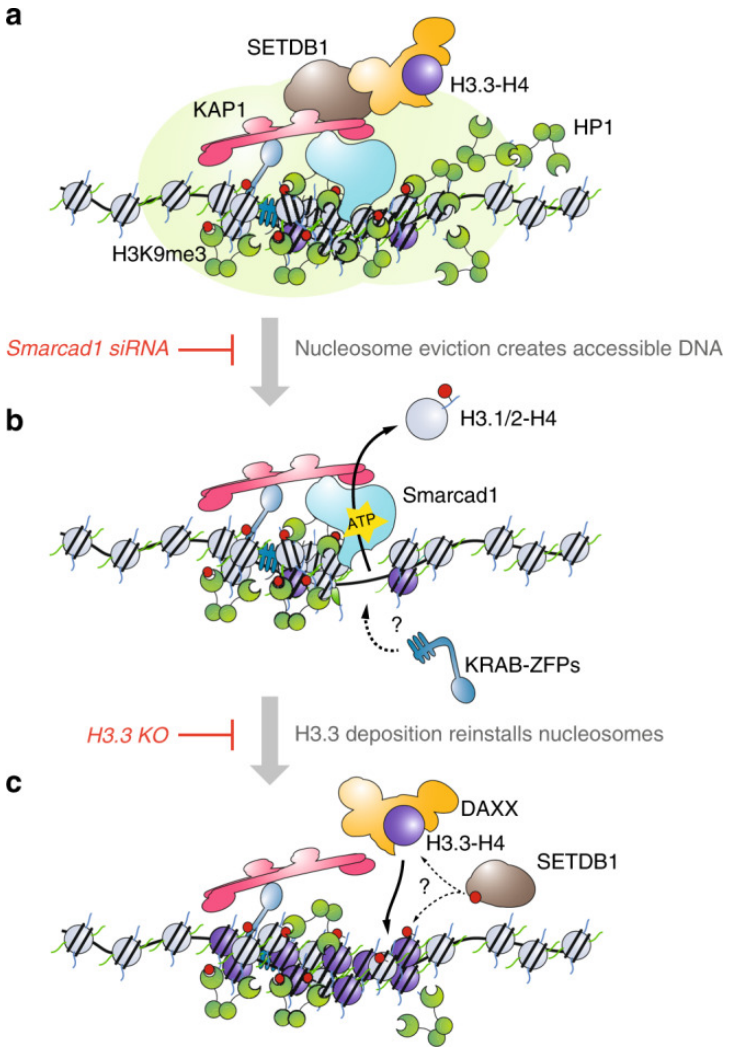
Histone variant H3.3 is enriched at class I and class II ERVs, especially at early transposon (ETn)/MusD family and IAPs in ESCs. The H3.3 deposition at these ERVs is performed by the DAXX/ATRX complex, which interacts with the TRIM28/SETDB1 corepressor complex establishing the H3K9me3-mediated heterochromatin (Elsässer et al. 2015; Navarro et al. 2020). More precisely, genome-wide distribution analysis revealed a colocalization of DAXX/ATRX with TRIM28 at class I and II ERVs, in addition, to a co-immunoprecipitation of DAXX with TRIM28 (Elsässer et al. 2015). Furthermore, depletion of H3.3 led to a reduced level of H3K9me3 and an ineffective recruitment of DAXX and TRIM28 to these ERVs. These data clearly propose that H3.3 plays a role in ERV silencing that cannot be compensated by canonical H3 isoforms, and therefore the methylation of H3K9 is rather H3.3K9 at ERVs. Therefore, it is reasonable that H3.3 mutation K27M and K36M de-repress TEs by perturbating antagonistic chromatin marks (Chaouch et al. 2021). Nevertheless, the role of H3.3 in ERVs regulation is trivial (Wolf et al. 2017), especially when loss of ATRX, DAXX, or H3.3 in mESCs reveal only a minor impact on ERV silencing (Elsässer et al. 2015; Sadic et al. 2015).

Distinct sets of histone chaperones may be involved in loading histone variants at ERVs. The chromatin assembly factor 1 (CHAF1A, also known as p150 subunit of the CAF-1), a histone chaperone specific for histones H3/H4 (Volk and Crispino 2015), was identified in an siRNA screen as a repressor for class I and II ERVs by engaging TRIM28 and for class III ERVs through interaction with KDM1a and HDAC2 (Yang et al. 2015). In addition, CHAF1a was found to interact with HP1 via a specific motif at its N-terminus and SETDB1 (Lechner et al. 2000; Thiru et al. 2004; Yang et al. 2015). In line with the observation that CHAF1A participates in ERVs silencing, a recent unpublished work from our lab provided evidence for a direct physical interaction between DAXX and CHAF1A, which together target histone H3.3 to the X-chromosome and repetitive elements.

It would be an omission not to mention that apart from the already highlighted role of H3K9me3 at ERV silencing, which loss dissolve mouse heterochromatin organization (Montavon et al. 2021), the methylation of H3K27 and H4K20 are characteristic heterochromatic histone marks frequently found on nucleosomes of TEs. In particular, it has been shown that polycomb-repressive complexes (PRC) act redundantly to stably suppress ERVs expression by adding H3K27me3 marks in mESCs (Leeb et al. 2010).

(4) RNA-mediated silencing

The RNA-mediated silencing of ERVs is another transcription repression mechanism identified in mammals. Short interfering RNAs (siRNAs) have been reported to suppress IAPs as well as non-LTR retrotransposons such as LINE-1 (J. Li et al. 2014; Stein et al. 2015). The most know example of RNA-dependent gene silencing is the X chromosome inactivation mediated by the long noncoding RNA Xist (Augui, Nora, and Heard 2011; Żylicz et al. 2019). An important binding repressor protein of Xist is Spen (Chu et al. 2015; Dossin et al. 2020; McHugh et al. 2015:3; Minajigi et al. 2015). Recent study showed that loss of Spen reactivates a subset of ERVs in mESCs. In addition, Spen binds directly to ERV-derived RNA and recruits several chromatin remodeling factors such as histone deacetylases (Carter et al. 2020). Another example of RNA-mediated ERVs silencing is the Piwi-interacting RNA (piRNA) pathway. piRNAs, in complex with the Piwi proteins, degrade retrotransposon-derived mRNA. Moreover, piRNAs induce chromatin changes and DNA methylation (Seto, Kingston, and Lau 2007). Depletion of Piwi proteins led to derepression of IAPs and LINE-1 in mice (Aravin et al. 2007, 2008), while piRNAs induced silencing through H3K9me3 formation in *Drosophila* (Pal-Bhadra et al. 2004).



To sum up, the current notion is that ERVs are governed by dynamic heterochromatin and the proposed model is summarized in Figure 34.

Figure 34: Dynamic heterochromatin model in ERV sequences by (Navarro et al. 2020). (A) KRAB-ZFPs (blue) bind to ERVs via their zinc fingers and recruit KAP1 (also known as TRIM28). Then, KAP1 orchestrates heterochromatin formation and maintenance, recruiting amongst others histone methyltransferase SETDB1 (brown), histone H3.3 (purple) chaperone DAXX (yellow) and chromatin remodeler Smarcad1 (light blue). Through SETDB1-mediated histone H3K9me3 methylation (red), HP1 (green) is recruited and presumably contributes to heterochromatin formation by compaction/phase separation. (B) Smarcad1 ATP-dependent remodeling activity leads to a disassembly of nucleosomes and permanent eviction of existing histones. The histone eviction probably give access to KRAB-ZFPs to amplify KAP1 recruitment. (C) In wild type cells, DNA is

only accessible transiently, since nucleosomes are immediately reformed using H3.3, not canonical histone H3.1/2. The deposited H3.3 may be newly synthesized, thus does not carry H3K9me3 methylation. Hence, SETDB1 needs to reestablish H3K9me3 on the dynamic nucleosome substrates. In the absence of H3.3, nucleosome reassembly is impaired, leaving DNA accessible. In the absence of Smarcad1, nucleosomes are not evicted, alleviating the requirement for H3.3 as a substrate for new nucleosomes and suspending nucleosome dynamics at heterochromatin.

d) ERV in cancer

Many TEs, and especially ERVs, have been reported to escape epigenetic silencing in cancer cells, as a result of the global epigenetic dysregulation that occurs in chromatin during tumorigenesis (Geis and Goff 2020; Grundy, Diab, and Chiappinelli 2022). In human, ERV-K expression is linked to many types of cancers, both hematological and solid, such as melanoma, breast cancer, ovarian cancer, pancreatic cancer, lymphoma, and prostate cancer (Büscher et al. 2005; Grabski et al. 2019; Katoh et al. 2011; Li et al. 2017; Löwer, Löwer, and Kurth 1996; Reiche, Pauli, and Ellerbrok 2010; Reis et al. 2013; Schmitt et al. 2013; Serafino et al. 2009; Stengel et al. 2010; Wang-Johanning et al. 2001, 2003, 2007). For example, in melanoma the demethylation of ERV-K LTRs leads to its transcriptional increase (Stengel et al. 2010), and in ovarian cancer it is observed the simultaneous expression of multiple ERV Env proteins: ERV-E, ERV-K, and ERV3 (Wang-Johanning et al. 2007). In addition, ERV-W is also frequently expressed in human cancer cells, such as testicular cancer (Gimenez et al. 2010). The Env protein of ERV-W, syncytin-1, which normally facilitates trophoblast fusion and the formation of the placenta, also plays a critical role in the pathogenesis and prognostic impact of several cancers. Syncytin-1 expression has been detected in several leukemia and lymphoma (Maliniemi et al. 2013; Sun et al. 2010), where it promote invasion and metastasis via activation of EMT in endometrial carcinoma (C. Liu et al. 2019), and works as a prognostic indicator in patient with breast and colon cancer (Bjerregaard et al. 2006; Larsen et al. 2009; Larsson, Holck, and Christensen 2007). Moreover, ERVs expression have been found reactivated in many neurodegenerative diseases (Giménez-Orenga and Oltra 2021) as well as in pHGGs (Krug et al. 2019). Many studies also report that ERVs knockdown via short hairpin RNA (shRNA) or siRNA targeting reduces tumor growth (Li et al. 2017; Mangeney et al. 2005; Zhou et al. 2016). In conclusion, these are few examples of ERVs expression reactivation in various malignancies due to epigenetic dysregulation of TEs.

ERVs can also contribute to transformation and tumor growth by functioning as promoters, enhancers through their LTRs regions, apart from their role at protein level. LTRs serve as a hub for DNA-binding transcription factors, and their binding can influence nearby host genes apart from Env expression. In particular, more than one third of the p53 binding sites are enriched at the LTR regions of ERVs (Wang et al. 2007:53), and p53-deficient cancer cells are predisposed to elevated expression of ERVs. Thus, it is not a surprise that this tumor suppressor factor, with the many pleiotropic functions (Figure 17), regulate ERVs. Similarly, interferon gamma (IFNγ)-inducible transcription factor binding sites were enriched in 27 TE families (Chuong, Elde, and Feschotte 2016). Furthermore, binding sites for inflammatory transcription factors, such as NF-κB or IRF family members, have been found at the regulatory elements of TEs (Manghera et al. 2016; Manghera and Douville 2013). These findings suggest that ERV functions were co-opted to regulate the host immune system. In conclusion, ERVs have a critical role in the cancer immune-response.

(1) Possible anticancer therapies

For many years, ERVs reactivation has been linked to cancerogenesis. However, it is now clear that drug-induced ERVs derepression also induces a state of cellular activation through a process called viral mimicry (Figure 35) and contribute to cancer immunotherapy (Jones et al. 2019; Petrizzo et al. 2021). In particular, the reactivated ERVs generate double-stranded RNA (dsRNAs) recognized by TLR3 and MDA5, dsRNAs sensors, which activate type I or type III interferon responses (canonical interferon signaling).

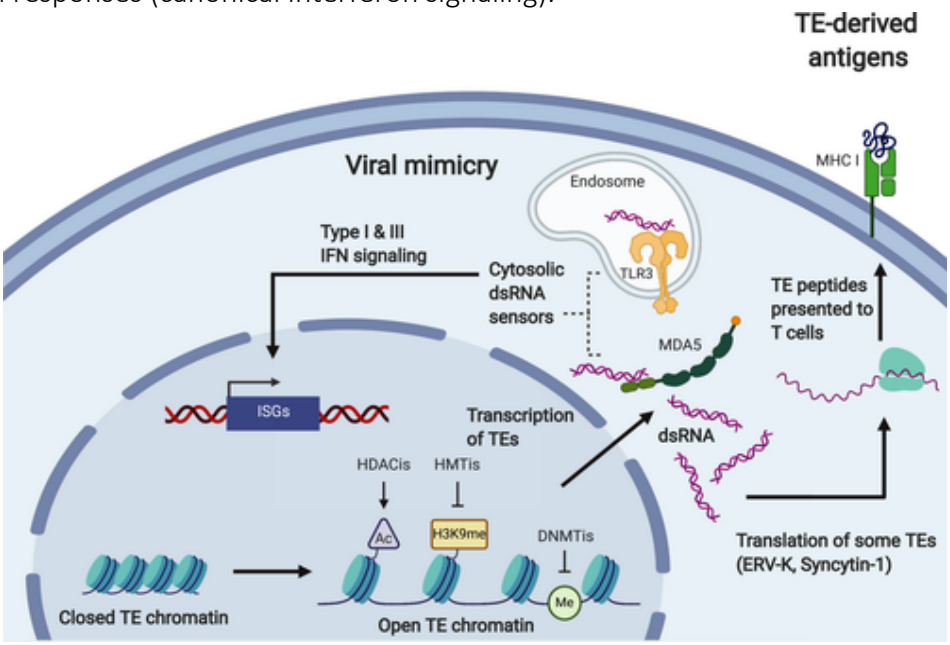


Figure 35: Anticancer therapeutic strategies using viral mimicry and TE-derived antigens by (Grundy et al. 2022). Treatment of tumor cells with DNMTis, HDACis, and HMTis induces transcription of TEs. The dsRNA are detected by the cytosolic dsRNA sensors, MDA5 and TLR3. This detection induces interferon response by upregulation of interferon-stimulated genes (ISGs), a process called "viral mimicry". The TE-derived dsRNA can also be translated into peptides, as is the case for ERV-K and Syncytin-1. These peptides can bind to major histocompatibility complex (MHC) I on the surface of the tumor where they can be recognized by T cells. Upon recognition of these tumor-associated antigens, the tumor cells can be targeted and killed by immune cells.

Treatment of cancer cells or mice with DNA methyltransferase inhibitors (DNMTi), such as Azacytidine (Aza) (Tsai et al. 2012) and 5-aza-2'-deoxycytidine (Dac) (Kaminskas et al. 2005) reduce global DNA methylation and result to interferon response through viral mimicry (Chiappinelli et al. 2015; H. Li et al. 2014; Roulois et al. 2015; Saito, Nakaoka, and Saito 2017). The interferon response induced by DNMTi can be increased by adding HDAC inhibitors (HDACi) (Brocks et al. 2017; Stone et al. 2017), inhibitors of H3K9 methyltransferases (Liu et al. 2018), or Vitamin C, which is a cofactor for the TET DNA demethylases (Liu et al. 2016). Furthermore, it is possible to induce viral mimicry in cancer cell lines with loss of the H3K9 methyltransferase SETDB1 and the H3K4me1/2 demethylase LSD1 (Cuellar et al. 2017; Karakaidos, Verigos, and Magklara 2019; Sheng et al. 2018). Moreover, cyclin-dependent kinases (CDKs) are therapeutically targeted to promote viral mimicry in cancer (Carnero 2002; Dickson and Schwartz 2009; Sherr and Roberts 1999). CDKs inhibitors (CDKis), especially for CDK4/6, not only halt the division of rapidly dividing cancer cells, but also suppress the DNMT1 expression, resulting in ERVs reactivation and induction of viral mimicry (Acevedo et al. 2016; Bourdeau and Ferbeyre 2016; Cingöz and Goff 2018; Goel et al. 2017).

Preclinical and clinical studies testing epigenetic inhibitors have shown promising results (Figure 36) (Cao and Yan 2020; Petrizzo et al. 2021). Mostly, these inhibitors are combined with monoclonal antibodies (Chiappinelli et al. 2016), such as anti-CTLA4 in melanoma (Di Giacomo et al. 2019) and anti-PD-1 in murine ovarian cancer (Stone et al. 2017). This drug combination increases anticancer efficiency and prevents immune checkpoint blockade. Nevertheless, results from the recent METADUR trial, a phase II trial that investigated the efficacy of CC-486, an oral form of Aza, with the T-cell survival promoter durvalumab (a monoclonal antibody against PD-1) in colorectal cancer, ovarian cancer, and breast cancer patients, found that viral mimicry is not induced, resulting in no clinical response. Therefore, it is really important to develop epigenetic therapies with greater stability and better pharmacokinetics.

Category	Target	Approved drug and indications	Drugs under clinical trials
DNMT inhibitor	DNA methylation writers	Azacitidine (myelodysplastic syndromes), decitabine (myelodysplastic syndromes), procainamide (cardiac arrhythmias), hydralazine (essential hypertension), procaine (local anesthetics)	Tioguanine, FdCyd, TdCyd, Aza-TdCyd, fluorocyclopentenylcytosine, guadecitabine
HDAC inhibitor	Histone acetylation erasers	Vorinostat (cutaneous T cell lymphoma), romidepsin (cutaneous T cell lymphoma), belinostat (peripheral T cell lymphoma), panobinostat (multiple myeloma), valproic acid (seizures), chidamide (peripheral T-cell lymphoma, by CFDA)	Tacedinaline, mocetinostat, abexinostat, entinostat, pracinostat, resminostat, givinostat, quisinostat, kevetrin, tefinostat, nanatinostat, domatinostat, ricolinostat, ME-344, CG200745, CUDC-101, AR42
KMT6A inhibitor	Histone methylation writer	Tazemetostat (epithelioid sarcoma)	SHR2554, CPI-1205, GSK2816126, PF-06821497, MAK683
SIRT activator	Histone acetylation erasers	None	SRT2104
BET inhibitor	Histone acetylation readers	None	Mivebresib, molibresib, birabresib, INCB057643, ZEN003694, FT-1101, GSK2820151, CC-90010, CPI-0610, PLX51107, ABBV-744, BAY1238097, BI 894999, BMS-986158, GS-5829
PRMT5 inhibitor	Histone methylation writer	None	JNJ-64619178, PF-06939999, GSK3326595
PRMT1 inhibitor	Histone methylation writer	None	GSK3368715
KDM1A inhibitor	Histone methylation eraser	None	Seclidemstat, IMG-7289, tranylcypromine, GSK2879552, INCB059872, phenelzine sulfate
KMT4 inhibitor	Histone methylation writer	None	Pinometostat

Figure 36:Epigenetic anti-cancer drugs approved or under clinical trials by *(Cao and Yan 2020)*.

Finally, ERV-encoded peptides, such as the envelop ERV protein, can also serve as cancer antigens (also known as tumor-associated antigens: TAAs), presented on the surface of cancer cells, to be recognized by chimeric antigen receptors (CAR) on T-cells to trigger immune-response (Figure 35). Recent studies have not only identified ERV-specific T cells in cancer patients with glioblastoma and AML, but also observed that the corresponding ERV-derived peptides are presented in the context of MHC class I molecules on tumors, confirming the potential of TEs as tumor-associated antigens (TAAs) (Kong et al. 2019; Saini et al. 2020). As for viral mimicry, TAAs may work better in combination with epigenetic therapies. The development of effective vaccine approaches using TAAs is an emerging field with great therapeutic potential (Ishak, Classon, and De Carvalho 2018; Lin et al. 2022; Melief et al. 2015).

To sum up, the aforementioned examples show that ERVs, on one hand, would promote the pathological progression of various cancers, neurological disorders, or autoimmune diseases and, on the other hand, would help to detect and specifically target cancer cells through the immune-response.

Preliminary results

II. Preliminary results: Functional link between H3.3, DAXX and p53

1. p53 is a member of the endogenous DAXX complex in MEFs.

The histone chaperone DAXX was previously found to form a stable complex with the chromatin remodeling factor ATRX and to target H3.3 to heterochromatic foci such as pericentromeres and telomeres (Drané et al. 2010). However, how DAXX exerts its function at these regions remains elusive.

To get more insight into the function of DAXX, we generated by CRISPR/Cas9 a mouse embryonic fibroblast cell line (MEF) with C-terminal Flag- and HA-epitope-tagged DAXX (e-DAXX). As expected, e-DAXX expression patterns show a specific staining throughout the nucleoplasm, forming numerous dots corresponding to the DAPI staining of heterochromatic foci and PML bodies (Figure 37A). Epitope-tagged DAXX complex was then purified by double immunoaffinity from MEF soluble nuclear extract by sequential immunoprecipitations with anti-Flag antibody followed by anti-HA antibody. Proteins associated with DAXX were separated by SDS-containing 4%-12% polyacrylamide gradient gels and subsequently silver-stained (Figure 37B, upper panel). Immunoblotting and mass spectrometry analyses allowed the identification of ATRX and H3.3 as specific components of the DAXX complex (Figure 37B, lower panel) as our laboratory has shown previously (Drané et al. 2010; Salem and Hamiche 2019). Surprisingly, additional unexpected partners have been found in this complex, such as the specific heterochromatin marker HP1α, the ubiquitin ligase Trim28, in line with (Elsässer et al. 2015), the potential transcription factor ADNP and more importantly the oncogenic transcription factor p53 (Figure 37B).

Having identified the tumor suppressor transcription factor p53 as one of the main oncogenic factors associated with the H3.3 deposition machinery, we next analyzed the interaction of p53 with DAXX and H3.3. Recombinant His-H3.3/H4, HA-DAXX and V5-p53 were co-expressed in E. Coli B21 cells and assayed for interaction by pull-down assays. As expected, His-H3.3 was able to pull-down DAXX and p53 while His-H3.1 could only pull-down p53 (Figure 37C). In the reverse assay, HA-DAXX was able to pull down H3.3 and p53 but not H3.1 (Figure 37D). Further analysis revealed that p53 is able to interact with both H3.1 and H3.3 (data not shown). GST-pull down assays using GST-p53 deletion mutants identify the C-terminal part of p53 (244-393 aa) as the main domain interacting with DAXX (Figure 37E). The reverse experiment identifies the N-terminal domain of DAXX (1-302 aa) as the p53 interacting domain (data not shown). These data identify p53 as one of the main oncogenic factors associated with the H3.3 deposition machinery and show that p53 forms *in vitro* a stable complex with DAXX and H3.3.

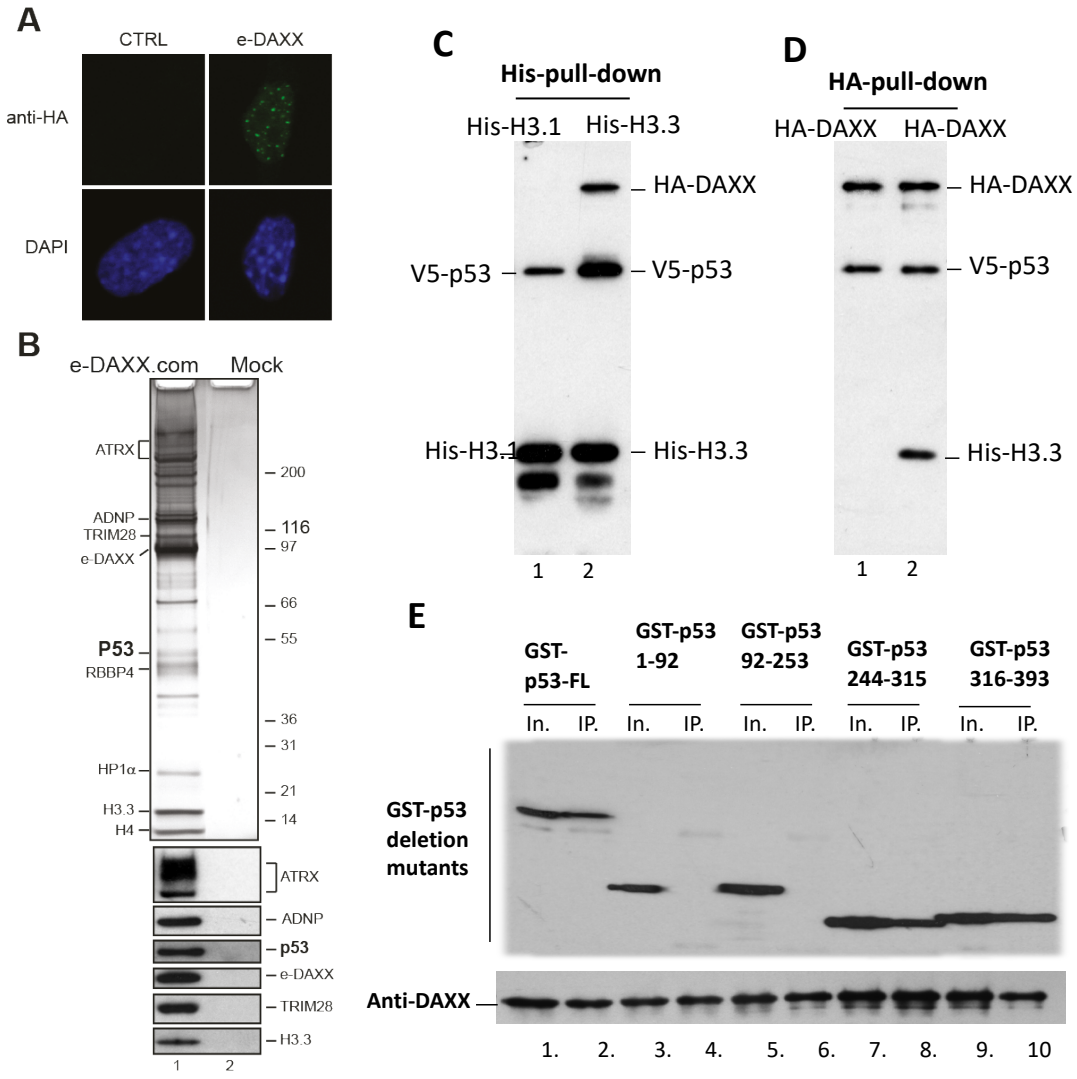


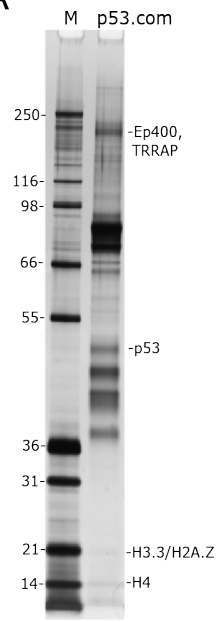
Figure 37: Purification of e-DAXX complex and identification of the oncogenic transcription factor p53 as one of the main associated protein partners by *(Salem and Hamiche 2019)*. (A). Immunostaining with an anti-HA antibody of the e-DAXX MEF cell line. (B). Upper panel, Silver staining of e-DAXX and control (mock) complexes isolated from MEF cells stably expressing e-DAXX or from naive MEF cells, respectively; Lower panel, western blotting analysis of the e-DAXX complex and mock purifications shown in the upper panel. (C). His-pull down assay using His-tagged H3.1 or H3.3 and recombinant V5-p53 and HA-DAXX. (D). HA-pull down assay using HA-tagged DAXX and recombinant V5-p53 and His-3.1 or H3.3. (E). GST-pull down assay using GST-tagged p53 deletion mutants and recombinant HA-DAXX.

2. Characterization of the endogenous p53 complex in MEFs.

To validate the association of p53 with DAXX and H3.3, we generated MEF cells expressing endogenous C-terminal Flag-HA tagged p53 using the CRISPR/Cas9 technology. The purification of the endogenous p53 complex from MEF cells further confirmed the association of H3.3 with p53 and identified several novel oncogenic and epigenetic factors associated with p53 among them the histone variant H2A.Z, the oncogenic chromatin remodeling factor Ep400/TRRAP, the cell cycle transcription factors E2F1/RBl1 and the condensin SMC2/SMC4 complex (Figure 38). However, the purification of the endogenous p53 complex did not confirm the p53 interaction with DAXX. The association of H3.3 and H2A.Z with the p53 complex is unexpected and can shed light at the molecular mechanism of H3.3 mutations transformation. A speculation is that H3.3/H2A.Z nucleosomes mark nucleosome-depleted regions of active response elements, as

the H3.3/H2A.Z nucleosome are less stable than the replication-coupled nucleosome providing a structure to chromatin that favors binding of transcription factors, like p53, and subsequently allows ERVs expression (Chen, Wang, and Li 2014; de Dieuleveult et al. 2016; Jin et al. 2009; Jin and Felsenfeld 2007).

A



B

Protein	MWT (KDA)	Unique PeptidesENS	Total Peptides ENS	Unique Peptides ENI	Total Peptides ENI	
Trrap	436.94	39	62	6	6	Chromatin remodeling/ Transcriptional activation
Trrap	291.37	61	90	17	20	
Ep400	336.97	35	43	1	1	
Ruvbl2	51.08	10	11	5	6	ATPase & DNA helicase activity
Ruvbl1	50.18	9	9	3	3	
Smc2	134.16	22	29	5	6	Condensin complex
Smc4	146.80	22	29	2	2	
Smchd1	225.51	21	25	0	0	
Trim28	88.79	12	16	3	3	KAP1
Rbl1	119.38	28	49	7	7	cell cycle
Tp53	43.13	14	104	10	34	
E2f4	43.81	7	7	0	0	
E2f3	48.73	5	5	0	0	
E2f2	48.47	2	2	0	0	
E2f1	46.29	2	3	0	0	
Cul7	192.17	43	142	11	22	Ubl conjucation
Cul1	89.63	15	17	0	0	
Cul9	209.03	2	3	1	1	
Cul2	86.82	2	2	0	0	
Fbxw8	67.91	0	0	9	11	

Figure 38 : Purification of e-p53 complex and identification of several novel oncogenic and epigenetic factors as p53 interactors. (A) Silver staining and (B) mass spectrometry analysis of e-p53 complex isolated from MEF cells stably expressing e-p53. MWT: Molecular weight, ENS: Soluble nuclear extract, ENI: insoluble nuclear extract.

3. Effect of oncogenic H3.3 K27M and G34R mutations on ERVs expression in ESC.

Histone H3.3, KAP1 (TRIM28) and DAXX are required for ERV silencing in ESC, while ERV expression is commonly prevented by DNA methylation and other epigenetic control mechanisms in tissues or differentiated cells (see section: ERV regulation and Figure 34). Furthermore, the introduction of H3.3 K27M mutation led to a derepression of ERVs in a pHGG mouse model (Krug et al. 2019). Thus, H3.3 seems to have an essential role in the establishment and maintenance of ERVs silencing during early development, and in ERV de-repression in cancer development.

To investigate the effect of oncogenic H3.3 K27M and G34R mutations on ERVs expression in pluripotent cells, we have generated genetically modified mouse ES cells, in which one allele of H3.3A gene was replaced by a tagged HA-FLAG-H3.3 fusion allele (knock-in) bearing or not H3.3 K27M or G34R mutations using CRISPR-Cas9 technology (Vidal and Hamiche 2019). Transcriptomic analysis of the wild-type and mutant ES cells revealed major differences in the transcriptomic profiles of not only coding genes but also repetitive elements such as transposons and major satellites (Figure 39). H3.3 K27M mutation led to 393 up-regulated genes and 158 down-regulated genes (Figure 39A) while G34R mutation had a much stronger impact and resulted in 1482 up-regulated genes and 720 down-regulated genes (Figure 39B). H3.3 G34R also had a more pronounced effect on repetitive elements families than H3.3 K27M,

with 49 and 25 repeat families deregulated, respectively (Figure 39C, D). Interestingly, H3.3 K27M and G34R mutations had 12 common ERVs upregulated, and H3.3 G34R mutation upregulated 30 additional ERVs families (Figure 39E, F). Collectively, our data suggest that H3.3 mutations are driving major changes in gene expression and repetitive elements repression.

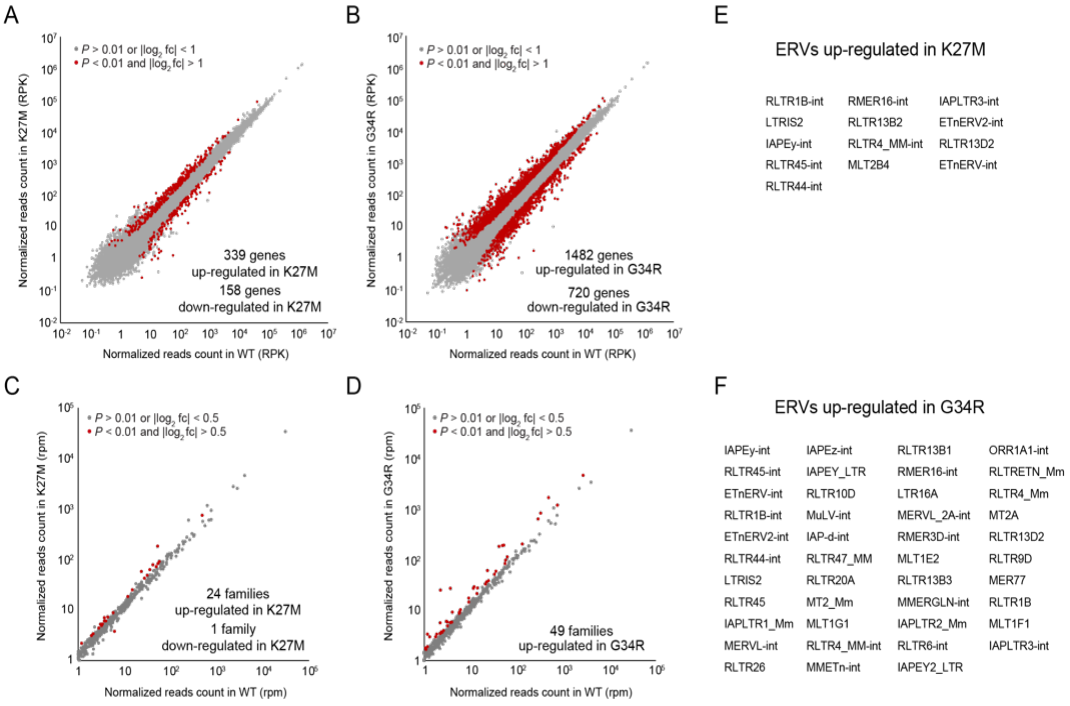


Figure 39: Transcriptomic analysis of the wild-type (WT) *versus* H3.3 mutants K27M or G34R in mESCs. A-B. Scatter plots comparing global gene expression levels between WT and H3.3 K27M (A) or G34R (B) mutants in mESCs. C-D. Scatter plots comparing global transcription of repetitive elements between WT and H3.3 K27M (A) or G34R (B) mutants in mESCs. Up and down regulated transcripts are highlighted in red. List of ERV families up-regulated in H3.3 K27M (E) and G34R (F) mutants in mESCs.

Our preliminary results suggest that the H3.3 heterochromatin deposition machinery is associated with the tumor suppressor factor p53 and that H3.3 mutations drive ERV derepression.

Aim of the study

III. Aim of the study

The histone variant H3.3 is an essential epigenetic player and its high rates of mutation and lethal phenotype in various tumors including pediatric high-grade glioma (pHGG) indicate its importance in cancer. Since 2012, H3.3 point mutations K27M and G34R/V have been highlighted as drivers of oncogenesis. The specific overlap of H3.3 mutations with the p53 and ATRX/DAXX mutations in pHGG (Schwartzentruber et al. 2012; G. Wu et al. 2012) indicates a functional link among these proteins. Our laboratory has shown that the death domain-associated protein DAXX and the chromatin-remodeling factor ATRX (alpha-thalassemia/mental retardation syndrome protein) are specifically associated with the H3.3 deposition to heterochromatic foci, such as pericentromeres, telomeres and transposable elements. DAXX functions as a *bona fide* histone chaperone involved in the replication-independent deposition of H3.3 (Drané et al. 2010). Our preliminary result (see section:p53 is a member of the endogenous DAXX complex in MEFs.) also suggests that the H3.3 deposition machinery (ATRX /DAXX/TRIM28) is associated with the tumor suppressor transcription factor p53. Moreover, H3.3 is required for endogenous retrovirus silencing in mESCs (Elsässer et al. 2015). Recent studies report that H3.3 K27M mutation led to a de-repression of ERVs in a pHGG mouse model (Krug et al. 2019). In line with this, we also observed ERVs derepression at H3.3 K27M and G34R mutations in mESCs (see section: Effect of oncogenic H3.3 K27M and G34R mutations on ERVs expression in ESC.). In addition, more than one third of the p53 binding sites are enriched at the LTRs regions of ERVs (Wang et al. 2007), and p53-deficient cancer cells are predisposed to elevated expression of ERVs.
Considering the aforementioned bibliographic observations and preliminary results, we speculated that the underlying functional link between p53 and H3.3 impacts heterochromatin formation and endogenous retrovirus silencing in cancer.
Thus, the aims of my Ph.D. project were first to dissect the molecular mechanism through which the histone variant H3.3 and the tumor suppressor factor p53 contribute to oncogenesis by regulating the expression of endogenous retrovirus and second to address the potential role of ERVs on p53-mediated tumor suppressor activity.

The first part of this manuscript presents the results showing that p53 is required for the expression of the murine ERV1 family MMERGLN-int (abbreviated GLN) and elucidates the molecular mechanism of activation of GLN expression by p53, which induces a change in the chromatin landscape along the retroviral sequence that promotes GLN expression, using cutting-edge genomic technics.
The second part addresses the most impactful result of this study, the tumor suppressor activity of endogenous retrovirus GLN envelop protein which contributes to the tumor suppressor activity of p53 using an allograft study and the cutting-edge technology of in vivo bioluminescence tumor detection.

Our study represents a breakthrough in the field of functional genomics and cancer. In particular, our work will contribute to decoding the complex crosstalk between cancer cells and the immune system, probably mobilized by ERVs envelop protein, and highlights GLN envelop protein as an excellent candidate for cancer immunotherapy combined with existing epigenetic inhibitors, thanks to the elucidation of the molecular mechanism that regulates ERV expression.

Material and Methods

IV. Material and Methods

A. Experimental Models

1. Mice

Trp53$^{-/-}$ mice purchased from the Jackson Laboratory (002547 - C3Ou.129S2(B6)-*Trp53*^{tm1Tyj}/J) and had been crossed with C57BL6/J. Homozygous mice for the *Trp53*^{tm1Tyj} mutation show no visible phenotype but most develop tumors (principally lymphomas and sarcomas) at 3-6 months of age. Heterozygous mice develop tumors at about 10 months of age. This mutant allele, *Trp53*^{tm1Tyj}, was produced by a targeted neo insertion into the Trp53 locus. More specifically A neomycin cassette replaced 40% of the coding sequences beginning with exon 2 (upstream of the translation start site) and extending into exon 6. These mice model some of the features of human Li-Fraumeni syndrome (Correa 2016; Gargallo et al. 2020; Li et al. 2020), a form of familial breast cancer with mutations in TRP53. Homozygous mice may produce a litter before succumbing to tumors.

2. Cell lines

Embryonic stem cells

Mouse ESCs (Fucci *Trp53*$^{+/+}$ & *Trp53*$^{-/-}$) were a kind gift of Menno Ter Huurne (Department of Molecular Biology, Faculty of Science, Radboud University, 6525GA Nijmegen, the Netherlands) as described in (Ter Huurne et al. 2020). Mouse ESCs were cultured on feeder-free 0.1% gelatin coated plates in a 37°C humidified incubator with 5% CO_2. Serum medium consists of DMEM with GlutaMax (Gibco #31966-021) containing 15% fetal bovine serum (Hyclone #SV30160.03), LIF (1000U/mL, Millipore #ESG1107), sodium pyruvate (1x, Gibco#11360-070) and β-mercaptoethanol (50 µM, Millipore#8.05740.0250).

Mouse embryonic fibroblasts

Primary MEFs were isolated from individual embryos at embryonic day 13.5 (E13.5) bearing *Trp53*$^{+/+}$ and *Trp53*$^{-/-}$ genotypes. The head were used for genotyping, internal organs were removed, and the torso was minced for cell extraction. Cells were then gently pelleted, re-suspended in DMEM containing 10% FBS, penicillin-streptomycin (100 units/ml), and plated for growth. For p53 genotyping, the following primers has been used:

Primer_p53_1	TGG ATG GTG GTA TAC TCA GAG C
Primer_p53_2	CAG CCT CTG TTC CAC ATA CAC T
Primer_p53_3	AGG CTT AGA GGT GCA AGC TG

For rescue experiments, cDNA containing the complete coding sequence of the envelope protein (Env) of the MMERGLN family (GLN_EnvFL), or truncated N-terminal deletions (GLN_EnvΔ1-50 and GLN_EnvΔ1-320) were subcloned into the XhoI-NotI sites of the pREV-XhoI/NotI-Flag HA tagged C_terminal-CD4^{+} retroviral vector, using standard technics. Full length or mutants Env proteins fused to C-terminal Flag- and HA-epitope tags (GLN_e-EnvFL/GLN_e-EnvΔ1-50/ GLN_e-EnvΔ1-320, see protein detailed sequence below) were stably expressed in primary p53$^{-/-}$ MEFs by retroviral transduction (Obri et al. 2014; Ouararhni et al. 2006:2; Shuaib et al. 2010).

GLN_e-EnvFL, GLN_e-EnvΔ1-50 and GLN_e-EnvΔ1-320 p53$^{-/-}$ cell lines were then infected with lentivirus. More specifically, we produced lentiviral particles by transient transfection of 293T cells with the expression vectors carrying H-Ras-Luc/EGFP (pSico-CAG-v-H-Ras-IRES-Luciferase/EGFP, addgene plasmid 58959) or H-Luc/V5 (pLX311, addgene plasmid 117735), pMDLg/pRRE (addgene plasmid 12251), psPAX2 (originally generated by Didier Trono, Addgene plasmid 12260) and pCMV-VSVG (originally generated by Robert Weinberg, Addgene plasmid 8454) using the Effectene Kit (QIAGEN 301427). Transfection medium was replaced the next day with fresh complete medium. Viral supernatant was collected 48h post transfection and filtered through 0.45um filters. GLN_e-EnvFL, GLN_e-EnvΔ1-50, GLN_e-EnvΔ1-320 *Trp53*$^{-/-}$ primary MEFs were infected with the viral supernatant at around 70% confluence. Infection medium was replaced the next day and cells were selected with 10μg/ml blasticidin or GFP presence using flow cytometry. Cells were analyzed for Luciferase efficiency 15 days later.

GLN_e-EnvFL:

MMSGLWRRLLILLSCACFVGAIPKDFNPHSPVQQTWEVLNEGGRAVWTIAEVHPLWTWWPDLFPDICKLAIGAPP
RWDLEGYSDIQRAPLTPPPYVEKHLRDPWGGCSNQRDRSMLRTHPFYVCPGPHQSQSLNPTCGGKADFFCKSWG
CETSGTARWKPSSSWDYIRVTANYSLASYVPGGFDLDECTDWCHPLRVTFTEPGKRALGWTRGYTWGLRIYKERYD
EGLLFTIRLKIETPYNPLGPPTKFTPLTHTITQPTPVIADPLNMAAITQPPTPQVPLTITPAIPSRQRMFNLVRGAFYALN
RTDPSATEDCWLCLSLGPPYYEGIAFNGDFNRTSSHTSCSWGTGQKLTLTEVSARNPGLCIGTPPSTHKHLCGQIQSV
SRTEANYYLVPSPVGWWACNTRLTPCVSTKVFNSSHDFCVMIQLLPRVYYHPASSLEESYAGRRSKREPITLTLAAFM
GIGMAVGVGTGVSALIEGRQGIQSLRDAVNEDLAAIEKSIDALEKSLTSLSEVVLQNRRGLDLLFLKEGGLCAALKEEC
CFYADHTGIVRDSMQKLRERLERRKRERDAQRGWFESWFESRPSWITSLISAVAGPILMICLALVFGPCIINRGMAFI
QSKIDTVKLMVLQRQYQPIVQVDEELGDTNLAAAGGDYKDDDDKSAAGGYPYDVPDYA

GLN_e-EnvΔ1-50:

MAEVHPLWTWWPDLFPDICKLAIGAPPRWDLEGYSDIQRAPLTPPPYVEKHLRDPWGGCSNQRDRSMLRTHPFY
VCPGPHQSQSLNPTCGGKADFFCKSWGCETSGTARWKPSSSWDYIRVTANYSLASYVPGGFDLDECTDWCHPLRV
TFTEPGKRALGWTRGYTWGLRIYKERYDEGLLFTIRLKIETPYNPLGPPTKFTPLTHTITQPTPVIADPLNMAAITQPPT
PQVPLTITPAIPSRQRMFNLVRGAFYALNRTDPSATEDCWLCLSLGPPYYEGIAFNGDFNRTSSHTSCSWGTGQKLTL
TEVSARNPGLCIGTPPSTHKHLCGQIQSVSRTEANYYLVPSPVGWWACNTRLTPCVSTKVFNSSHDFCVMIQLLPRV
YYHPASSLEESYAGRRSKREPITLTLAAFMGIGMAVGVGTGVSALIEGRQGIQSLRDAVNEDLAAIEKSIDALEKSLTSL
SEVVLQNRRGLDLLFLKEGGLCAALKEECCFYADHTGIVRDSMQKLRERLERRKRERDAQRGWFESWFESRPSWITS
LISAVAGPILMICLALVFGPCIINRGMAFIQSKIDTVKLMVLQRQYQPIVQVDEELGDTNLAAAGGDYKDDDDKSAAG
GYPYDVPDYA

GLN_e-EnvΔ1-320:

MPVLGSGPPYYKGIAFNGDFNRTSSHTSCSWGTGQKLTLTEVSARNPGLCIGTPPSTHKHLCGQIQSVSRTEANYYLV
PSPVGWWACNTRLTPCVSTKVFNSSHDFCVMIQLLPRVYYHPASSLEESYAGRRSKREPITLTLAAFMGIGMAVGVG
TGVSALIEGRQGIQSLRDAVNEDLAAIEKSIDALEKSLTSLSEVVLQNRRGLDLLFLKEGGLCAALKEECCFYADHTGIVR
DSMQKLRERLERRKRERDAQRGWFESWFESRPSWITSLISAVAGPILMICLALVFGPCIINRGMAFIQSKIDTVKLMV
LQRQYQPIVQVDEELRDTDLAAAGGDYKDDDDKSAAGGYPYDVPDYA

Flag tag and HA tag are depicted in blue and green color, respectively. All lines were regularly tested negative for mycoplasma contamination.

B. Methods details and analyses

Allograft assays

Pilot study: 50.000, 500.000 or 5 million of $p53^{-/-}$ primary MEFs expressing H-Ras-Luc/EGFP or H-Luc/V5 were injected subcutaneously or intravenously in 6 weeks old wild type C57BL/6J mice. Three mice for each experimental condition were used, i.e. a total of 36 mice (2x2x3x3). The pilot study lasted in total 6 weeks/ 42 days. Once we established the best experimental conditions for tumor development (cell line, cell numbers and injection site), we performed the following main study.

Main study: 5 million $p53^{-/-}$ primary MEFs expressing H-Ras-Luc/EGFP and GLN_e-Env full-length or mutants (GLN_e-EnvFL, GLN_e-EnvΔ1-50, GLN_e-EnvΔ1-320 or transduction control) were injected subcutaneously in 6 weeks old WT C57BL/6J mice. Twelve mice had been used for each experimental condition, i.e. a total of 48 mice (4X12). The main study lasted in total 7 weeks/ 50 days.

Bioluminescence: Bioluminescent imaging (BLI) was initiated 1 (main study) or 2 (pilot study) days after implantation of cells and conducted weekly thereafter. Each mouse received an intraperitoneal injection of 150 mg D-luciferin (PerkinElmer Part Number #122799) /kg body weight and maintained under isoflurane gas anesthesia for 5 min before imaging. Bioluminescent images were obtained 10 min after D-luciferin intraperitoneal injection and 26 min in total, using IVIS spectrum *in vivo* imaging system (PerkinElmer, Waltham, MA, USA) available at IGBMC.
Quantification of tumor bioluminescent activity was performed with Living Image 4.7.4 software (PerkinElmer, Waltham, MA) by taking an auto-exposure and recording the total photons in a set region of interest that was kept consistent throughout the duration of study. Bioluminescent activity is presented as photons per second (p/s).

At the end of the study or when a mouse reached the study's end point (weight loss greater than 20%, pain scoring, necrosis of tumor/s, tumor size reaching 1 cm3, detection of concealed metastases), a bioluminescent image was obtained 10 min after D-luciferin intraperitoneal injection, blood sample was collected post-mortem, subcutaneous tumors were extracted and snap-freeze in liquid nitrogen for future uses, and internal organs had been tested for tumor micro-metastasis using IVIS spectrum in vivo imaging system, max 30 min after D-luciferin intraperitoneal injection.

Caliper and body weight measurements were collected 3 times per week.

Antibodies

Antibody	Technique	Quantity	Producer	Reference
Rat anti-HA high affinity clone 3F10	IF, WB	1:200,1:1000	Roche Diagnostics	11867423001
Cy3 Goat Anti-Rat (H+L)	IF	1:400	JIR	112-165-167
Alexa Flur488 Goat Anti-Rat secondary	IF	1:400	Invitrogen	A-11006

Alexa Flur568 Goat Anti-Rabbit secondary	IF	1:400	Invitrogen	A-11011
GAPDH	WB	1:10000	Abcam	Ab8245
Goat anti-rat HRP	WB	1:20000	Sigma Aldrich	A9037
Goat anti-mouse HRP	WB	1:30000	Sigma Aldrich	A2304
p53	X-ChIP-seq, WB	15µg,1:4000	Novocastra	NCL-L-p53 CM5p
5mC	DIP-seq	2 µl per 20 µg DNA	Active Motif	39649
5hmC	DIP-seq	4 µl per 20 µg DNA	Active Motif	39791
H2A.Z	CUT&Tag	1 µg per IP	Active Motif	39113
H3K9me3	CUT&Tag	1 µg per IP	Abcam	Ab8898
H3K27ac	CUT&Tag	1 µg per IP	Active Motif	39133
H3K27me3	CUT&Tag	1 µg per IP	Active Motif	39155
H3k36me3	CUT&Tag	1 µg per IP	Abcam	Ab9050
SMC2	CUT&Tag	1 µg per IP	Abcam	10399
SMC4	CUT&Tag	1 µg per IP	Abcam	17958
Trim28	X-ChIP-seq	4 µg per IP	Abcam	ab10483
H3.3	X-ChIP-seq	4 µg per IP	Abnova	H00003021-M01
H3.3	X-ChIP-seq	4 µg per IP	Abcam	ab176840
H3.3	X-ChIP-seq	4 µg per IP	Merck	cat 17-10245

Western blot

Samples were separated by SDS-PAGE on 10 % gels and transferred to a nitrocellulose membrane 0,45 µm. Membrane blocking was performed with 5 % skim milk in phosphate buffered saline with Tween 20 (PBS supplemented with 0,1 % Tween20, PBST) 1h at room temperature (RT). Membranes were incubated with primary antibody (see Antibodies table) in 5 % skim milk in PBST overnight at 4°C. Membranes were washed 3 times in PBST, and incubated with HRP-conjugated secondary antibody 1h at RT (see Antibodies table). Membranes were then washed 3 times in PBST, one time in PBS, and the signal was resolved with Immobilon® Forte Western HRP Substrate (Merck) and detected using Amersham HyperfilmTM ECL (GE Healthcare) on a Kodak X-OMAT 3000RA Processor.

Immunofluorescence

Immunofluorescence used standard procedures. Briefly, Cells were cultivated on coverslips in 6-wells plate for at least 18 hours. Cells were washed twice 5 min with cold PBS and fixed with 4 % paraformaldehyde solution in PBS for 20 min at RT. The following steps were all performed at RT, and each wash performed for 5 min. After 2 washes with PBS, 2 washes with PBS-0,1M Glycine pH 8.5, 1 wash with PBS-BSA 1 %-FBS 1 %, cells were permeabilized with PBS-0,05 % Triton X-100 for 15 min. After 2 washes with cold PBS, blocking was performed in PBS-BSA 1 %-FBS 1 % for 1h, followed by primary antibody incubation in PBS-BSA 1 %-FBS 1 %, 3 washes with PBS-BSA 1 %-FBS 1 % and secondary antibody incubation in PBS-BSA 1 %-FBS 1 % for 1h. As primary antibody, rat anti-HA antibody (Roche, 11867423001) has been used at 1/200 dilution; as secondary antibody, goat anti-Rat IgG coupled to Alexa Fluor 488 (Invitrogen, A-11006) or goat anti-Rat IgG coupled to Cy3 (JIR, 112-165-167) have been used at 1/400 dilution. After 3 washes with PBS-BSA 1 %-FBS 1 % and 1 wash with PBS, coverslips were incubated with DAPI 1 µg/mL in PBS for 10 min and washed with PBS before mounting on microscope slides using Aqua-Poly/Mount mounting medium (Polysciences, Inc.).

Double immunoaffinity purification

Extracts were prepared as described in (Drané et al. 2010) using a modification of the Dignam protocol (Dignam 1990). Briefly, cells were lysed in hypotonic buffer (10 mM Tris-HCl at pH

7.65, 1.5 mM $MgCl_2$, 10 mM KCl) and disrupted by Dounce homogenizer. The cytosolic fraction was separated from the pellet by centrifugation at 4°C. The nuclear-soluble fraction was obtained by incubating the pellet in high-salt buffer (to get a final NaCl concentration of 300 mM). Tagged proteins were immunoprecipitated with anti-Flag M2-agarose (Sigma), eluted with Flag peptide (1 mg/mL), further affinity-purified with anti-HA antibody-conjugated agarose, and eluted with HA peptide (1 mg/mL). The HA and Flag peptides were first buffered with 50 mM Tris-HCl (pH 8.5), then diluted to 4 mg/mL in TGEN 150 buffer (20 mM Tris at pH 7.65, 150 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.01% NP40), and stored at -20°C until use. Between each step, beads were washed in TGEN 150 buffer. Complexes were resolved by SDS-PAGE and stained using the Silver Quest kit (Invitrogen).

Mass spectrometry

Mass spectrometric analysis of proteins was accomplished using an ion-trap mass spectrometer (Thermo Finnigan LTQ-Orbitrap Velos) or by the Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, Massachusetts, USA) as described previously by (Obri et al. 2014).

Native ChIP-seq (N-ChIP-seq)

For Flag-HA native ChIP-seq, DNA was purified from the elution of the double-immunoaffinity purification (see section above) on the nuclear insoluble fractions. DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. Libraries were prepared using the Diagenode MicroPlex Library Preparation kit v2, and sequenced on Illumina Hiseq 4000 sequencer as single-end 50 bp reads following Illumina's instructions. Image analysis and base calling were performed using RTA 2.7.3 and bcl2fastq 2.17.1.14. Adapter dimer reads were removed using DimerRemover v0.9.2 (https://sourceforge.net/projects/dimerremover/). Reads were mapped to the mouse genome (mm9) using Bowtie2 (Langmead et al., 2009) v2.4.5 with default parameters.

Cross-linked ChIP-seq (X-ChIP-seq)

H3.3 and Trim28 X-ChIP experiments were performed as previously described (Obri et al. 2014). Briefly, cells were crosslinked with 0.4% paraformaldehyde for 10 min at room temperature. The reaction was stopped by adding glycine to a final concentration of 0.125 M for 10 min. Cells were rinsed with PBS 1X, pelleted and re-suspended in lysis buffer (10 mM EDTA, pH 8, 50 mM Tris-HCl pH 8, SDS 1%). Lysate was sonicated for 18 min using a Covaris focused-ultrasonicator and centrifuged at 13,000 r.p.m. (18,000*g*) for 30 min. The cleared supernatant was used immediately in ChIP experiments. 50 µg of sonicated chromatin isolated from sub-confluent MEFs were immunoprecipitated using 4 µg of each antibody (see antibody table). One independent chromatin immunoprecipitation per antibody was sent for sequencing analysis. Libraries were prepared and sequenced on the Illumina Hiseq 2500 as single-end 50 bp reads following Illumina's instructions. Image analysis and base calling were performed using RTA 1.17.21.3 and CASAVA. Reads forming adapter dimers were removed using an in-house script. Reads were mapped to the mouse genome (mm9) using Bowtie v1.0.0 with the following arguments: -m 1 --strata --best -y -S –l 40 -p 2.

CUT&Tag

Cells were harvested as described in (Kaya-Okur et al. 2019) using a modification of nuclei preparation as described by (Kaya-Okur and Henikoff 2020). Briefly, cells were harvested and

centrifuged for 3 min at 600*g* at room temperature. Nuclei were extracted using NE1 buffer (20 mM HEPES–KOH, pH 7.9, 10 mM KCl, 0.1% Triton X-100, 20% Glycerol, 0.5 mM Spermidine and Protease Inhibitor EDTA-Free). Aliquots of 500,000 nuclei were bounded to concanavalin A coated magnetic beads. Primary antibody (see antibodies table) incubation was performed on a nutator overnight at 4 °C. In total, 1µg of primary antibody have been used. Guinea Pig anti-Rabbit IgG secondary antibody incubation was performed for 1 hour at room temperature and tagmentation for 1 hour at 37°C.To amplify libraries, 30 µL DNA was mixed with 2,5 µL of a universal i5 and a uniquely barcoded i7 primer, using a different barcode for each sample. For more details, the CUT&Tag-IT Assay Manual kit (Active Motif #53160) had been used.

Libraries were sequenced on an Illumina HiSeq 4000 sequencer as paired-end 100 base reads. Image analysis and bas calling were performed using RTA version 2.7.7 and bcl2fastq version 2.20.0.422. Data were preprocessed with Cutadapt (Martin 2011) v3.7 to trim adapter sequences (Nextera Transposase Sequence) from 3' end of reads. Cutadapt was used with the following parameters '-a CTGTCTCTTATA -A CTGTCTCTTATA -m 25:25'. Reads were mapped to Mus musculus genome (mm9) using Bowtie2 (Langmead and Salzberg 2012) v2.4.5 with default parameters except for "–end-to-end –very-sensitive –no-mixed –no-discordant -I 10 -X 700".

RNA-seq

Total RNAs were purified from subconfluent MEFs or homogenized mice tissues (cortex, liver and kidney) using TRIzol reagent. More specifically, the soft tissue homogenizing CK14 precellys lysing beads (P000912-LYSK0-A) had been used for liver and kidney homogenization. Primary MEFs were kept in culture for no more than three to six passages (p3-p6) before RNA-seq analyses. The library was created using the TruSeq Stranded Total RNA SamplePrep kit (Illumina) and sequenced on Illumina Hiseq 4000 sequencer as Single-Read 50 base reads following Illumina's instructions. Reads were preprocessed in order to remove adapters, polyA and low-quality sequences (Phred < 20) and reads shorter than 40 bases were discarded for further analysis (cutadapt version 1.10). Reads were mapped to spike sequences using bowtie2 (version 2.2.8) and reads mapping to spike sequences were removed for further analysis. Reads were mapped onto the mm9 assembly of *Mus musculus* genome using STAR (version 2.5.3A). Quality control on the reads was performed with FastQC (version 0.11.5) and quality control on the alignments with RSeqQC (version 2.6.4).

Gene expression quantification was performed from uniquely aligned reads using HTSeq-count (version 0.6.1p1), with annotations from Ensembl version 67 and "union" mode. Only non-ambiguously assigned reads have been retained for further analyses. Read counts have been normalized across samples with the median-of-ratios method proposed by (Anders and Huber 2010) to make these counts comparable between samples. Differential expression comparisons were performed using the Wald test proposed by (Love, Huber, and Anders 2014) and implemented in the Bioconductor package DESeq2 version 1.16.1. P-values were adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg 1995).

RNA-seq repeat analyses were performed as described in (Papin et al. 2017) with slight modifications in order to discriminate signal coming from genes expression from the one coming from repeat expression. Reads were aligned to repetitive elements in two steps. In the

first step, reads were aligned to the non-masked mouse reference genome (NCBI37/mm9) using BWA v.0.6.2 (Li and Durbin 2009). Reads which sense was of the same sense as overlapping transcript were removed. Prior to this step, genomic coordinates of transcripts were extended 3Kb upstream of TSS and 10Kb downstream of TTS to remove reads arising from transcriptional readthrough. Positions of the reads mapped uniquely to the mouse genome were cross-compared with the positions of the repeats extracted from UCSC (rmsk table in the UCSC data- base for mouse genome mm9), and reads overlapping a repeat sequence were annotated with the repeat family. In the second step, reads not mapped or multimapped to the mouse genome in the previous step were aligned to RepBase v.18.07 (Jurka et al. 2005) repeat sequences for rodent. Reads mapped to a unique repeat family were annotated with their corresponding family name. Finally, we summed up the read counts per repeat family of the two annotation steps. Data were normalized based upon library size. Differential analysis of repeat families was performed using the Wald test proposed by (Love et al. 2014) and implemented in the Bioconductor package DESeq2 version 1.16.1. P-values were adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). To avoid over- or underestimating fold enrichments due to low sequence representation, repeat families with less than 100 mapped reads per RNA were excluded from further analysis.

MeDIP-seq and hMeDIP-seq

Twenty micrograms of DNA was used as input, then 2 µl 5mC monoclonal antibody (Active Motif, 39649), 2 µl monoclonal immunoglobulin G (IgG) control antibody (Abcam, ab81032), 4 µl 5hmC polyclonal antibody (Active Motif, 39791) or 4 µl IgG polyclonal antibody (Abcam) were used to immunoprecipitate DNA. DNA and antibodies were incubated at 4°C overnight in a final volume of 1 ml DIP buffer (10 mM sodium phosphate pH 7.0, 140 mM NaCl, 0.05% Triton X-100). The bound material was recovered after incubation with 60 µl blocked protein A and G Dynabeads (beads washed three times with 1 ml DIP buffer and incubated for 4 hours minimum with BSA 1 mg/ml and yeast tRNA 0.5 mg/ml). The beads were washed three times with 1 ml DIP buffer, then treated overnight with RNase A at 65°C in the presence of 300 mM NaCl and then treated for 4 hours with proteinase K at 55°C. Immunoprecipitated DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. For each condition, 5mC, 5hmC, and IgG control DIP assays were performed using the same batches of genomic DNA (Ibrahim et al. 2021:2). Libraries were prepared using the SMART cDNA Library Construction Kit and sequenced on Illumina HiSeq 4000 sequencer as single-end 50-bp reads following Illumina's instructions. Image analysis and base calling were performed using RTA 2.7.3 and bcl2fastq 2.17.1.14. Data were preprocessed with Cutadapt (Martin 2011) v3.7 to remove the first nine nucleotides and to remove sequences with a trailing polyT of at least 10 Ts. Cutadapt was used with the following parameters: "-u 9 -a T [10] --discard-trimmed". Reads were mapped to the mouse genome (mm9) using Bowtie v2.4.5 with default parameters.

Sequencing read alignment of published datasets

Published datasets were downloaded from GEO in SRA format and converted to FASTQ format using the fastq-dump program in the sratoolkit (version 2.1.9). For ChIP-seq and DIP-seq datasets, reads were mapped to the mouse genome (mm9) using Bowtie v1.0.0 with the following setting: -p 3 -m 1 --strata --best --chunkmbs 128 -S. For RNA-seq datasets, reads were mapped onto the mm9 assembly of the mouse genome by using Tophat (Trapnell, Pachter, and Salzberg 2009) v2.0.14 and Bowtie (Langmead et al. 2009) v2-2.1.0. Quantification of gene

expression was performed using HTSeq (Anders and Huber 2010) v0.6.1 and gene annotations from Ensembl release 67.

Computational analyses

Heatmaps and quantifications of the ChIP-seq/DIP-seq/MNase-seq/RNA- seq data were performed running seqMINER (Ye et al. 2011), using datasets normalized to 10 million uniquely mapped reads. As reference coordinates, we used the annotated Repeatmasker (RMSK) database (for repetitive elements) or the CGIs coordinates from the UCSC genome browser (CpG islands track, mm9 assembly). In order to attribute one single-gene per proximal CGI, the nearest TSS for each CGI was identified using Homer (http://biowhat.ucsd.edu/homer/ngs/annotation.html). In a second time, the reverse analysis was performed, searching the nearest CGI for each TSS, using the "Fetch closest non-overlapping feature for every interval" tool in Galaxy. One single gene per one single CGI was attributed when both methods resulted in identical CGI/TSS association (n = 9,151).

Reanalysis of published datasets

Mark	Cell	Type	GEO accession	Ref
H2A.Z	ESCs	X-ChIP-seq	GSM984544	(Subramanian et al. 2013)
H3.3	ESCs	X-ChIP-seq	GSM2417116	(Chronis et al. 2017)
H3K4me1	ESCs	X-ChIP-seq	GSM1516085	(Chronis et al. 2017)
H3K4me2	ESCs	X-ChIP-seq	GSM2417084	(Chronis et al. 2017)
H3K4me3	ESCs	X-ChIP-seq	GSM2417080	(Chronis et al. 2017)
H3K9ac	ESCs	X-ChIP-seq	GSM2417092	(Chronis et al. 2017)
H3K9me3	ESCs	X-ChIP-seq	GSM2417112	(Chronis et al. 2017)
H3K14ac	ESCs	X-ChIP-seq	GSM3566928	(Zhang et al. 2019)
H3K27ac	ESCs	X-ChIP-seq	GSM2417096	(Chronis et al. 2017)
H3K27me3	ESCs	X-ChIP-seq	GSM2417100	(Chronis et al. 2017)
H3K36me3	ESCs	X-ChIP-seq	GSM2417108	(Chronis et al. 2017)
H3K79me2	ESCs	X-ChIP-seq	GSM2417104	(Chronis et al. 2017)
Trim28	ESCs	X-ChIP-seq	GSM1406445	(Castro-Diaz et al. 2014)
p53	ESCs	X-ChIP-seq	GSE141179	(Ter Huurne et al. 2020)
RNA	ESCs	RNA-seq	GSE141178	(Ter Huurne et al. 2020)
5mC	ESCs	DIP-seq	GSM1036278	(Shen et al. 2013)
5hmC	ESCs	DIP-seq	GSM1036277	(Shen et al. 2013)
5fC	ESCs	DIP-seq	GSM1036276	(Shen et al. 2013)
5aC	ESCs	DIP-seq	GSM1036275	(Shen et al. 2013)

Results and Discussion

V. Results and Discussion

A. p53 directly activates ERVs of the MMERGLN family in mice

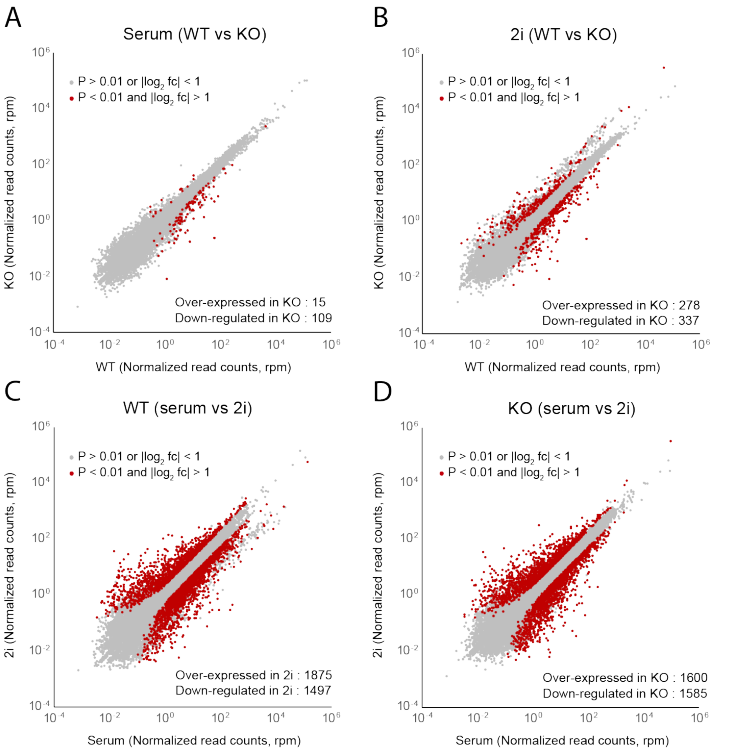
1. p53 activates GLN-ERVs in stem cells.

a) GLN-ERVs are down-regulated in the absence of p53 in ESCs.

Embryonic stem cells (ESC) are initially derived from the inner cell mass (ICM) of blastocysts. Since these pluripotent stem cells possess unique properties of self-renewing while maintaining potential to differentiate into any somatic cell lineages (Hanna et al. 2010), they are widely used as an *in vitro* cellular model for early mammalian developmental studies. mESC cultured in presence of two kinase inhibitors (known as 2i conditions) have a globally hypomethylated genome that closely resemble the methylome of ICM cells in pre-implantation blastocysts, whereas serum-grown mESC have a hypermethylated genome similar to that of post-implantation embryos (Wu and Zhang 2014). mESCs grown in serum-supplemented condition give rise to different flavors of ESCs that reflect developmental states (primed mESCs), while mESCs that transition from serum to serum-free 2i medium have a unrestricted developmental potential and represent the pluripotent naïve ground state (Habibi et al. 2013; Marks et al. 2012; Schlesinger and Meshorer 2019).

We set out to decipher the distinct roles of p53 in gene expression regulation in ground state 2i and serum conditions. To this end, we reanalyzed ribo-zero RNA-seq datasets obtained in mESCs WT and KO for p53, cultured in both serum and 2i conditions (Ter Huurne et al. 2020). The genome-wide transcriptome analysis between WT and KO cells identified 124 and 615 significantly mis-regulated genes in serum and 2i conditions, respectively (Figure 40A, B) with most affected genes being up- or down-regulated not much greater than 2-fold. Gene ontology analysis (GO) of the mis-regulated genes did not reveal any significant biological process deregulated in the absence of p53. We concluded that p53 function has very little impact on global transcription.

In agreement with available data (Kolodziejczyk et al. 2015; Marks et al. 2012), differential expression analyses revealed that the transition from serum to 2i induces a vast transcriptome reprogramming, with hundreds of genes being either upregulated (1875 genes) or down-regulated (1497 genes). The primed to naïve state conversion induced the deregulation of genes implicated in lysosome processes and cell differentiation (Figure 40E). In p53 KO ESCs, 1600 genes were up-regulated and 1585 down-regulated. Up- and down-regulated genes were the same in WT and KO cells, indicating that the vast majority of genes are normally regulated upon serum to 2i transition in the absence of p53 (Figure 40C, D). We concluded that p53 is not required to induce, stabilize and maintain the naïve ground state in mESC.



E

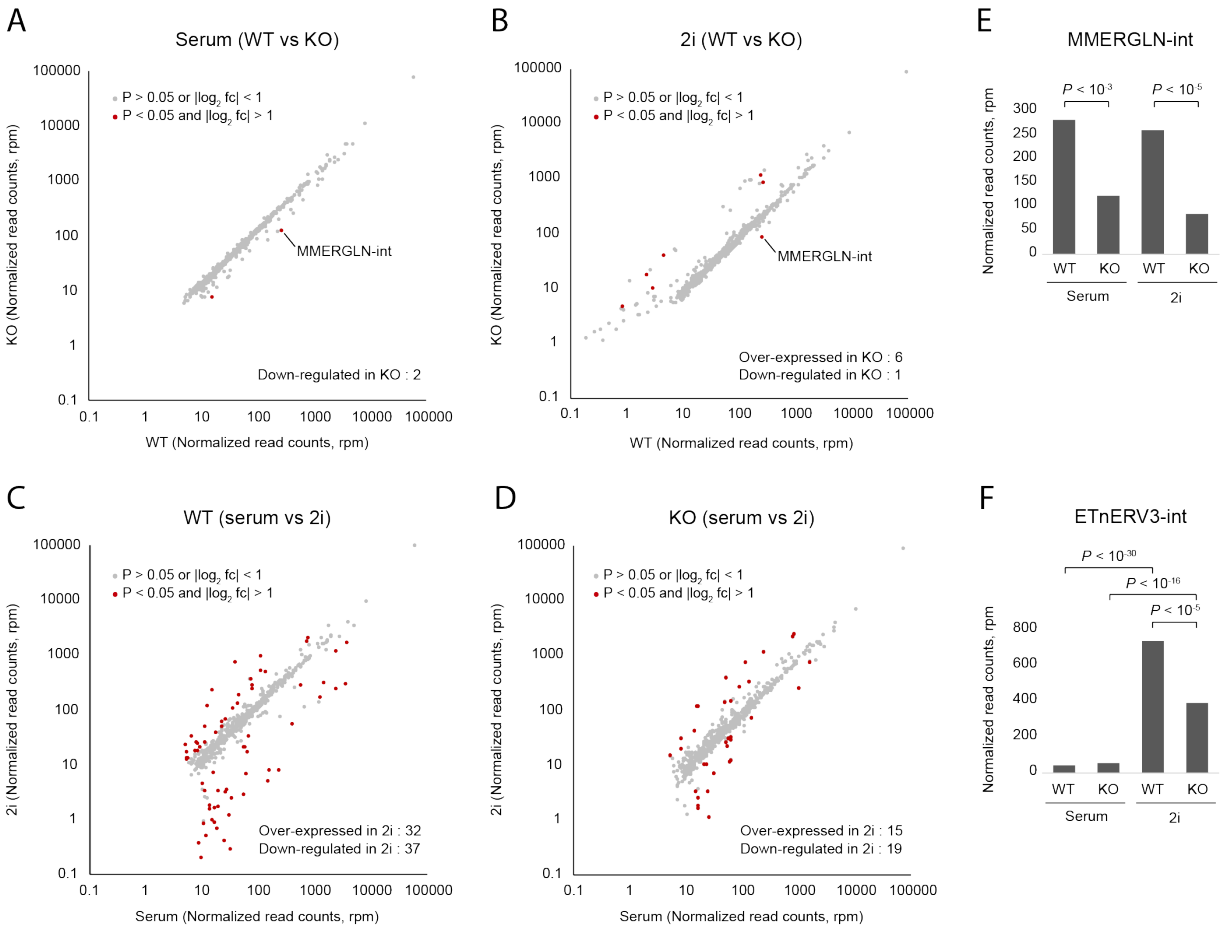
Functional clustering :

	cluster	enrichment score	*P* value
Down-regulated genes in 2i	lysosome	8.05	$9.6\ 10^{-9}$
Over-expressed genes in 2i	cell differentiation	12.64	$8\ 10^{-15}$
	cell junction	9.2	$1.5\ 10^{-12}$
	transcription	7.69	$6.2\ 10^{-17}$
	actin binding	6.9	$7.5\ 10^{-8}$

Figure 40: A-B. Scatter plots comparing global gene expression levels between WT and KO mESCs cultured in serum (A) or 2i (B) conditions. C-D. Scatter plots comparing global gene expression levels between serum and 2i conditions in WT (C) and KO (D) mESCs. (E) Functional annotation clustering of differentially expressed genes upon serum to 2i transition.

Repetitive elements, in particular ERVs, have been reported to actively shape the p53 transcriptional network in human cells (Wang et al. 2007). We specifically analyzed DNA repetitive elements transcription (Figure 41). Interestingly, we showed that p53 is required for the expression of the murine ERV1 family MMERGLN-int (abbreviated GLN). GLN-ERVs are found specifically down-regulated in p53 KO mESC cultured in both serum and 2i conditions (Figure 41A, B, E, G). We conclude that the p53-mediated GLN-ERVs expression is independent of the DNA methylation status of chromatin in pluripotent cells, as GLN is expressed in both hypermethylated (serum) and hypomethylated (2i) chromatin environment.

In line with the global DNA demethylation observed during primed to naïve state conversion, a large over-expression of the ERVs (24 families up-regulated) (Figure 41C, G) and a wide transcriptome reprogramming (Figure 40C, D) were observed during the serum-to-2i transition. We detected a global decrease in ERVs awakening efficiency in naïve ESCs KO for p53 (Figure 41D, G), as illustrated for the EtnERv3-int family (Figure 41F). Furthermore, it should be noted that the expression of GLN-ERVs was not affected by the switch from serum-to-2i. In conclusion, these results indicate that p53 contributes to the efficiency of ERV awakening, but is not required, during conversion from primed to naïve state.



G

Serum (WT vs KO)	WT (serum vs 2i)			KO (serum vs 2i)		
MMERGLN-int *	ETnERV3-int *	RLTR44-int *	tRNA-Lys-AAA	ETnERV3-int *	RMER21B *	
(TTAA)n	RLTR13B2 *	Charlie10	tRNA-Gln-CAG	IAPEy-int *	L1Md_F3	
	IAPEy-int *	Arthur1	tRNA-Ala-GCY	RLTR13B2 *	tRNA-Phe-TTY	
	RLTR1B-int *	RLTR27 *	tRNA-Tyr-TAC	RLTR1B-int *	tRNA-Lys-AAA	
	IAPEY_LTR *	Tigger10	tRNA-Asn-AAC	U17	FAM	
	LTRIS2 *	RLTR31A_Mm *	(C)n	IAPEY_LTR *	tRNA-Tyr-TAC	
	RMER16-int *	RLTR16 *	tRNA-Val-GTG	LTRIS2 *	tRNA-Arg-AGA	
	RLTR13D4 *	MLT2B5 *	(G)n	RLTR10D *	tRNA-Val-GTG	
	RLTR10D *	B2_Mm1t *	tRNA-Arg-AGG	(T)n	tRNA-Leu-CTA_	
	IAP-d-int *	RMER13B *	tRNA-His-CAY_	BGLII	tRNA-Leu-TTA	
	RLTR45-int *	FordPrefect	tRNA-Arg-AGA	RLTR13D4 *	(ATAAG)n	
	RLTR17 *	(TTAA)n	tRNA-Ile-ATT	(A)n		
	LTRIS3 *	B2_Mm1a	tRNA-Met-i	RMER16-int *		
	RLTR9C *	(CCCCCA)n	tRNA-Ile-ATA	RLTR10A *		
	RLTR13B1 *	FAM	tRNA-Leu-CTG	IAP-d-int *		
	(CTG)n	tRNA-Ser-TCY	tRNA-Thr-ACY_	RMER13B *		
	BGLII	(TGGGGG)n	tRNA-Val-GTA	tRNA-Ser-TCY		
	RLTR10A *	RMER21B *	tRNA-Leu-CTA_	RLTR6-int *		
	(T)n	L1Md_F3	tRNA-Asp-GAY	HAL1		
	RLTR13C2 *	tRNA-Gly-GGA	tRNA-Leu-TTG	L1Md_A		
	IAPLTR1_Mm *	4.5SRNA	tRNA-Leu-CTY	tRNA-Gln-CAG		
	(A)n	U8	(ATAAG)n	RLTR4_MM-int *		
	IAPLTR2b *	tRNA-Phe-TTY	tRNA-Leu-TTA	(CCCCG)n		
2i (WT vs KO)						
MMERGLN-int *						
FordPrefect						
tRNA-Gln-CAG						
U17						
tRNA-Ile-ATT						
tRNA-His-CAY_						
tRNA-Leu-TTG						
Down-regulated in KO				Up-regulated in 2i		
Up-regulated in KO				Down-regulated in 2i		
ERVs indicated with *				ERVs indicated with *		

Figure 41: A-B. Scatter plots comparing global transcription of repetitive elements between WT and KO mESCs cultured in serum (A) and 2i (B) conditions. C-D. Scatter plots comparing global transcription of repetitive elements between serum and 2i conditions in WT (C) and KO (D) mESCs. Bar graphs representing the expression levels of MMERGLN-int (E) and ETnERV3-int (G) ERV family. (F) List of up-regulated and down-regulated repetitive elements in WT and KO p53 mESCs transitioning from serum to 2i condition.

b) p53 binds specifically at the LTRs of the MMERGLN family in stem cells.

For simplicity, we will collectively further refer to the internal domains containing the coding sequence of the endogenous retroviral proteins (gag, pol and env), necessary for the life cycle of the integrated viruses as ERV-int, and the ERV external domains containing the regulatory regions of the long terminal repeats as LTRs.

In 1993, an immune selection procedure detected p53 binding at the LTR of the GLN ERV family (Zauberman et al. 1993). To identify the genome wide p53 distribution, we reanalyzed p53 ChIP-seq in serum- and 2i-cultured mESCs (Ter Huurne et al. 2020). We showed that p53 is specifically enriched at the LTR (RLTR1B family) of the GLN family (Figure 42A, B, C). The quantification of p53 profiles revealed a 4-fold decrease in p53 density at GLN-LTRs in 2i condition compared to serum condition, suggesting that the p53 binding to their response element is enhanced by DNA methylation. Collectively, our analyses reveal that p53 activates specifically the GLN family in stem cells through direct binding to their LTRs.

c) Dissecting the molecular mechanism of GLN regulation by p53 in mESCs.

Our next goal was to dissect the molecular mechanism that regulates GLN expression by p53 having in mind that p53 is functionally linked with H3.3, DAXX, Trim28 in ERVs regulation, as previously mentioned (see section: Preliminary results: Functional link between H3.3, DAXX and p53). Since ERVs are highly expressed in stem cells, these cells constitute an excellent *in cellulo* model to study the molecular mechanisms underlying the repression of GLN-ERV family in the absence of p53 during early mammalian development.

- *Deciphering a combinatorial epigenetic code at ERVs in mESCs.*

To better understand the epigenetic control of ERVs, our goal was first to decipher the distribution of the main epigenetic marks (5mC, 5hmC, 5fC, 5caC, H2A.Z, H3.3, H3K4me1, H3K4me2, H3K4me3, H3K9ac, H3K9me3, H3K14ac, H3K27ac, H3K27me3, H3K36me3, H3K79me) along ERVs (Figure 42A). To this end, we reanalyzed several public epigenomic datasets (MeDIP-seq and ChIP-seq) obtained in serum cultured cells. DNA repetitive elements have always presented computational challenges for sequence alignment of NGS datasets due the ambiguity of mapping short reads to non-unique sequences. To circumvent this problem, standard ChIP-seq alignments discard any read that cannot be mapped to a single location in the genome, leaving coverage gaps wherever the underlying sequence is found in multiple copies throughout the genome. To overcome this computational challenge and improve the read coverage at DNA repeats in general and at ERVs in particular, we included multi-hit reads randomly assigning them to one of the best alignments. Read densities were then collected in 50-bp sliding windows spanning 2 kb (divided in 20 bins) of the length-normalized ERV-int (divided in 60 bins), and heatmaps generated on full-length ERV-int (with a 6-kb cut-off) to eliminate truncated and degenerated ERVs.

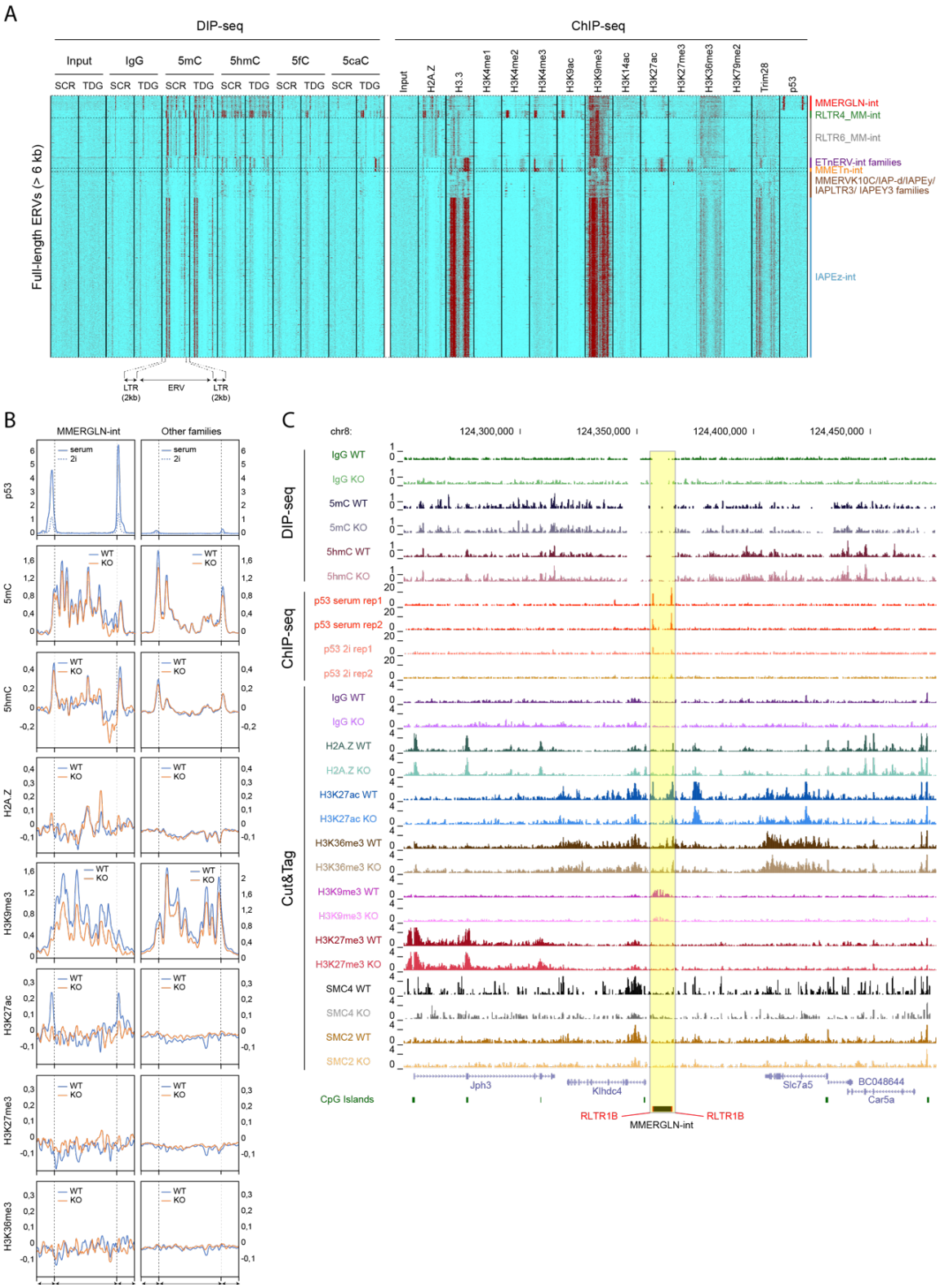


Figure 42: p53 activate the expression of the GLN family in mESCs through direct binding to their LTR. (A) Heatmaps of average chromatin modifications densities at full-length ERVs families (length > 6 kb, n = 1295) in serum-cultured cells. The 6-kb cut-off was selected to eliminate truncated and degenerated ERVs. Tags densities were collected in 50-bp sliding windows spanning 2 kb (divided in 20 bins) of the length-normalized ERV-int (divided in 60 bins). ERVs were sorted by families. p53 is found specifically at the long terminal repeats of the RLTR1B family surrounded the GLN family. (B) Normalized epigenetic modification densities (reads per million) at GLN family in WT and KO cells. (C) Genome browser view at representative locus on chromosome 8, showing epigenetic marks distributions (5mC, 5hmC, p53, H2A.Z, H3K9me3, H3K27ac, H3K36me3, H3K27me3, SMC2, SMC4, normalized in reads per million) in mESCs WT and KO for p53.

Interestingly, we observed that each ERV family has its own distinct epigenomic profile. For example, the IAPEz-int family accumulates repressive marks (5mC, H3.3, H3K9me3, H3K36me3 and Trim28) in line with (Turelli et al. 2014), while the RLTR4_MM-int family accumulates active marks (5hmC, H2A.Z, H3K4me2, H3K4me3 and H3K9ac). Moreover, our analyses revealed that the GLN family is found in a specific chromatin context characterized by the presence of both active (5hmC, H3.3 and H3K27ac enrichments on their LTRs) and repressive marks (accumulation of 5mC and H3K9me3 along their internal domain) (Figure 42A, B).

Our data uncover a combinatorial epigenetic code at ERVs in stem cells, that is in line with the dynamic DNA methylation code described by (Papin et al. 2017) at repetitive elements. Each ERV family has its distinct epigenomic profile, suggesting that the regulation of their expression is not limited to DNA methylation and H3K9me3 marks (Matsui et al. 2010; Rowe, Friedli, et al. 2013; Rowe, Kapopoulou, et al. 2013; Turelli et al. 2014), but rather through a more sophisticated mechanisms, implicating different combinations of transcription factors and epigenetic marks, imposed by a dynamic chromatin context.

- *The H3.3-mediated epigenetic regulation of GLN expression is lost in the absence of p53.*

Our analyses reveal a specific epigenetic profile at the GLN family characterized by the presence of both active marks (5hmC, H3.3 and H3K27ac) at their LTRs (which colocalize with p53) and repressive marks (5mC and H3K9me3) along the central part of the retroviral sequence. To confirm these results and to highlight the molecular mechanisms underlying the repression of GLN-ERVs in the absence of p53, our next goal was to understand how the absence of p53 impacts the chromatin landscape at ERVs. To this end, we performed DIP-seq, ChIP-seq and CUT&Tag experiments to characterize the profiles of several epigenetic marks including 5mC, 5hmC, H2A.Z, H3K9me3, H3K27me3, H3K27ac and H3K36me3 in mESCs WT and KO for p53 (Figure 42B, C). While we do not observe any change in the distribution of 5mC, 5hmC, H2A.Z, H3K27me3 and H3K36me3 in the absence of p53 at ERVs, our data highlight a specific and overall decrease in the amount of H3K9me3 along GLN-ERVs associated with a specific defect in H3K27ac deposition at their LTRs (Figure 42B, C).

Since H3K27ac and H3K9me3 is found at H3.3-enriched regions at ERVs (Figure 42A), and knowing that ERV expression is regulated by H3.3 in ESCs (Elsässer et al. 2015), we postulate that H3.3 is the main H3 family member targeted by K27ac and K9me3 modifications at ERVs. Thus, the loss of active (H3K27ac) and repressive (H3K9me3) epigenetic marks at GLN-ERVs might be a direct consequence of H3.3 deposition defect in the absence of p53. To confirm this hypothesis, we performed H3.3 and TRIM28 ChIP-seq experiments in p53 WT and KO cells, libraries are being sequenced at the time of writing this manuscript.

- *p53 targets condensin at transcribed CpG-rich promoters in mESCs.*

The condensin SMC2/SMC4 complex had been identified to interact with p53 (see section: Characterization of the endogenous p53 complex in MEFs.). Knowing that p53-tetramer preferentially binds to looped DNA (Brázda and Coufal 2017:3; Coufal et al. 2013; Jett et al.

2000; Stros et al. 2004; Vaughan et al. 2014) and that Condensin complex promotes loop extrusion (Davidson and Peters 2021; Ganji et al. 2018), we assumed that p53 tetramer might interact with SMC2/SMC4 subunits forming a p53-Condensin complex that facilitates ERV LTRs looping and subsequently ERV expression. To investigate this hypothesis, we performed SMC2 and SMC4 CUT&Tag using light cross-linking condition [0,1% PFA, 2 min (Kaya-Okur and Henikoff 2020)] in mESCs WT and KO for p53.

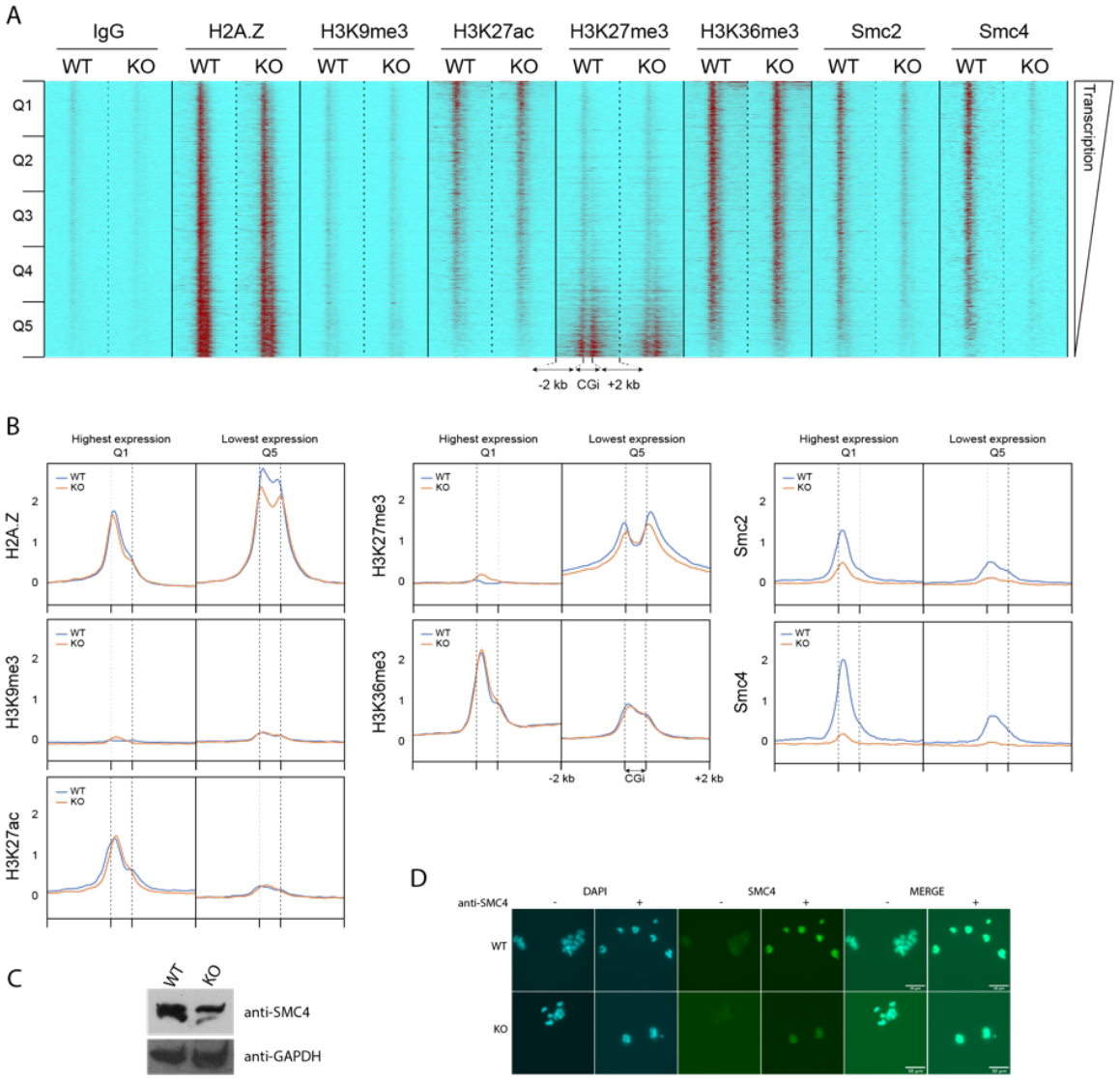


Figure 43: CGIs dictate the histone code at promoters in ESCs. (A) Heatmaps representing epigenetic marks densities around proximal CpG islands (CGIs). CGIs were length-normalized, transcription-oriented and sorted according to the expression level of their associated genes as fully detailed in methods of (Papin et al. 2021). CGIs were sorted in five quantiles according to the transcription level of their associated genes. The epigenetic marks distribution analysis based on oriented and length-normalized CGIs allows i) to identify a distinct profile for each epigenetic mark dictated by CGI boundaries and ii) to distinguish two specific chromatin signatures for active and repressed CGI promoters. (B) Average epigenetic marks signals (reads per million) at proximal CGIs in highly expressed (quintile Q1, left) and repressed (quintile Q5, right) promoters. (C) Western blot and (D) immunofluorescence analyses of SMC4 expression level in WT and KO cells.

While the SMC proteins were not found at ERVs (Figure 42C), our genome-wide analysis detected an enrichment of Condensin at CpG-rich promoters (containing CpG islands) that is positively correlated with transcription (Figure 43A). Of note, we measured a 3- to 10-fold

decrease in SMC2 and SMC4 densities, respectively, in KO cells, suggesting that p53 is required to target condensin on expressed CpG-rich promoters (Figure 43A, B). To address if this phenotype is related to modification of the chromatin state, we analyzed the distribution profiles of H2A.Z, H3K9me3, H3K27ac, H3K27me3 and H3K36me3 around CpG-rich promoters in the presence or in the absence of p53. We found that the incorporation of H2A.Z is mainly imposed by their CG content rather than their transcriptional activity (Figure 43A, B), while H3K27ac and H3K36me3 marks marked active CpG-rich promoters as previously described (Papin et al. 2021). Our data showed that inactive CGI promoters are not decorated by H3K9me3 in pluripotent cells. This result is in agreement with recent studies showing that H3K9me3 at gene promoters appears just after embryonic implantation to repress lineage-inappropriate genes, where it constitutes a major epigenetic barrier for reprogramming cell identity (Nicetto et al. 2019; Wang et al. 2018). The epigenetic marks profiles around proximal CGIs are similar in both WT and KO cells, suggesting that the defective recruitment of Condensin arising upon p53 loss, is not related to local chromatin state variation. This in line with our transcriptomic data showing that p53 functions have very little impact on global transcription in pluripotent cells (Figure 40). We further confirmed a decrease in the SMC4 expression level upon p53 loss by western blot and immunofluorescence analyses (Figure 43 C, D). The defective recruitment of Condensin on chromatin observed in the absence of p53 is an interesting phenotype that needs further validation by performing CUT&RUN or ChIP-seq experiments targeting the SMC2 and SMC4 subunits.

2. p53 activates specifically GLN-ERVs in MEFs.

Knowing that p53 activates specifically GLN-ERVs through direct binding to their LTRs in pluripotent cells, we asked whether this regulation mechanism is preserved in differentiated cells such as MEFs. To this end, we isolated WT and KO p53 primary MEFs and performed ribo-zero RNA-seq experiments from three biological replicates for each condition. Differential gene expression analysis confirmed that the p53 pathway are specifically down-regulated in KO cells (Figure 44A, B, C). Furthermore, DNA repeats transcription analysis confirmed the role p53 in the regulation of GLN-ERVs expression in MEFs (Figure 44D, E, F, G). With aim to decipher the genome-wide distribution of p53 in fibroblasts, we then used MEFs expressing endogenous Flag-HA tagged p53 (e-p53), and performed native ChIP-seq experiment. e-p53 was found localized at a subset of its target genes associated with Pol2 (data not shown) and at the LTR (RLTR1B family) of the GLN family (Figure 44F, G). We concluded that GLN-ERVs expression is activated by direct p53 binding to their LTRs in embryonic fibroblasts, suggesting that the GLN-related function of p53 is conserved during early mouse development from pluripotent to differentiated cells.

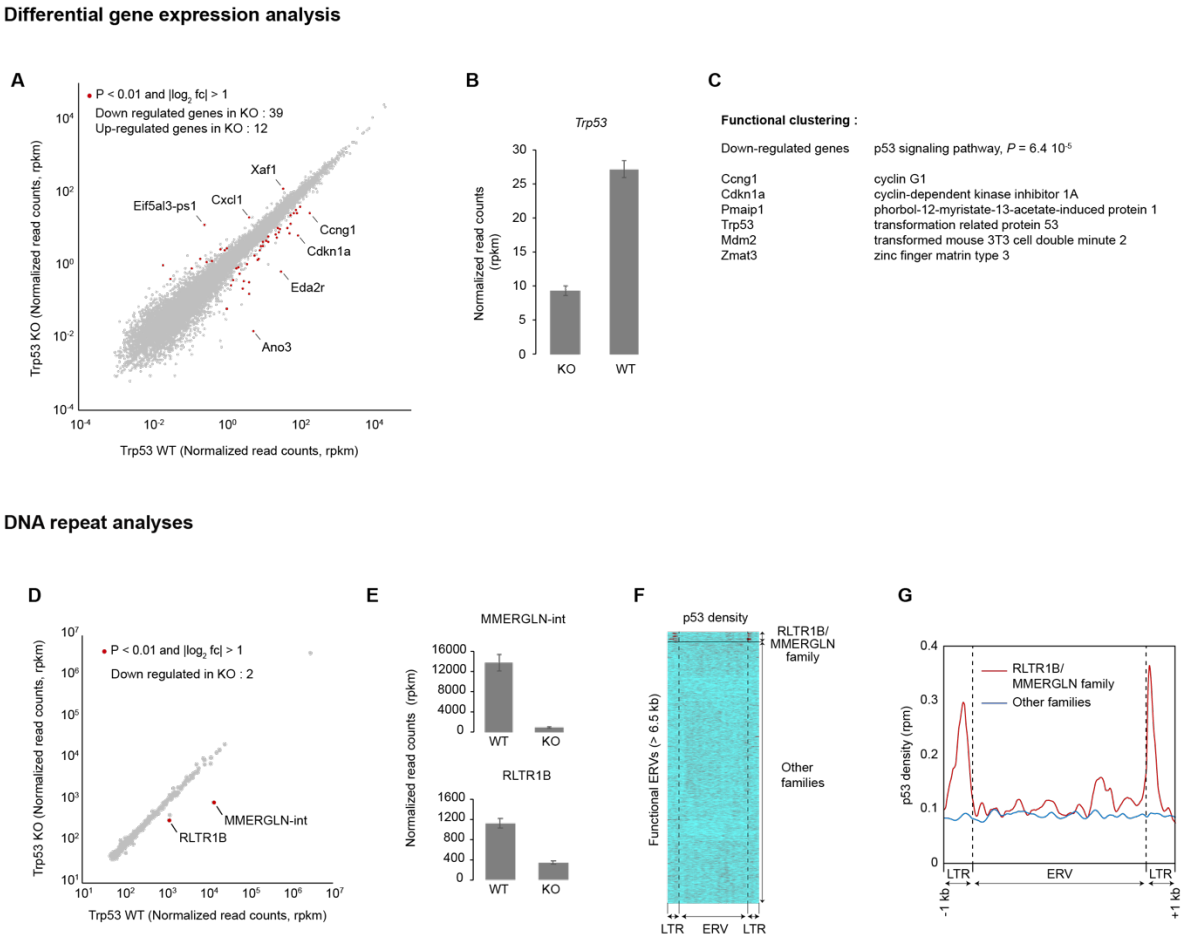


Figure 44: p53 binds at the LTR of GLN-ERVs and regulates their expression in primary mouse embryonic fibroblasts (MEFs). (A) Scatter plots comparing global gene expression levels between WT and KO p53 primary MEFs. 51 genes are significantly mis-regulated in the absence of p53 ($|\log_2$ fold change$| > 1$ and P value $< 10^{-2}$). (B) Bar graphs representing the expression level of *Trp53* gene in WT and KO p53 primary MEFs. (C) Functional annotation clustering of differentially expressed genes in the absence of p53. As expected, the p53 pathway is found down-regulated ($P < 10^{-4}$). (D) Scatter plots comparing global transcription of repetitive elements in WT and KO p53 primary MEFs ($|\log_2$ fold change$| > 1$ and P value $< 10^{-2}$). The RLTR1B/MMERGLN family (abbreviated GLN), is specifically down-regulated upon p53 loss. (E) Bar graphs representing the expression level of GLN ERV family in WT and KO primary MEFs. (F) Heatmap of e-p53 at full-length ERVs families (length > 6.5 kb, n = 1295) in e-p53 MEFs. e-p53 is found specifically at the long terminal repeats of the RLTR1B family surrounded the GLN family. (G) Quantification of p53 at full-length RLTR1B/MMERGLN family in e-p53 MEFs.

3. The p53-mediated regulation of GLN expression is preserved in adult tissues.

We then asked if p53 acts as an activator of GLN-ERVs expression in adult murine tissues. To this end, we analyzed the transcriptome of liver, kidney and cortex isolated from $p53^{+/+}$ and $p53^{-/-}$ mice (9 weeks old). Importantly, while no transcriptomic phenotype was observed in terms of gene expression, we observed a specific down regulation of ERVs of the GLN family in both kidney and liver (Figure 45). The absence of GLN expression in cortex is probably related to the distinct DNA methylation patterns and TE expression in the brain compared to the other tissues (Ecco et al. 2016; Erwin, Marchetto, and Gage 2014; Lister et al. 2013; Playfoot et al. 2021).

In conclusion, the GLN ERV expression mediated by p53 is a conserved process from early mouse embryonic development (in pluripotent and differentiated cells) to adult tissues (in liver

and kidney). The fact that the absence of p53 suppresses specifically the expression of GLN-ERVs and has minor (mESCs, MEFs) to zero (kidney, liver) effects on gene expression at the genome-wide level further highlights the critical role of GLN-ERVs across murine development.

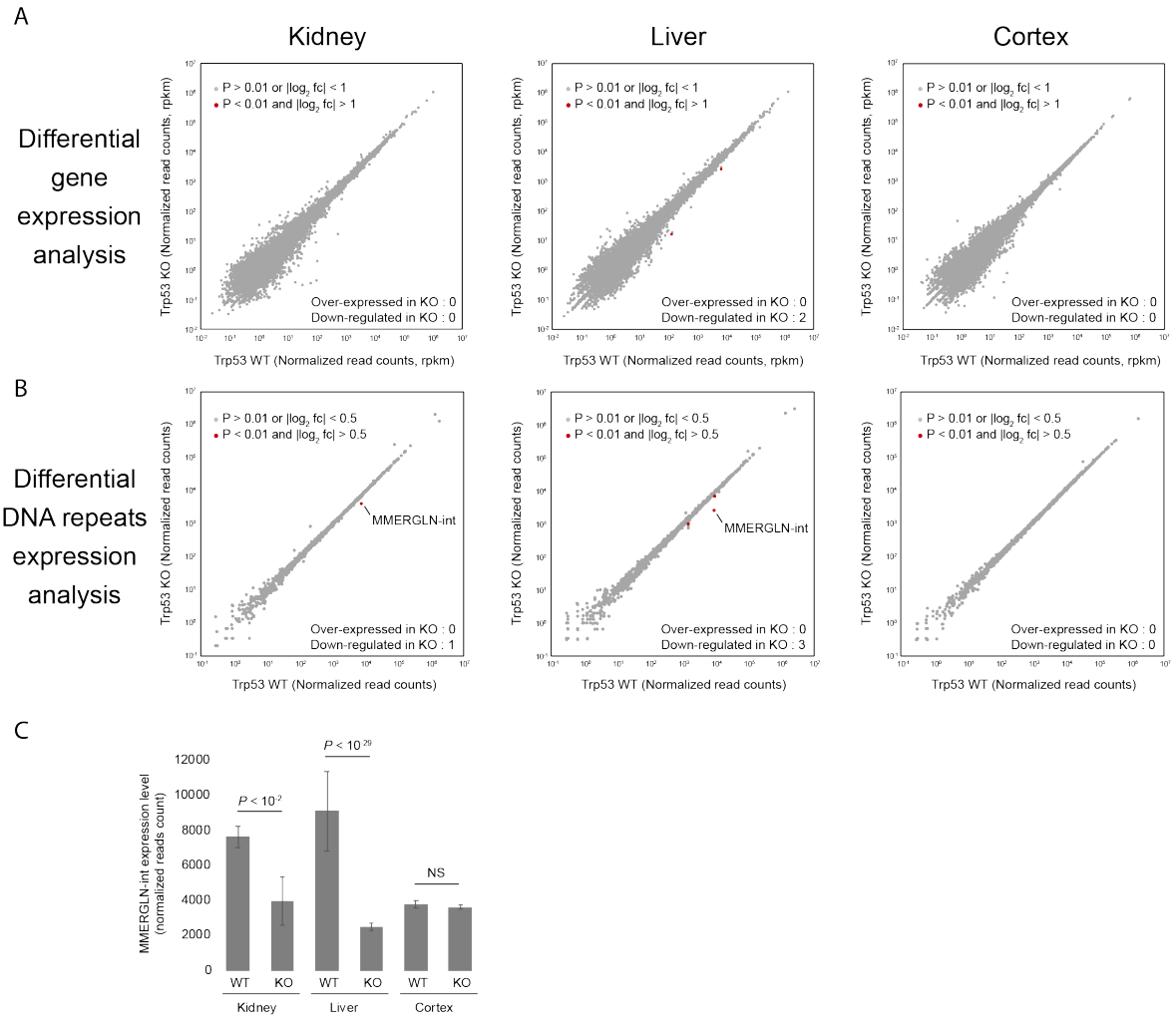


Figure 45: p53 activates GLN-ERVs expression from early development to adult tissues. Scatter plots comparing (A) global gene expression levels and (B) global transcription of repetitive elements in tissues (kidney, liver and cortex) between WT and KO p53 mice ($|\log_2$ fold change$| > 1$ and *P* value $< 10^{-2}$). (C) Bar graphs representing the expression level of the MMERGLN-int ERV family in tissues (kidney, liver, cortex) isolated from WT and KO mice.

4. Discussion

To sum up, our study elucidates the molecular mechanism underlying the functional link between p53 and H3.3 in the regulation of ERVs. We show that p53 binds directly to the LTRs of the murine ERV1 family GLN and promotes its expression, a mechanism preserved across murine development, based on our results in mESCs (Figure 41, Figure 42), MEFs (Figure 44) and adult tissues (Figure 45). Our data suggest that p53 is responsible for the recruitment of H3.3 at GLN-ERVs, and that, in the absence of p53, the H3.3-mediated epigenetic regulation of GLN-ERVs (through K27ac and K9me3 modifications) is lost. Thus, we postulate that p53 can access its binding sites within a nucleosome at the LTRs of GLN-ERVs, which are governed by dynamic heterochromatin (Figure 34) and are non-permissive to most DNA-binding transcription factors, by acting as a pioneer transcription factor ((Bao et al. 2017; Nishimura et

al. 2020:53; Sammons et al. 2020; Yu and Buck 2019)). In particular, recent studies show that the p53 binding sites, highly enriched in the ERV1 family, exhibit an unusual combination of chromatin patterns: high nucleosome occupancy and, at the same time, high DNase-I sensitivity (Bao et al. 2017), as well as a location at the entry/exit sites of the nucleosomal DNA, next to the H3-H4 tetramer (Nishimura et al. 2020:53; Yu and Buck 2019). Moreover, in line with these observations, a recent study has shown an increased p53 occupancy at methylated binding sites (Kribelbauer et al. 2017), which strengthens the notion of the p53 pioneer's ability to access its target sites in a methylated/non-permissive chromatin environment. Concluding, we believe that the pioneer p53 transcription factor recognizes and binds its binding site at GLN-ERVs, inducing a change in the chromatin landscape that favors the H3.3 nucleosome recruitment and its PTMs, K27ac and K9me3.

Our findings suggest an essential role of GLN-ERVs in host homeostasis. The facts that (i) GLN-ERVs were the only ERV family expressed irrespective of DNA methylation chromatin status in mESCs (Figure 41) (ii) that loss of p53 has minor (mESCs, MEFs) to zero (kidney, liver) effects on gene regulation (Figure 40, Figure 44, Figure 45) and (iii) that p53 specifically regulates GLN-ERVs expression across murine development, highlight its importance.

B. GLN-Env protein inhibits tumor growth *in vivo*

As mentioned in the introduction (see section: Chapter 3: Endogenous retrovirus and their dual role in cancer), although retrovirus infection has generally a deleterious effect on the host, the discovery of retrovirus genes that have been integrated into the genome for millions of years reveals that these elements may have been domesticated to play a fundamental role in the biology of the host. A representative example is the syncitin protein which is encoded by the envelope gene of a recently identified human endogenous defective retrovirus, HERV. Syncytin is required for the formation of mammalian placental architecture, by facilitating trophoblast cell fusion, but it also plays a critical role in the pathogenesis and prognostic impact of several cancers (see section: ERV in cancer). Therefore, we assumed that the envelope protein encoded by the GLN family (GLN-Env) may have such a dual role in cancer.

The GLN-Env protein is a transmembrane glycoprotein which is composed of two polypeptides, an external heavily glycosylated polypeptide (SU) and a membrane-spanning protein (TM). Together the two polypeptides form a heterodimer on the surface of the virion. These polypeptides are synthesized in the form of a polyprotein precursor which is glycosylated and proteolytically processed during its maturation in the secretory pathway. The polyprotein precursor is targeted to the cytoplasmic membrane by a signal peptide at its N-terminal end and then is divided into the two polypeptides by the cellular furin protease which recognizes its RSKR cleavage site. Env-GLN has a highly conserved motif of four amino acids, CWLC, which has been identified in most known oncoretroviral SU proteins, about two-thirds of the distance from the amino terminus (Gu et al. 1995; Ribet et al. 2008) (Figure 47A).

1. Working hypothesis

We hypothesized that GLN-Env could contribute to the tumor suppressor activity of p53, probably by activating the immune-response through an unknown co-opted mechanism that might implicate viral vesicles formation (Ribet et al. 2008:5; Zeilfelder et al. 2007). We

postulated that the GLN-Env proteins, which is under p53 control, is used by the cells to provide cell identity and prevent cell proliferation and/or migration during oncogenesis. Normal cells expressing GLN-Env proteins are contained by the immune system within their natural tissue perimeter. Oncogenic cells accumulating p53 mutations or deletions are unable to express GLN-Env proteins and hence lose their cell identity. They are not sensed by the immune system as invaders, which allows them to cross the extracellular matrix and to leave their original tumor microenvironment to form metastasis. The ability of cancer cells to disseminate from the primary site and form distant metastases is the leading cause of cancer-related morbidity in patients with solid tumors.

To validate our working hypothesis, we examined the potential function of GLN-Env in the repression of tumor development using an allograft assay in mice.

2. Allograft assay

Since p53 is required for GLN-ERVs expression in MEFs (Figure 44), we first asked if the injection of p53 KO MEFs could be an adapted tumorigenic cell line for allograft assays. In 1994, it was mentioned that KO p53 primary MEFs are polyploid, but unable to induce tumor formation in WT C57BL6 mice (Jacks et al. 1994), without the presence of a driver mutation such as the expression of H-RasV12 (Jia et al. 2012). Nevertheless, the tumor detection methods used were of low sensitivity, and more recent technics, such as luciferase-based bioluminescence (BLI) methods, can be used to detect tumors at preliminary stages. We then decided to perform allograft assays, using p53 KO MEFs expressing firefly luciferase (Luc) gene allowing us to directly track cells *in vivo* by BLI. The expression of luciferase was induced alone or together with v-H-Ras (Harvey murine sarcoma virus p21 transforming protein), by lentivirus transduction using two separate lentivirus expression vectors (H-Luc/V5 or H-Ras-Luc/EGFP, respectively). We first carried out a pilot study to address the tumorigenic potential of p53 null MEFs in WT C57BL6 mice and determine the best experimental conditions for tumor development (cell line, cell numbers and injection site).

a) Pilot study

6 weeks old WT C57BL/6J mice were injected subcutaneously or intravenously with p53 KO MEFs expressing H-Ras-Luc/EGFP or H-Luc/V5. Furthermore, 50,000, 500,000 or 5 million cells were injected to identify the minimum number of cells necessary for tumor development in a time window of 42 days. The mice injected with p53 null MEFs expressing H-Luc/V5 did not form tumors under any of the experimental conditions tested. Mice receiving subcutaneously or intravenously p53 null MEFs overexpressing v-H-Ras, generated tumors in 2-3 weeks but only when 5 million cells were injected (data not shown). In the intravenously injected mice, we observed lung tumors without any micro-metastasis (3 out of the 3 mice of the group), while the subcutaneously injected mice developed tumors at the injection site (3 out of the 3 mice of the group). For 1 of the 3 mice, micro-metastasis in gut, liver, spleen, kidneys and lungs were observed (data not shown).

We conclude that the optimal experimental condition to induce tumor was the subcutaneously injection of 5 million p53 null MEFs expressing H-Ras-Luc/EGFP. Our result confirms that p53

loss function *per se* is not oncogenic enough to detect tumor - even using sensitive BLI method – however, together with v-H-Ras overexpression, could dramatically promote tumorigenesis.

b) Multiple GLN isoforms are expressed in embryonic fibroblasts.

Since p53 is required for GLN-ERVs expression in MEFs (Figure 44), and p53 loss of function promote tumorigenesis when associated with v-H-Ras expression in fibroblasts, if GLN-Env contributes to the tumor suppressor activity of p53, we should observe an inhibition of tumors growth induced by p53 null MEFs injection, in which GLN-Env expression will have been rescued.

To address this question, we first wondered which GLN-Env isoforms are expressed in WT MEFs. We performed a locus analysis of our RNA-seq datasets obtained in MEFs WT and KO for p53, by focusing our attention on functional GLN-ERVs with a length of more than 7.5 kb. This size-based selection process eliminates the most truncated and highly degenerated ERVs, but do not exclude the full-length ERVs encoding truncated retroviral proteins due to small-scale mutations such as point mutations, small deletions or small insertions. The mouse genome contains 28 individual full-length GLN-ERVs, which encode N-terminal, C-terminal truncated or full-length GLN-Env proteins (Figure 46). We showed that 23% of the total full-length GLN-ERVs transcripts code for full-length envelope proteins (Env WT), 44% for a N-terminal deleted isoforms (Env Δ1-320), and 33% for other various deleted mutants (Figure 46). Of note, p53 activates the transcription of all individual GLN family members regardless of the Env isoforms they encode.

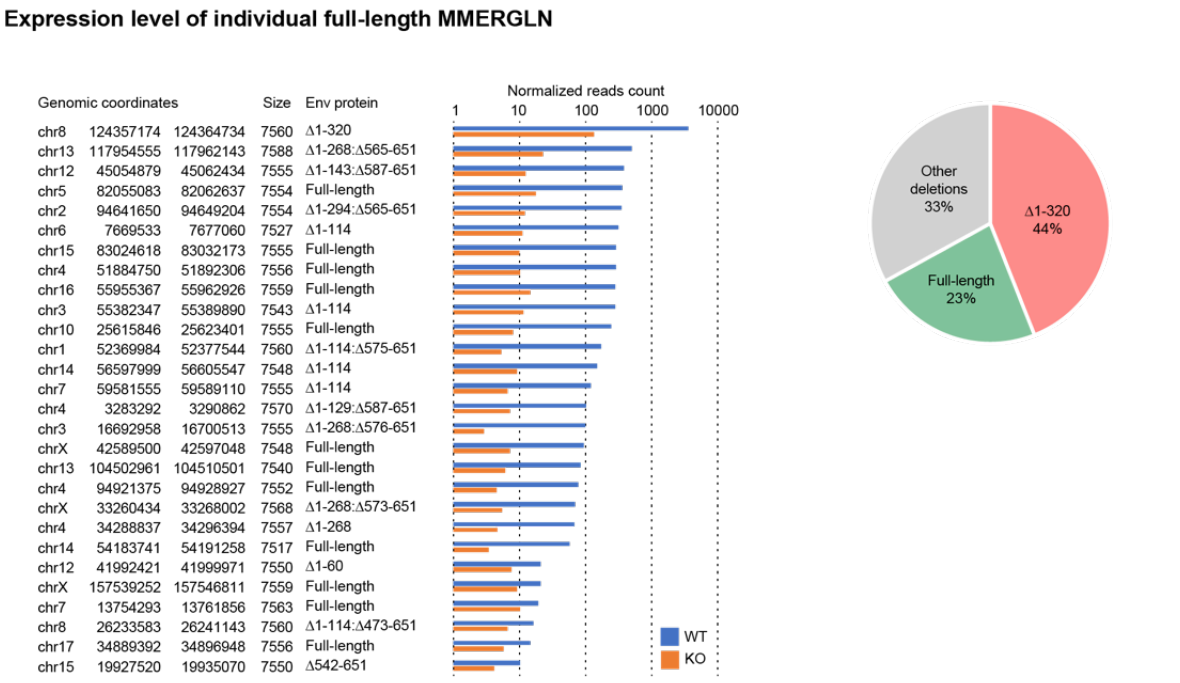


Figure 46: Expression levels of individual full-length GLN-ERVs in primary MEFs. Only full-length GLN-ERVs (>7.5 kb) were considered to eliminate truncated and degenerated elements. (A) List of the full-length GLN-ERVs. Their genomic coordinates (mm9), their size, and the isoform of the envelope protein (Env) encoded are indicated. The bar graphs represent the expression levels of the individual member of the GLN family in WT (blue) and KO (orange) cells. (B) Venn diagram depicting the percentage of full-length, Δ1-320 and other deleted isoforms of the GLN-Env protein that are expressed in WT MEFs.

c) Rescue experiments

Our transcriptomic locus analysis on GLN-ERVs showed that the deletion mutant Env Δ1-320 and the full-length Env protein (Env WT) are the major GLN-Env isoforms expressed in p53 null MEFs. For rescue experiment, we then decided to stably express Env WT and Env Δ1-320 isoforms in p53 KO MEFs, together with the deletion mutant Env Δ1-50 (lacking the signal peptide) as controls. Full length or N-terminal truncated Env proteins fused to C-terminal Flag- and HA-epitope tags were stably expressed in primary p53 KO MEFs by retroviral transduction. Expression of the different GLN-Env isoforms in p53-KO rescue cell lines were confirmed by immunofluorescence and western blot analyses (Figure 47).

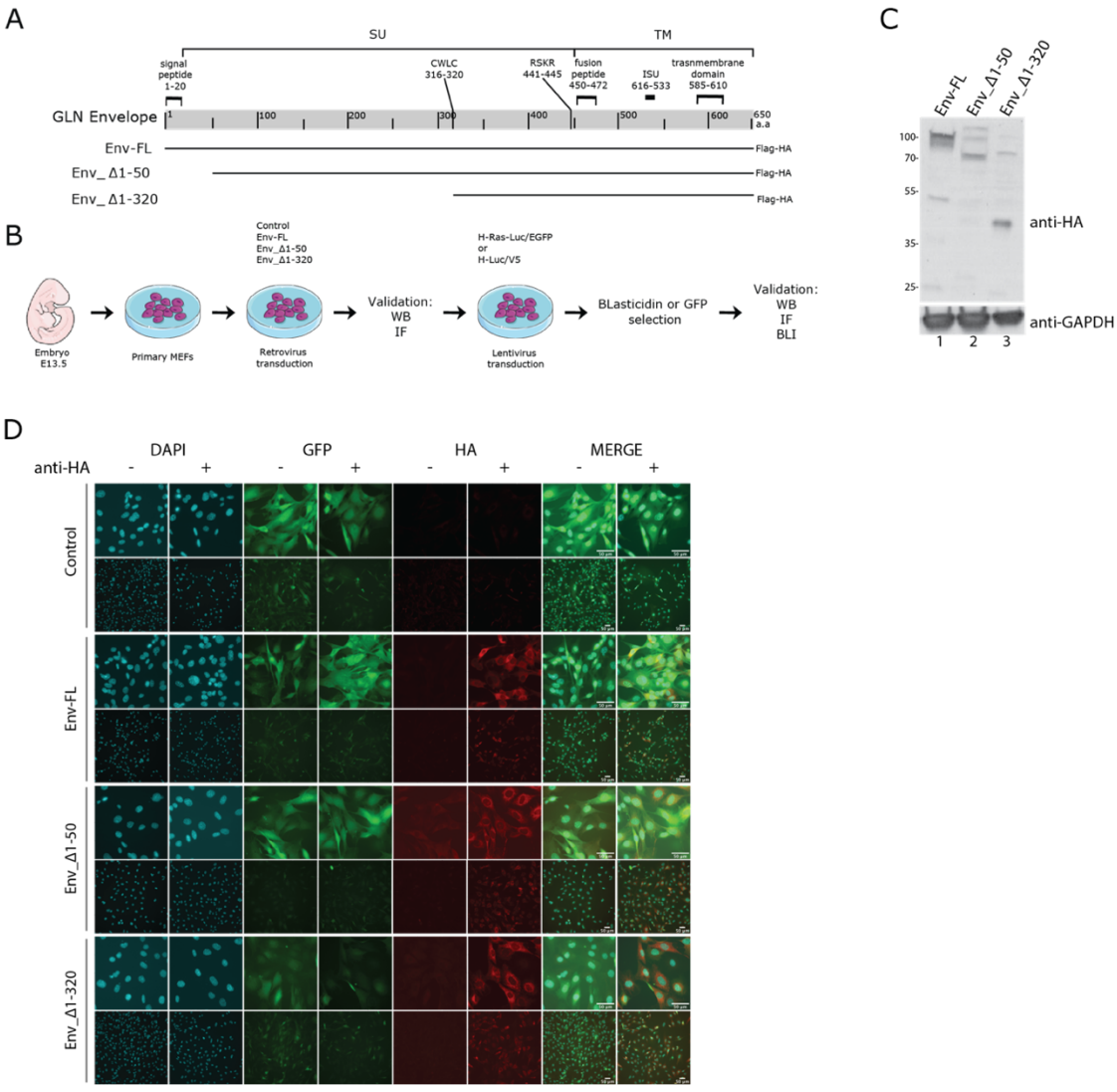


Figure 47: GLN-Env isoforms rescue experiments in p53 null primary MEFs. (A) Schematic representation of the different GLN-Env isoforms used. Structural organization of the GLN-Env protein is canonical, with a signal peptide at the N-terminal end and an R-X-(K/ R)-R consensus cleavage site for the cellular furin protease, splitting the Env protein into surface (SU) and transmembrane (TM) subunits. TM contains a hydrophobic fusion domain and a hydrophobic transmembrane anchor domain at their N- and C-terminal boundaries, respectively. The abbreviations Env-FL, Env Δ1-50 and Env Δ1-320 corresponds to the protein sequences GLN_e-EnvFL, GLN_e-EnvΔ1-50, GLN_e-EnvΔ1-320 described in 'Material and Methods' section. (B) Outline of retro- and lentivirus transduction process and cell line validation. (C-D) Western blot (C) and immunofluorescence (D) analyses of p53-KO rescue fibroblasts.

d) Main study

If GLN-Env contributes to the tumor suppressor activity of p53, we should observe inhibition and/or slowing of tumor growth induced by injection of p53 null MEFs, in which GLN-Env expression will have been rescued. To test this hypothesis, we repeated allograft assays with the optimal experimental conditions deduced from the pilot study, and evaluated the tumorigenic potential of p53 KO MEFs, after restoration or not of the full-length envelope protein (Env WT) or deletion mutants (Env Δ1-50 and Env Δ1-320).

6 weeks old WT C57BL/6J mice were injected subcutaneously with 5 million p53 KO MEFs expressing H-Ras-Luc/EGFP rescued with the different GLN-Env isoforms (Env WT or Env Δ1-50 or Env Δ1-320) or not (control). Tumors formation was evaluated using BLI, caliper and body weight monitoring for 50 days (Figure 48 A). BLI was initiated one day after implantation of cells and conducted weekly thereafter (days 1, 8, 15, 22, 29, 36, 43 and 50), while caliper and body weight measurements were conducted 3 times *per* week (Figure 48A). To have statistically significant results, 12 mice were used per condition.

Our allograft assays showed that restoration of the full-length GLN-Env protein significantly delays tumor progression during the first 3 weeks following injection. Comparing to control, a 2-fold decrease in tumors volume ($p < 0.05$) was calculated with caliper at days 15 and 17 in mice injected with GLN-Env rescued fibroblasts (Figure 48B, C, D). Of note, while the expression of the Env Δ1-50 isoform did not affect tumor progression, the expression of the deletion mutant Env Δ1-320 leads to a drastic acceleration of the tumor growth. Throughout the time-window, tumor progression was assessed using a caliper. We measured to 2-to-3 fold increase in tumor size in mice injected with p53 KO MEFs expressing the Env Δ1-320 mutant.

BLI monitoring (Figure 48E) did not detect any micro-metastasis at any organ and at any of the aforementioned conditions. We assumed that the metastasis observed in the pilot study was due to the injection process and not to the metastatic potential of p53-KO cells.

Similarly to the caliper tumor growth evaluation, BLI signal quantification also showed that (i) the full-length GLN-Env protein slowed tumor growth, (ii) the deletion mutant Env Δ1-320 accelerated tumor growth, while (iii) Env Δ1-50 isoform had no effect on tumor growth compared with control for the first 3 weeks after injection (Figure 48F, G). Nevertheless, BLI is essentially qualitative technique and ideal tool in exploring metastatic spread, but attempts to quantify the BLI signal can be misleading given that the signal strength is dependent on several factors including duration of exposure, anesthetic technique, time elapsed after injecting luciferin, etc. (Sinha, Pieterse, and Kaur 2018). Therefore, we considered only the caliper measurement for the quantitative assessment of tumor growth.

Our results showed that the full-length GLN-Env has tumor suppressor activity, as restoration of its expression slowed p53-null MEF-induced tumor progression. Interestingly, mice injected with MEFs expressing GLN-Env Δ1-50 had a 2-fold increase in tumor volume ($p < 0.05$) during the 3 weeks after injection (Figure 48B, C, D). This result suggests that the GLN-Env signal peptide, located at its twenty-first N-terminal amino acids, is necessary for targeting the cytoplasmic membrane and is required for the tumor suppressor activity of GLN-Env. Furthermore, the observation showing accelerated tumor growth of the Env Δ1-320 deletion mutant, which lacks the N-terminal amino acids located before the highly conserved CWLC motif, indicates that it is a gain-of-function mutation. In particular, at murine leukemia virus (MLV), the CWLC motif is responsible for the labile disulfide bond between SU and TM controlling (i) the retrovirus membrane fusion, or (ii) the endosome's fusion at the plasma

membrane (Pinter et al. 1997; Wallin, Ekström, and Garoff 2005). Thus, we speculate that the truncated GLN-Env Δ1-320 fail to interact with the SU domain or change the stoichiometry between SU and TM in the plasma membrane resulting to an aberrant oncogenic inter-cellular signaling. Nevertheless, the oncogenic potential of the GLN Env deletion mutants (Δ1-50, Δ1-320) is not definite, as we are not sure that GLN Env deletion mutants are translated to proteins in normal cells. They might function only at the post-transcriptional level. Thus, we concluded that GLN-Env protein contributes through its function to the tumor suppressor activity of p53 and has a vital role in cellular homeostasis as its mutations may have deleterious effects on tumor progression.

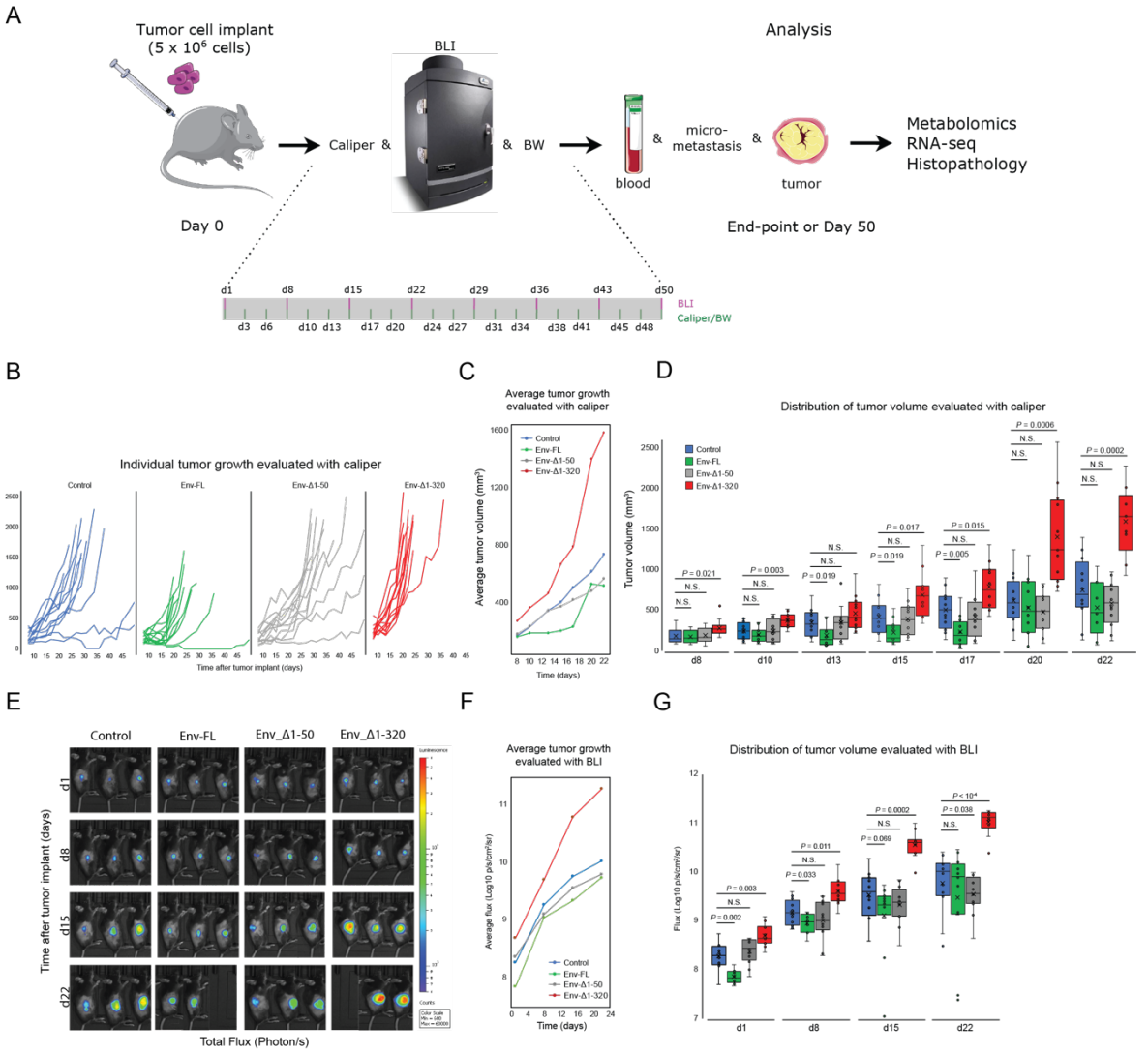


Figure 48: GLN-Env protein inhibits tumor growth contributing to the tumor suppressor activity of p53 *in vivo*. (A) Outline and timeline of experimental design. (B) Individual, (C) average and (D) distribution of tumors growth calculated by caliper measurements at the indicated time point (*P* value < 0,05). (E) Bioluminescent images of the mice over time. Only 3 mice per experimental conditions are shown. Average (F) and distribution (G) of tumor growth calculated by bioluminescence at the indicated time point (*P* value < 0,05).

Taken as a whole, our *in vivo* experiments confirm our initial hypothesis that the full-length GLN-Env contributes to the suppressor activity of p53. However, this study does not elucidate the exact mechanism through which the envelope protein encoded by GLN-ERVs inhibits tumor

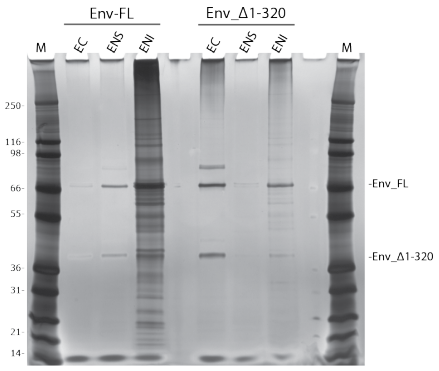
formation and is “sensed” by the immune system. Further experiments are needed to answer this question.

3. Protein interactors of GLN: SLC receptors

In 2019, SCL19A1, the reduced folate carrier, was identified as the receptor of Env GLN protein using a gain-of-function brain cDNA library screen (Tsang et al. 2019). To get more insight into the function of GLN-Env isoforms, full-length (Env WT) and deletion mutant Env Δ1-320, were used as bait to identify their protein interacting partners. More precisely, Flag-HA-epitope-tagged full-length and Δ1-320 Env complexes were purified from cytoplasmic and insoluble nuclear fractions of rescued KO p53 MEFs using double immunoaffinity sequential immunoprecipitations with anti-Flag antibody followed by anti-HA antibody. Proteins associated with the Env WT or Env Δ1-320 were separated on SDS-PAGE and subsequently silver-stained (Figure 49A).

Mass spectrometry analyses identified ER vehicles trafficking factors as GLN-Env protein interactors for both isoforms (Figure 49B). This was an expected result, as GLN-Env is a transmembrane protein that reaches the cytoplasmic membrane through ER vehicles trafficking (Stalder and Gershlick 2020). Interestingly, we identified numerous transmembrane transporters of the SLC family (Figure 49C) responsible primarily for the influx and secondarily for the efflux of a very wide variety of substrates such as ions, amino acids, metals, sugars, nucleotides, vitamins, hormones or neurotransmitters, but not the reduced folate carrier, SCL19A1. This difference in identifying the SLC receptors may derive from the fact that distinct DNA methylation profiles (Lister et al. 2013) and TE expressions (Erwin et al. 2014; Playfoot et al. 2021) are established during brain development compared to other tissues, derived from MEFs. Moreover, the first 320 N-terminal amino acids of GLN-Env seem important for its interaction with some SLC receptors (Figure 49C). In conclusion, our data shed light on GLN function by identifying SLC transporters as potential receptors for Env-GLN proteins in different tissues and cell lines. We assume that the Env-SLC interaction helps the immune system to distinguish tumorigenic cells from healthy cells by a mechanism that remains to be discovered.

A



B

Functional clustering :

cluster	Env-FL enrichment score	Env-FL *P* value	Env_Δ1-320 enrichment score	Env_Δ1-320 *P* value
Endoplasmic reticulum (ER)	160.41	$3.3\ 10^{-184}$	113.02	$2.4\ 10^{-128}$
Membrane / transmembrane	45.28	$5.1\ 10^{-104}$	39.91	$7.4\ 10^{-97}$
Lipid metabolism	24.5	$2.4\ 10^{-25}$	20.20	$1.3\ 10^{-20}$
Protein transport	22.25	$2.4\ 10^{-25}$	13.84	$5\ 10^{-20}$
ER to Golgi vesicle-mediated transport	15.9	$7.4\ 10^{-20}$	10.98	$6.7\ 10^{-10}$
Mitochondrion	13.68	$1.5\ 10^{-15}$	16.44	$2.6\ 10^{-44}$
Protein folding	11.54	$2.3\ 10^{-12}$	5.8	$3.7\ 10^{-12}$

C

Protein	MWT (KDA)	Env-FL Unique Peptides ENI	Env-FL Total Peptides ENI	Env_Δ1-320 Unique Peptides ENI	Env_Δ1-320 Total Peptides ENI
GLN Envelope	75.56	32	208	11	41
Slc27a4	72,27	13	16	7	9
Slc25a4	32,88	7	13	10	27
Slc25a13	74,42	1	1	10	14
Slc25a3	39,61	4	7	8	21
Slc25a12	74,52	0	0	7	9
Slc3a2	58,3	6	7	1	1
Slc25a11	34,13	2	2	4	4
Slc33a1	61,04	4	4	3	3
Slc25a24	52,87	1	1	4	4
Slc25a5	32,91	3	4	3	5
Slc44a2	80,06	3	3	1	2
Slc39a7	50,63	3	4	0	0
Slc1a5	58,45	2	3	0	0
Slc16a1	53,23	2	3	0	0
Slc12a4	120,55	2	2	0	0
Slc27a3	72,92	2	2	0	0
Slc30a7	41,76	2	2	0	0
Slc27a1	71,23	1	2	0	0
Slc35a2	40,74	1	2	0	0
Slc6a6	69,81	1	1	0	0
Slc41a3	53,22	1	1	0	0
Slc1a5	58,39	1	1	0	0
Slc39a13	38,48	1	1	0	0
Slc39a11	35,44	0	0	1	1
Slc2a1	53,95	1	1	0	0
Slc25a20	33	0	0	1	1

Figure 49 : Purification of Env-GLNs complexes and identification of SLC transporters as their potential receptors. (A) Silver staining of GLN-Env complexes isolated from MEFs stably overexpressing full length (Env_FL) or Env_Δ1-320 isoforms. (B) Functional annotation clustering of enriched pathways identified by the mass spectrometry analyses of Env GLNs complexes, WT and Env Δ1-320. (C) Mass spectrometry analysis providing a selectively list with identified SLC transporters. MWT: Molecular weight, ENS: Soluble nuclear extract, ENI: insoluble nuclear extract.

4. Discussion

The vast majority of endogenous retroviruses, which are not subject to selection pressure, have been progressively rendered inactive by simple accumulation of mutations or deletions during evolution. As a result, among all the copies of an ERV family, only a few elements have retained their ability to generate infectious viral particles (Ribet et al. 2008). Some members of the class I endogenous retroviruses GLN family have remained infectious. The GLN-ERV family, designated due to an unusual primer-binding site sequence corresponding to $tRNA^{GLN}$, was first identified over two decades ago (Itin and Keshet 1986; Obata and Khan 1988). Based on the available bibliography, GLN-ERVs seems to have an oncogenic role. In particular, knock-down (KD) of GLN-ERVs suppresses cell growth in several mouse cell lines, while GLN-ERVs KO induces G1/S phase arrest and inhibits cell proliferation in NIH/3T3 cells (Y. Wu et al. 2012). GLN-ERVs expression is repressed by H3K9me3 mediated by SETDB1 (Karimi et al. 2011a), while GLN-ERVs function as a cis-regulatory element capable of regulating H3K27ac enrichment and transcription of neighboring loci (Xuemeng Zhou et al. 2021).

In this study, although, we prove that endogenous retroviral proteins do not only have pathological roles, but that they can be 'domesticated' to acquire a physiological function beneficial to the host. Specifically, we prove that the full-length GLN-Env contributes to the tumor suppressor activity of p53 (Figure 48). Our data highlight a vital role of GLN-Env in host homeostasis as (i) its mutations may have deleterious effects on tumor progression (Figure 48), (ii) it is the only ERV family expressed independent of DNA methylation in mESCs (Figure 41) with minor (pluripotent and differentiated cells; Figure 40, Figure 44,) to zero (tissues; Figure 45) effects on gene regulation, (iii) p53 act as pioneer transcription factor and regulates GLN-ERVs expression across murine development. Moreover, our results suggest that the tumor suppressor function of p53 is mediated in part by the GLN envelope glycoprotein, which “senses” the extracellular environment and may crosstalk with the immune system.

The identification of SLC transporters in Env-GLN-associated protein complexes indicates that proteins of the SLC family may act as potential receptors for envelope glycoproteins encoded by ERVs (Figure 49). In the future, it will be important to elucidate whether the tumor suppressor activity of GLN envelope proteins is mediated by SLC proteins, and whether it mobilizes the immune system. If so, addressing the molecular mechanisms by which an Env-SLC interaction can promote the immune system to distinguish tumorigenic cells from healthy cells, and studying whether such a function is found in humans, will be key objectives to better understand the mechanisms of tumorigenesis.

Conclusion and Perspectives

VI. Conclusion and Perspectives

Most of the current genome-wide studies do not consider repetitive elements in their analysis due to alignment problems and poor mappability, even though they represent more than half of the human genome and the abundance of transcription binding sites at ERV LTRs indicates a critical role in genome integrity and functionality. In this study, we showed that p53 directly binds to the LTRs of GLN-ERVs, induced a change in the chromatin landscape along the retroviral sequence and activated their expression, a mechanism preserved across murine development. This result is in line with the high occupancy of p53 binding sites observed at the LTRs of class I ERVs (Bao et al. 2017; Wang et al. 2007:53), to which GLN-ERV family belong. In addition, we showed that p53 is responsible for the recruitment of H3.3 to GLN-ERVs. As a result, the post-translational modifications carried by H3.3 (K27ac and K9me3) are lost in the absence of p53. We hypothesize that the function of p53 is to bridge the 5'LTR and 3'LTR of GLN ERV to mediate the formation of a looped chromatin structure that controls the transcription process. The 3D looping between the two GLN LTRs will be stabilized by the Condensin (SMC) complex, which is known to promote loop extrusion (Davidson and Peters 2021; Ganji et al. 2018). To validate this hypothesis, we will design an in vivo experiment to test the ability of GLN LTRs to form a DNA loop. More precisely, we will use distinct fluorescent sgRNA to label the two GLN LTRs using the Cas9-mediated fluorescence in situ hybridization (CASFISH) technic (Deng et al. 2015) in p53 WT and KO primary MEFs cells. In p53 WT cells, we expect to observe a colocalization of the fluorescent signal due to LTRs' looping. In contrast, we expect to see two distant and distinct fluorescent signals in p53 KO cells since p53 is required to recruit the Condensin to the ERV LTRs. Another possibility to prove our hypothesis is to check the GLN LTRs looping formation in vitro using a recombinant p53 protein bound to a synthetic GLN LTRs sequence. We will visualize the GLN LTRs' looping formation either by gel shift assays (EMSA) (Coufal et al. 2013) or electron microscopy (Jett et al. 2000; Stansel, Subramanian, and Griffith 2002). Similarly, we will assay the preference of p53 to bind to methylated over non-methylated synthetic GLN LTRs sequence, as suggested by in vivo experiments showing an increased p53 occupancy at methylated binding sites (Kribelbauer et al. 2017).

The most exiting result of this study is undoubtedly the tumor suppressor activity of the full-length GLN envelop protein. This result suggests that the tumor suppressor function of p53 is mediated in part by the envelope glycoprotein encoded by GLN-ERVs. This work provides evidence that endogenous retroviral proteins do not only have pathological roles, but they can be 'domesticated' to acquire a physiological function beneficial to the host. Our next goal is to elucidate the mechanism of GLN envelope glycoprotein recognition by the immune system through metabolomics, transcriptomics, and immunological analysis of tumors with restored GLN envelop expression in the absence of p53. A special attention will be payed to the subcutaneous tumors where the GLN-Env expression is restored, as they constitute an excellent model to explore the molecular function of the GLN-Env in vivo. Therefore, the subcutaneous tumors will be extracted post-mortem (Figure 48A) and used for transcriptomics, metabolomics, and histopathologic analysis. Additionally, mice blood collection will be performed post-mortem (Figure 48A) to identify the hematopoietic cell populations that help circulating tumor cells (CTCs) to disseminate (Pereira-Veiga et al. 2022). These experiments have been already started but data processing and analysis will performed later. We are confident that the identification of the SLC transporters as possible GLN envelop

receptors will shed light on whether the tumor suppressor activity of the envelope protein is mediated by SLC proteins, and whether it mobilizes the immune system. Thus, our upcoming work will contribute to decoding the complex crosstalk between cancer cells and the immune system and will elucidate the molecular mechanisms by which an Env-SLC interaction may promote the immune system to distinguish tumorigenic cells from healthy cells. Moreover, investigating whether such a function exists in human cells will be our next challenge to better understand the basis of tumorigenesis and to develop targeted anti-cancer therapies.

Another future experiment is the restoration of the GLN envelop expression in *Trp53*$^{-/-}$ mice, already available in our laboratory. The *Trp53*$^{-/-}$ mice develop tumors (principally lymphomas and sarcomas) at 3-6 months. We assume that if we restore the Env GLN envelop expression under a constitutive promoter, the tumor development will be delayed or suppressed. This experiment will further validate the tumor suppressor activity of GLN envelop protein in a different model system.

Undoubtedly, the tumor suppressor activity of GLN envelop protein makes it an excellent candidate for cancer immunotherapy. The therapeutic potentials are numerous, such as using epigenetic inhibitors that will restore the post-translational modifications of H3.3 (K27ac and K9me3) at GLN-ERVs and, consequently, the expression of GLN envelop protein in malignant cells to inhibit cancer progression. Thus, elucidation of the molecular mechanism that regulates GLN ERV expression is of paramount importance, especially for the development of targeted epigenetic therapies. In addition, the GLN envelop protein can serve as a tumor-associated antigen (TAA) for developing effective vaccine approaches against cancer (Kraus et al. 2013; Melief et al. 2015; Sahin and Türeci 2018; Saxena et al. 2021).

It will be important to investigate whether a similar GLN/p53 mechanism exist in humans. The fact that p53 binding sites are enriched at class I endogenous retrovirus both in human and mice (Bao et al. 2017; Wang et al. 2007:53) makes us confident that an equivalent mechanism exist in humans, although our preliminary GLN ERV homology analysis did not highlight any interesting human homolog. Thus, our future goal is to identify the equivalent GLN ERV in humans using human fibroblasts knock-out for p53. It is important to note that human tumor samples derived from p53 mutations are not a good option due to the tremendous clonal heterogeneity and the accumulation of additional mutations. We are confident that our future work will identify the GLN ERV human homolog regulated by p53 and will significantly contribute to the development of innovative anti-cancer therapies.

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VII. References

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Fonctions des protéines p53 et H3.3 dans la suppression des tumeurs

Résumé

Le variant d'histone H3.3 est un acteur épigénétique majeur dont les mutations sont a l'origine de plusieurs cancers. Elles entraînent la dérépression des rétrovirus endogènes (ERVs) et se superposent aux mutations de p53 et d'ATRX/DAXX dans les gliomes pédiatriques. Cependant, le lien fonctionnel entre p53 et H3.3 reste inconnu. Nous présentons ici le mécanisme moléculaire de p53 et H3.3 dans la régulation des ERVs, qui est préservé au cours du développement murin. Nous montrons que p53 est responsable du recrutement de H3.3 au niveau des GLN-ERVs, et qu'en absence de p53, la régulation épigénétique des GLN-ERVs médiée par H3.3, à travers les modifications K27ac et K9me3, est perdue. De plus, nous démontrons que la protéine de l'enveloppe, Env, des GLN ralentit le développement tumoral en contribuant à l'activité de suppresseur de tumeurs de p53. Ce travail suggère que les protéines rétrovirales endogènes " domestiquées " fournissent des fonctions anticancéreuses bénéfiques à l'hôte.

Mots clés : p53, H3.3, rétrovirus endogène, ERV, MMERGLN-int, GLN, enveloppe, cancer

Summary

The histone variant H3.3 is an essential epigenetic player, and its mutations are drivers of oncogenesis. H3.3 point mutations drive endogenous retroviruses (ERVs) derepression and overlap with the p53 and ATRX/DAXX mutations in pediatric high-grade glioma. However, the functional link between p53 and H3.3 remain unknown. Here, we present the molecular mechanism of p53 and H3.3 in ERVs regulation, which is preserved across murine development. We show that p53 is responsible for the recruitment of H3.3 at GLN-ERVs, and that, in the absence of p53, the H3.3-mediated epigenetic regulation of GLN-ERVs, through K27ac and K9me3 modifications, is lost. Moreover, we demonstrate that the GLN envelope protein, Env, slows tumor development contributing to the tumor suppressor activity of p53. This work suggests that 'domesticated' endogenous retroviral proteins provide beneficial anti-cancer functions to the host.

Key words: p53, H3.3, endogenous retrovirus, ERV,MMERGLN-int, GLN, envelope, cancer