Caractérisation du complexe NuA4/TIP60 et ses liens avec le variant d'histone H2A.Z

Thèse

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Résumé

L'organisation des génomes eucaryotes sous forme de chromatine constitue un élément de régulation essentiel de tous les processus cellulaires dépendants de l'ADN. Les facteurs intervenant sur cette organisation jouent donc un rôle crucial dans le bon fonctionnement et le maintien de l'identité des cellules et l'intégrité du matériel génomique.

Le complexe NuA4/TIP60 est capable d'agir sur l'organisation de la chromatine de deux façons distinctes : premièrement en acétylant les histones H2A et H4 via sa sous-unité KAT5/Tip60, conduisant à une structure chromatinienne plus relâchée et accessible; deuxièmement en incorporant le variant d'histone H2A.Z dans la chromatine, conférant des propriétés particulières aux régions du génome concernées. NuA4/TIP60 joue ainsi un rôle central dans la régulation de nombreux processus cellulaires, en particulier l'expression des gènes et la réparation des dommages à l'ADN. Le complexe est composé d'au moins 17 sous-unités chez l'humain; les propriétés et fonctions de certaines de ces sous-unités restent à préciser dans le but de mieux comprendre comment NuA4/TIP60 régule l'organisation de la chromatine.

Dans la première partie de mes travaux de doctorat présentés ici, nous avons cherché à clarifier la fonction du chromodomaine de KAT5/Tip60, la sous-unité catalytique du complexe. En effet des observations contradictoires avaient été rapportées dans la littérature, en particulier en ce qui concerne la capacité du chromodomaine à reconnaître des marques d'histones spécifiques. Nos résultats suggèrent que ce domaine régule plutôt l'activité acétyltransférase du complexe indépendamment des marques d'histones. Nous avons également caractérisé des mutations de KAT5/Tip60, dont l'une dans le chromodomaine, liées à un syndrome neurodéveloppemental chez plusieurs patients.

Dans une deuxième partie, nous nous sommes intéressés à l'incorporation du variant d'histone H2A.Z au sein de la chromatine par NuA4/TIP60 et par un autre complexe, SRCAP. Nos résultats suggèrent que NuA4/TIP60 favorise l'un des

paralogues de H2A.Z, H2A.Z.2, par rapport à H2A.Z.1, contrairement à SRCAP. Nous avons également identifié des partenaires spécifiques pour chaque paralogue de H2A.Z qui permettent d'expliquer une partie des rôles différents joués par ces paralogues dans la régulation de la transcription.

Dans leur ensemble ces travaux contribuent à améliorer notre compréhension de la façon dont le complexe NuA4/TIP60 affecte l'organisation chromatinienne, et comment des perturbations de cette fonction peuvent entraîner des conséquences pathologiques sérieuses.

Abstract

Eucaryotic genomes take the shape of chromatin, the organization of which affects all DNA-based cellular processes. Hence, factors involved in this organization are critical for maintaining proper cell function, identity, and genome integrity.

The NuA4/TIP60 complex affects chromatin organization through two different mechanisms: first by acetylating histones H2A and H4 in chromatin, increasing its relaxation and accessibility; second by incorporating the histone variant H2A.Z into chromatin, assigning distinct properties to given genomic regions. NuA4/TIP60 therefore acts as a central regulator of many cellular processes, in particular gene expression and DNA damage repair. NuA4/TIP60 comprises at least 17 subunits, the functions and properties of many of which still need elucidating in order to better understand how the complex regulates chromatin structure.

In the first part of my PhD project presented hereby, we aimed to clarify the function of KAT5/Tip60, the catalytical subunit of NuA4/Tip60. Contradictory results had been previously reported regarding the chromodomain ability to bind specific histone marks. Our results suggest that this domain instead regulates the acetyltransferase activity of NuA4/Tip60 independently of histone marks. We have also characterized mutations in KAT5/Tip60, one of them inside the chromodomain, linked to a rare neurodevelopmental syndrome.

In the second part, we were interested in the incorporation of the histone variant H2A.Z in chromatin by NuA4/TIP60 as well as another complex, SRCAP. Our results suggest that NuA4/TIP60 favors one of the two H2A.Z paralogs, H2A.Z.2, over H2A.Z.1, as opposed to SRCAP which binds both equally. We also identified specific interactors for each paralog, which could explain in part how H2A.Z.1 and H2A.Z.2 regulate gene expression differently.

Overall this work contributes to a better understanding of how NuA4/TIP60 regulates chromatin organization, and how disruption of these functions can lead to serious pathological outcomes.

Table des matières

Résumé ii
Abstract iv
Table des matières v
Liste des figures ix
Liste des tableaux xi
Liste des abréviations, sigles, acronymes xii
Remerciements xviii
Avant-propos xx
Introduction 1
I.1 Chromatine et épigénétique : Généralités, définitions, historique 1
I.1.1 Dans 'épigénétique', il y a 'génétique': brève histoire de l'hérédité 1
I.1.2. Le 'dogme central' de la biologie moléculaire et ses limites 3
I.1.3. Structure et organisation de la chromatine 5
I.2 Mécanismes/facteurs de régulation de la chromatine 9
I.2.1 Les chaperons d'histones 10
I.2.2 Les complexes de remodelage de la chromatine ATP-dépendants 12
I.2.3 Les variants d'histones 14
I.2.4 Les modifications post-traductionnelles des histones 19
I.3 Le complexe NuA4/TIP60 31
I.3.1 Le module acétyltransférase 33
I.3.2 Le module d'incorporation de H2A.Z 35
I.3.3 Le sous-module TINTIN 37
I.3.4 Autres sous-unités 37
I.3.5 Aperçu des fonctions principales de NuA4/TIP60 38
I.4 Buts du projet de doctorat 43
Chapitre 1: KAT5/Tip60 chromodomain functions independently of histone marks to target chromatin for acetylation 45
1.1 Résumé 45
1.2 Abstract 46
1.3 Introduction 46

1.4 Results 48
KAT5 chromodomain does not display binding preference on peptides or nucleosomes 48
KAT5 chromodomain is essential for cell viability 49
KAT5 chromodomain is required for proper acetyltransferase activity of NuA4/TIP60 *in vitro* 49
Acetylation of lysine 52 in KAT5 chromodomain modulates NuA4/TIP60 activity 53
KAT5 is not required for ATM activation upon DNA damage 55
1.5 Discussion 56
1.6 Experimental Procedures 58
Recombinant protein purification 58
Large scale peptide and nucleosome-binding assays 58
Yeast Strains and Culture 58
Viability test, plasmid shuffling 59
Human Cell Culture and Transfection 59
Generation of Stable Cell Lines Producing Tagged KAT5 Variants and Affinity Purification of NuA4/TIP60 and ATM Complexes 59
In Vitro HAT Assays 60
Detection of K52 acetylation by Acetyl-lysine IP and Mass-spectrometry analysis 61
ChIP-Seq 63
ATM activation assay 64
Antibodies and immunoblotting 64
1.7 Acknowledgements 64
1.8 Supplemental Information 65
Chapitre 2: De Novo KAT5 Variants Cause a Syndrome with Recognizable Facial Dysmorphisms, Cerebellar Atrophy, Sleep Disturbance, and Epilepsy 67
2.1 Résumé 67
2.2 Abstract 68
2.3 Introduction 69
2.4 Material and Methods 70

Recruitment and Sequencing 70
Cell Culture and Transfection 70
Generation of Stable Cell Lines Producing Tagged KAT5 Variants and Affinity Purification of NuA4/TIP60 Complexes 71
In Vitro HAT Assays 71
RNaseq Methods 72
RT-qPCR 72
2.5 Results 73
Clinical Descriptions 73
Analysis of the Variants 74
Purification and Biochemical Analysis of KAT5 Variants 78
Transcriptomic Analyses 80
2.6 Discussion 82
2.7 Data and Code Availability 85
2.8 Supplemental Data 86
2.9 Acknowledgments 90
Chapitre 3: Integrated analysis of H2A.Z isoforms function reveals a complex interplay in gene regulation 92
3.1 Résumé 92
3.2 Abstract 93
3.3 Introduction 93
3.4 Results 95
H2A.Z.1 and H2A.Z.2 are major regulators of gene expression 95
H2A.Z.1 and H2A.Z.2 isoforms regulate both distinct and overlapping sets of genes 99
The presence of H2A.Z isoforms at TSS does not determine their transcriptional effect 108
H2A.Z.1 and H2A.Z.2 isoforms can have antagonistic roles 110
H2A.Z isoforms can replace each other at promoters 114
H2A.Z.1 and H2A.Z.2 are differently associated with specific proteins 116
PHF14 and SIRT1 are major H2A.Z1 and H2A.Z.2 effectors 117

PHF14 and SIRT1 can mediate the antagonistic relationship between H2A.Z.1 and H2A.Z.2 129
3.5 Discussion 132
3.7 Materials and methods 136
Cell cultures and transfections 136
Tagging of endogenous proteins in K562 cells 136
Generation of genome-edited cell clones in U2OS cells 137
Antibodies and immunoblotting 138
Tandem-affinity purification of endogenous H2AZ.1 and H2AZ.2 139
Mass-spectrometry analysis 139
Cell fractionation 140
RNA extraction and reverse transcription 142
ChIP experiments 142
Immunoprecipitation 143
RNA-Seq 144
ChIP-seq 144
RNA-Seq processing 144
RNA-Seq analysis and figures 145
ChIP-Seq processing and analysis 146
3.8 Data availability 146
3.9 Supplementary files 146
Conclusion 149
C.1 Le chromodomaine de KAT5/Tip60 : un ‘lecteur’ analphabète? 149
C.2 Chromatine et pathologies du cerveau 153
C.3 Incorporation de H2A.Z par NuA4/TIP60 et SRCAP 154
Bibliographie 158
Annexe A : Le domaine SANT de DMAP1 est requis pour l’interaction avec NuA4/TIP60 et SRCAP 203

Liste des figures

Figure I.1. Bases de la génétique mendélienne. 2
Figure I.2. Passage de l'ADN à la protéine. 4
Figure I.3. Structure des histones canoniques et du cœur du nucléosome (NCP). 7
Figure I.4. Niveaux de compaction de l'ADN au sein de la chromatine. 8
Figure I.5. Implication des chaperons d'histones dans l'assemblage de la chromatine. 12
Figure I.6. Les différentes familles et modes d'action des complexes de remodelage de la chromatine. 13
Figure I.7. Catalogue des variants associés à chaque histone canonique. 16
Figure I.8. Protéines identifiées à ce jour comme interagissant avec H2A.Z, organisées selon les processus cellulaires impliqués. 18
Figure I.9. Les trois classes de facteurs impliqués dans les modifications post-traductionnelles des histones. 20
Figure I.10. Principales modifications post-traductionnelles identifiées sur les quatre histones canoniques. 21
Figure I.11. Principales classes de domaines lecteurs de la chromatine. 22
Figure I.12. Représentation schématique de la réaction d'acétylation des lysines et de son effet sur la structure de la chromatine. 23
Figure I.13. Réaction de méthylation des lysines et des arginines. 28
Figure I.14. Principaux sites de méthylation des lysines sur les histones H3 et H4 et leurs KMTs et KDMs spécifiques chez la levure, la Drosophile et l'humain. 29
Figure I.15. La 'Famille Royale' des lecteurs de la méthylation. 30
Figure I.16. Modèle de l'évolution et de la composition des complexes NuA4, SWR1, TIP60 et SRCAP de la levure à l'homme. 33

Figure 1.1: KAT5 CHD displays no binding specificity to specific histone PTMs. 51
Figure 1.2. KAT5 chromodomain is essential for cell viability and acetyltransferase activity on chromatin, independently of pre-existing histone marks. 52
Figure 1.3. KAT5 K52 acetylation regulates NuA4/TIP60 activity. 54
Figure 1.4. KAT5 is not required for ATM activation in response to DNA damage. 56
Figure 1.S1. Structure of human KAT5/Tip60 chromodomain superposed with *Drosophila* HP1 chromodomain. 65
Figure 1.S2. Coomassie-stained gel of purified recombinant GST-KAT5-2-81. 65
Figure 1.S3. Generation of K562 cell lines stably expressing KAT5 mutants. 66
Figure 1.S4. In vitro HAT assays using indicated NuA4/TIP60 purified complexes and core histones as a substrate. 66

Figure 2.1. Clinical Images and Variant Details 76
Figure 2.2. Functional Impact of KAT5 Variants on the Native NuA4/TIP60 Acetyltransferase Complex 79
Figure 2.3. RNaseq Was Performed on Fibroblasts from Individuals 2 and 3 and Six Healthy Controls 81
Figure 2.S1. Generation of K562 cell lines stably expressing KAT5 mutants. 86
Figure 2.S2. Additional qPCR results. 87
Figure 2.S3. Snapshots of ChIP-seq from Jacquet et al. 2016 for NuA4/TIP60 subunit EPC1 in K562 cells (EPC1(1-584)-3Flag-2Strep integrated at the AAVS1 locus and empty vector control. 87

Figure 3.1. Effect of H2A.Z.1 and H2A.Z.2 depletion on gene expression. 97

Figure 3.1-figure supplement 1. Characterisation of U2OS cells genome-edited to express 3xFlag-2xStrep H2A.Z.1 or H2A.Z.2. 98
Figure 3.2 H2A.Z.1 and H2A.Z.2 regulate both distinct and overlapping sets of genes. .101
Figure 3.2-figure supplement 1. Validation of RNA Seq results. 102
Figure 3.2-figure supplement 2. Effect of a second siRNA H2A.Z.2 (siZ2#). 103
Figure 3.2-figure supplement 3. RNA-seq analysis after H2A.Z.1 and H2A.Z.2 depletion in U2OS cells. 104
Figure 3.2-figure supplement 4. Profiles of tagged H2A. 106
Figure 3.2-figure supplement 5. Analysis of H2A.Z.1 or H2A.Z.2 presence around TSS and enhancers. 107
Figure 3.3. H2A.Z isoforms exert an antagonistic regulation on gene expression....111
Figure 3.3-figure supplement 1. Effect of the double depletion of H2A.Z.1 and H2A.Z.2 on genes similarly regulated by H2A.Z.1 and H2A.Z.2. 112
Figure 3.3-figure supplement 2. Antagonistic regulation by H2A.Z isoforms in U2OS cells…. 113
Figure 3.4. H2A.Z.1 and H2A.Z.2 replace each other at genes promoters. 115
Figure 3.5. Identification of differential H2AZ.1 and H2AZ.2 interactors. 118
Figure 3.5-figure supplement 1. Tagging of H2A.Z isoforms by CRISPR/Cas9 in K562 cells used for characterisation of interactomes. 120
Figure 3.5-figure supplement 2. Mass spectrometry analysis of the H2A.Z.1/2 purifications shown in Figure 3.5B and validation in U2OS cells. 121
Figure 3.6. PHF14 and SIRT1 mediate H2A.Z.1 and H2A.Z.2 functions respectively. 124
Figure 3.6-figure supplement 1. Characterisation of U2OS cells expressing 3xFlag-2xStrep tagged PHF14. 126
Figure 3.6-figure supplement 2. Quantification and replicate of Figure 3.6A and B experiments. 127
Figure 3.6-figure supplement 3. Validation of siRNAs targetting SIRT1 and PHF14. 128
Figure 3.7. PHF14 and SIRT1 can mediate the antagonism between H2A.Z.1 and H2A.Z.2. 130
Figure 3.7-figure supplement 1. PHF14 mediates antagonistic effect of H2A.Z.1 and H2A.Z.2 on ADAMTS1 and COLEC12 mRNA expression. 131

Figure C.1. Structure du ‘cœur’ de NuA4 de S. cerevisiae lié à un nucléosome, en présence ou non du chromodomaine de KAT5/Esa1. 151
Figure C.2. Structure du chromodomaine de KAT8/MOF 153

Figure A.1. Purification des complexes natifs associés à DMAP1 endogène 203
Figure A.2. Implication du domaine SANT de DMAP1 dans l’interaction avec NuA4/TIP60 et SRCAP 204

Liste des tableaux

Tableau I.1. Liste des principales KATs chez la levure et l'homme, classées par familles, et des complexes qui les incluent.....24
Tableau I.2. Liste des principales KDACs chez la levure et l'homme, classées par classes, et des complexes qui les incluent. 25
Tableau I.3. Modules et sous-unités du complexe NuA4/TIP60 humain. 32

Table 2.1 Main Clinical Features.....78
Table 2.S1 Additional clinical features.....88
Table 2.S2 Sleep disorder characteristics 90
Table 2.S3 List of primers used for RT-qPCR. 90

Supplementary file 3.1. Genes upregulated upon H2A.Z.1 depletion in WI38 cells. 146
Supplementary file 3.2. Genes upregulated upon H2A.Z.2 depletion in WI38 Cells.....146
Supplementary file 3.3. Genes downregulated upon H2A.Z.1 depletion in WI38 cells.....147
Supplementary file 3.4. Genes downregulated upon H2A.Z.2 depletion in WI38 cells.....147
Supplementary file 3.5. Genes regulated upon the combined depletion of H2A.Z.1 and H2A.Z.2 in WI38 cells. 147
Supplementary file 3.6. Genes upregulated upon H2A.Z.1 depletion in U2OS cells. 147
Supplementary file 3.7. Genes upregulated upon H2A.Z.2 depletion in U2OS Cells. 147
Supplementary file 3.8. Genes downregulated upon H2A.Z.1 depletion in U2OS cells. .147
Supplementary file 3.9. Genes downregulated upon H2A.Z.2 depletion in U2OS cells. .147
Supplementary file 3.10. Genes regulated upon the combined depletion of H2A.Z.1 and H2A.Z.2 in U2OS cells. 147
Supplementary file 3.11. Genes regulated upon PHF14 depletion in WI38 cells. 147
Supplementary file 3.12. Genes regulated upon SIRT1 depletion in WI38 cells. 147
Supplementary file 3.13. List of siRNA and primers. 148

Liste des abréviations, sigles, acronymes

53BP1: Tp53 binding protein 1
AAVS1: adeno-associated virus integration site 1
Acetyl-CoA: acetyl coenzyme A
ACTL: actine like
ACTR: actine related
ADAMTS: a disintegrin and metalloproteinase with thrombospondin motif
ADN/DNA: acide désoxyribonucléique / desoxyribonucleic acid
AF-9: ALL1-fused gene from chromosome 9
AKAP: A-kinase anchor protein
ANP32E: acidic leucine-rich nuclear phosphoprotein 32 family member E
(T)AP: (tandem) affinity purification
ARID: AT-rich interaction domain
ARN/RNA: acide ribonucléique / ribonucleic acid
ARNm/mRNA: ARN messager / messenger RNA
ARP: actin related protein
Asf1: anti-silencing function protein 1
ATM: Ataxia telangiectasia mutated
ATP: adenosine triphosphate
ATR: Ataxia telangiectasia and Rad3 related
BAF: BRG1 associated factor
BAH: bromo adjacent homology
Bdf: bromodomain factor
bp/pb: paire de bases
BRCA: breast cancer
BRD: bromodomain
BRD8: BRD-containing protein 8
BRG1: Brahma related gene 1
CAF1: chromatin assembly factor 1
Cas9: CRISPR associated protein 9
CBP: CREB binding protein
CDKN1A: cyclin-dependent kinase inhibitor 1A
CDYL: chromodomain Y like
CENP-A: centromeric protein A
CHD: chromodomain
ChIP: chromatin immunoprecipitation
COLEC: collectin
CRISPR: clustered regularly interspaced short palindromic repeats
DINOL: damage induced long noncoding RNA
DMAP1: DNA methyltransferase 1-associated protein 1
DNA-PK: protein kinase DNA dependant
DSB: double strand break / cassure double brin (de l'ADN)
DOT1: disruptor of telomeric silencing 1
CREB: cAMP response element-binding protein
Eaf: esa1 associated factor
EP400: E1A-binding protein P400
EP400-NL: EP400-N-terminal like
EPC: enhancer of polycomb
Epl1: enhancer of polycomb like 1

Esa1: essential SAS2-related acetyltransferase 1
FACT: facilitates chromatin transcription/transactions
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GAS41: glioma amplified sequence 41
Gcn5: general control nonderepressible 5
GNAT: Gcn5 N acetyltransferase related
GST: glutathione S-transferase
HA: human influenza hemagglutinin
HAT: histone acetyltransferase
HBO1: histone acetyltransferase bound to ORC1
HDAC: histone deacetylase
HDM: histone demethylase
HIRA: histone cell cycle regulator
HMG: high mobility group protein
HMT: histone methyltransferase
Htz1: *S. cerevisiae* H2A.Z
HP1: heterochromatin protein 1
HR: homologous recombination
HSA: helicase SANT associated
HSS: hand-SANT-slide
ING: inhibitor of growth
Ino80: inositol requiring 80
IPO: importin
ISWI: imitation SWI
KAP1: KRAB-associated protein 1
KAT: lysine acetyltransferase
KD: knockdown
KDAC: lysine deacetylase
KDM: lysine demethylase
KMT: lysine methyltransferase
KO: knockout
L3MBTL: Lethal(3)malignant brain tumor-like protein
LSD: lysine specific demethylase
MBT: malignant brain tumor
MDM2: murin double minute 2
MIER: mesoderm induction early response
MLL: mixed-lineage leukemia
MOF: male absent on the first
MORF: monocytic leukemia zinc finger protein-related factor
MORF4: mortality factor 4
MOZ: monocytic leukemia zinc finger
MRG15/X: MORF related gene on chromosome 15/X
MRGBP: MRG binding protein
MS: mass spectrometry
MYST: MOZ, Ybf2/Sas3, Sas2, TIP60
NAP1L: nucleosome assembly protein like
NCAPD2: non-SMC condensin I complex subunit D2
NHEJ: non-homologous end joining
NuA4: nucleosome acetyltransferase of H4
NuRD: nucleosome remodeling deacetylase
NURF: nucleosome remodeling factor

PALB2: partner and localiser of BRCA2
PANDAR: promoter of CDKN1A antisense DNA damage activated RNA
PBAF: polybromo-associated BAF
PCAF: p300/CBP associated factor
PHD: plant homeodomain
PHF: PHD finger protein
PI3K: phosphoinositide 3-kinase
PIKK: PI3K related kinases
PLAT: plasminogen activator, tissue type
PLIP: cPLA2-interacting protein
Pol: RNA polymerase
PRC: polycomb repressive complex
PRMT: protein arginine methyltransferase
PTM: post-traductional modification
Rad: radiation (yeast genes)
RAI: retinoic acid induced
Rpd: reduced potassium dependency
RRM2: ribonucleoside-diphosphate reductase subunit M2
Rtt: regulator of Ty1 transposition protein
Ruv: recombination UV
RUVBL: RuvB-like
SAGA: Spt-Ada-Gcn5 acetyltransferase
SAM: S-adenosylmethionine
SANT: Swi3, Ada2, N-Cor, TFIIIB
Sas: something about silencing
SET: suppressor of variegation, enhancer of zeste, trithorax
SETD2: SET domain-containing protein 2
Sin3: Swi-independent 3
SIRT: sirtuin
SNF: sucrose non fermentation
SPT: suppressor of Ty
SRCAP: Snf2-related CREB-activator protein
STAGA: SPT3-TAFII31-GCN5 acetyltransferase
Swc: Swr1 complex
SWI: mating type switching
Swr1: Swi2/Snf2-related ATPase 1
TBP: TATA box binding protein
TCF: transcription factor
TFIID: transcription factor II D
TFIIIC: transcription factor III C
TINTIN: trimer independent on NuA4 involved in transcription interactions with nucleosomes
Tip60: Tat interacting protein 60kDa
Tra1: yeast homolog of TRRAP1
TRRAP: transformation/transactivation domain associated protein
TSS: transcription start site
Vps: vacuolar protein sorting
WT: wild type
Yaf9: yeast AF-9
YEATS: Ynl107, ENL, AF-9, and TFIIF small subunit
Yng: yeast homolog of mammalian ING1

ZDHHC: zinc finger DHHC-type palmitoyltransferase
ZNHIT1: zinc finger HIT-type containing 1

À mon père

"That is the beginning of knowledge- the discovery of something we do not understand."
Frank Herbert, God Emperor of Dune

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Avant-propos

Cette thèse présente les résultats obtenus lors de mon doctorat sous la forme de trois articles.

Le premier article est un manuscrit qui sera soumis prochainement. Salar Ahmad a réalisé l'expérience présentée à la figure 1.2A, Dorine Rossetto l'expérience présentée à la figure 1.3C, Karine Jacquet les expériences présentées aux figures 1.3A (avec la participation de Jean-Philippe Lambert et la mienne), 1.3D et 1.3E, et Anahita Lashgari a réalisé les expériences présentées à la figure 1.4. J'ai réalisé le reste des expériences et écrit le manuscrit avec Jacques Côté et Anahita Lashgari.

Le deuxième article a été publié en août 2020 dans l'*American Journal of Human Genetics* (https://doi.org/10.1016/j.ajhg.2020.08.002). J'en suis co-premier auteur en compagnie de Smrithi Salian et Periklis Makrythanasis. J'ai réalisé les expériences présentées aux figures 2 et S1. Les aspects cliniques et la mesure de l'expression des gènes ont été réalisés par les autres auteurs, principalement sous la direction de Philippe Campeau. J'ai participé à l'écriture du texte avec Jacques Côté et Philippe Campeau.

Le troisième article a été publié en février 2020 dans *eLife* (https://doi.org/10.7554/eLife.53375). J'en suis le deuxième auteur après Assala Lamaa. J'ai mis en place la méthodologie utilisée tout au long de l'article pour le tagging des deux paralogues de H2A.Z. J'ai réalisé les expériences présentées aux figures 5 et les figures supplémentaires associées, avec l'aide de Xue Cheng pour le tagging et la première purification de H2A.Z.2 dans les cellules K562. Samer Hussein a contribué à l'analyse des données de ChIP-seq aux figures 5S1E et F. Le reste des expériences a été principalement réalisé par Assala Lamaa dans le laboratoire de Didier Trouche avec l'aide de Marion Aguirrebengoa et Estelle Nicolas pour l'analyse des données. J'ai participé à l'écriture du texte avec Assala Lamaa, Jacques Côté et Didier Trouche.

Introduction

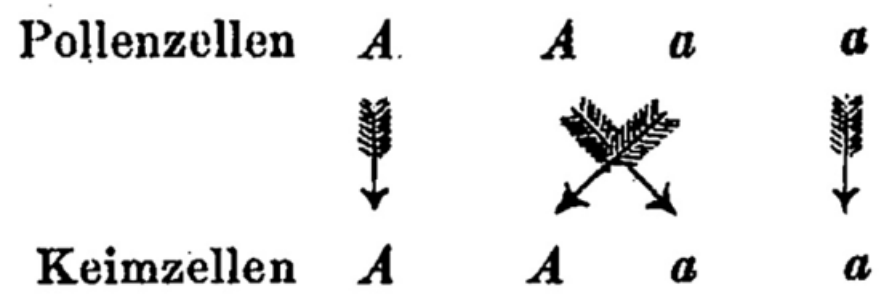
I.1 Chromatine et épigénétique : Généralités, définitions, historique

I.1.1 Dans ‘épigénétique’, il y a ‘génétique’: brève histoire de l’hérédité

La faculté à se reproduire et à transmettre un certain nombre de caractéristiques à sa descendance est une des propriétés majeures de ce que nous appelons la vie. Mais s’il semble que le concept d’hérédité soit apparu très tôt dans notre histoire de manière intuitive, et théorisé par de nombreux penseurs allant d’Aristote (Aristotle & Peck, 1943) à Charles Darwin (Darwin, 1859), ce n’est véritablement qu’au cours des deux derniers siècles que les mécanismes moléculaires sous-jacents ont été mis à jour pour permettre l’émergence d’une nouvelle discipline au sein de la biologie: la génétique.

Les premiers efforts expérimentaux à la base de la fondation de la génétique furent ceux de Gregor Mendel, qui parvint à l’aide de croisements de pois à prédire les proportions par lesquelles certains traits pouvaient être transmis à la descendance (Mendel, 1866). Malgré des simplifications et un certain nombre de controverses (Radick, 2022), les travaux de Mendel furent, suite à leur redécouverte à la fin du XIXe siècle, à la base de la fondation de la génétique, et les lois mendéliennes de l’hérédité sont encore aujourd’hui le socle de l’enseignement de la discipline (Figure I.1).

A



B

Croisement Aa/Aa	A	a
A	AA	Aa
a	Aa	aa

Figure I.1. Bases de la génétique mendélienne. (Adapté de Mendel, 1866)
A) En considérant A et a comme deux variations (allèles) d'un caractère observé (gène), les gamètes mâles (ici le pollen, Pollenzellen) comme femelles (ovules, Keimzellen) peuvent être porteurs de l'allèle A ou a, et les quatre combinaisons possibles peuvent être considérées comme également probables lors d'un croisement entre individus de génotype Aa.
B) Tableau (échiquier de Punnett) des résultats possibles lors d'un croisement entre individus de génotype Aa. Chaque individu produit des gamètes A et a en proportion égale, qui peuvent se combiner de quatre façons différentes pour former des descendants de génotypes AA, Aa (deux fois, Aa=aA) et aa. Si l'allèle A est dominant sur a, les trois-quarts de la descendance de ce croisement sera de phénotype (résultat visible de l'expression du gène) A, le reste de phénotype a.

Lors de la première décennie du XXe siècle, Wilhelm Johannssen (Johannsen, 1909) et William Bateson (Bateson, 1906) furent les premiers à parler respectivement de 'gènes' et de 'génétique' pour définir les traits hérités lors de la reproduction et la discipline dédiée à leur étude.

Une fois établie l'existence de gènes comme éléments discrets transmis lors de la reproduction selon des 'règles' précises, il restait à déterminer comment ces éléments théoriques étaient représentés et fonctionnaient concrètement. Pour cela il nous faut remonter quelque peu en arrière. Au cours des années 1870 et 1880,

Walther Flemming réalisa de nombreuses observations essentielles pour notre compréhension des mécanismes de la division cellulaire (mitose). En particulier, il mit en évidence au sein du noyau des cellules eucaryotes une structure fibreuse, facilement colorable et qu'il nomma donc chromatine (Flemming, 1882). Il soupçonnait que cette substance était à l'origine des filaments observés lors des différentes phases de la mitose, mais il fallut attendre 1888 pour que Heinrich Wilhelm Waldeyer leur donne le nom de chromosomes (Waldeyer, 1888). Les travaux, entre autres, de Theodor Boveri (Boveri, 1904), Walter Sutton (Sutton, 1902, 1903) et Thomas Morgan (Morgan, 1916) permirent d'établir que les gènes, porteurs des caractéristiques héritées lors de la reproduction, étaient en réalité situés sur les chromosomes, ces structures héritées lors de la division cellulaire.

Par la suite, de nombreuses découvertes ont permis d'affiner la compréhension des mécanismes génétiques: en particulier, il fut établi entre 1928 (Griffith, 1928) et 1944 (Avery et al., 1944) que le support de l'information génétique au sein des chromosomes était l'ADN (Acide Désoxyribo-Nucléique). Les célèbres de travaux de Rosalind Franklin, Raymond Gosling, James Watson et Francis Crick (Franklin & Gosling, 1953; Watson & Crick, 1953), mettant en évidence la structure en double hélice de l'ADN, pavèrent également la voie vers la génétique moléculaire moderne.

I.1.2. Le 'dogme central' de la biologie moléculaire et ses limites

Pour comprendre les phénomènes épigénétiques et ce qu'ils nous permettent d'expliquer, il est nécessaire de commencer par un bref rappel des concepts de base de la génétique et de la biologie moléculaire. L'information génétique est portée par la molécule d'ADN, composée de deux brins complémentaires formés d'une succession (séquence) des quatre nucléotides Adénine, Thymine, Guanine et Cytosine (A, T, G, C). Au sens classique du terme, un gène est une séquence d'ADN délimitée et liée à une fonction particulière. Cette fonction est accomplie par deux étapes principales: la première, la transcription, consiste à générer une molécule d'ARN, complémentaire de l'un des brins d'ADN du gène en question. Cet ARN messager est ensuite utilisé lors de la deuxième étape, la traduction, comme 'patron' pour la production de protéines par les ribosomes, la séquence de nucléotides étant

traduite en séquence peptidique selon le ‘code génétique’. C’est cette protéine qui confère sa fonction au gène, en influant sur des processus biologiques spécifiques (Beadle & Tatum, 1941; Horowitz, 1948; Jacob & Monod, 1961).

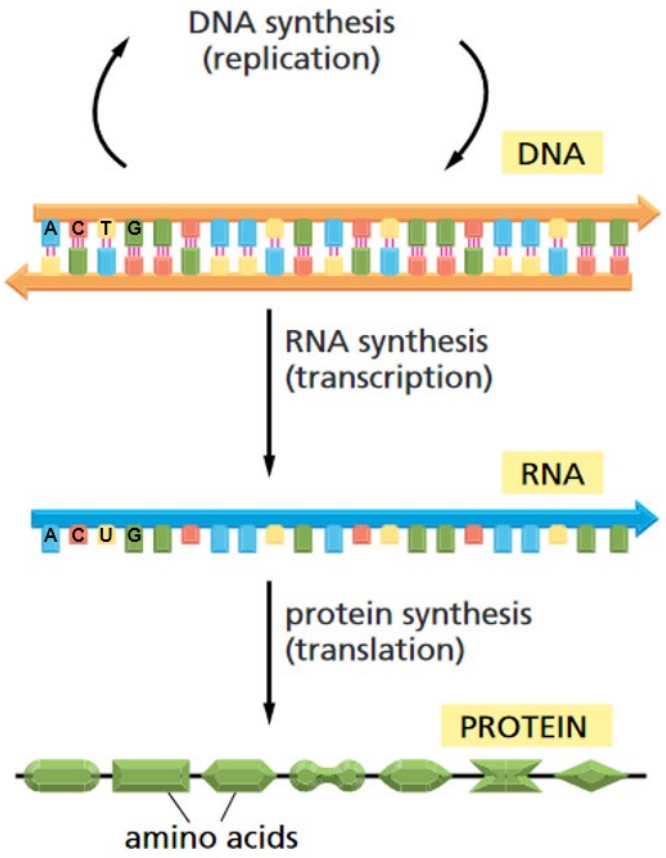


Figure I.2. Passage de l’ADN à la protéine. (Adapté de Alberts, 2008)

En vertu de ce modèle, il était séduisant de considérer que le génome d’un être vivant donné était porteur de toute l’information nécessaire à la compréhension de sa biologie ; l’espoir de ‘cracker’ le code du vivant, ainsi que les promesses qui en découlaient notamment en termes de bénéfices pour la santé humaine, ont certainement inspiré les efforts qui menèrent du premier gène séquencé (Jou et al., 1972) au séquençage complet du génome humain, achevé au début du XXIe siècle (Lander et al., 2001; Venter et al., 2001). Cependant, il apparut très vite que la réalité était plus complexe. En premier lieu, les gènes codant pour des protéines ne représentent qu’environ 1% du génome humain, et l’idée que le reste ne soit

composé que de séquences ‘inutiles’ (junk DNA) a été rapidement abandonnée (Elgar & Vavouri, 2008). Par ailleurs, il est nécessaire d’ajouter un degré de complexité lorsqu’il s’agit d’expliquer le phénomène du développement des organismes multicellulaires. En effet, toutes les cellules d’un organisme possèdent le même ADN, et il apparaît ainsi que des mécanismes régulateurs doivent exister pour permettre leur spécialisation au cours du développement. Enfin, et cela ne rentre pas dans le cadre de cette thèse, une vue trop simplifiée centrée sur les acides nucléiques et les protéines tend à négliger de nombreuses autres composantes de la biologie des cellules (Hewitt, 2020).

L’épigénétique (étymologiquement ce qui existe ‘en plus’ de la génétique), dans son acception la plus courante, s’intéresse à tous les mécanismes, hérités lors de la division cellulaire, qui ont un effet sur l’expression du génome mais sans impliquer de modification de la séquence d’ADN. Ces mécanismes peuvent être divisés en trois ‘classes’ principales chez les eucaryotes:

- Des modifications directement sur les molécules d’ADN (sans altérer la séquence nucléotidique), essentiellement par leur méthylation. Ce processus ne sera pas décrit en détail ici, se reporter aux revues suivantes: Greenberg & Bourc’his, 2019; Li & Zhang, 2014.

- L’organisation du génome sous forme de chromatine, et ses modifications et systèmes de régulation afférents, dont la description fait l’objet des sections suivantes.

- De nombreux ARNs non-codants agissant sur l’expression des gènes à différents niveaux (transcription, traduction, structure de la chromatine). Voir Sun et al., 2018; Thomas & Joan, 2014 pour revue.

I.1.3. Structure et organisation de la chromatine

En parallèle de la découverte de la chromatine à la fin du XIXe siècle, Albrecht Kossel identifia au sein du noyau des cellules eucaryotes des protéines appelées

histones (Kossel & Pringle, 1906), mais ce ne fut pas avant les années 1970 que les travaux entre autres de Roger Kornberg (Kornberg, 1974; Kornberg & Thomas, 1974) établirent définitivement que les histones étaient à la base de la composition des nucléosomes, l'unité de base de la chromatine telle que décrite dans la structure en 'collier de perles' visible au microscope électronique (Olins et al., 1975; Olins & Olins, 1974; Oudet et al., 1975).

Les histones sont une famille de petites protéines, très conservées chez tous les eucaryotes, composées de nombreux acides aminés basiques favorisant l'interaction avec les phosphates de la molécule d'ADN chargés négativement. D'un point de vue structural, les histones possèdent un domaine globulaire central (aussi appelé histone fold domain, Figure I.3b et c), et des extrémités N- et C-terminales peu structurées.

Le cœur du nucléosome (nucleosome core particle, NCP) est composé de 147 paires de bases (pb) d'ADN enroulé autour d'un octamère formé de deux copies de chacune des histones H2A, H2B, H3 et H4. Dénommées histones canoniques, ces quatre protéines sont codées par des gènes hautement exprimés lors de la phase S du cycle cellulaire, et sont ainsi incorporées au sein de la chromatine lors de la réplication de l'ADN (Kurat et al., 2014).

La détermination de la structure des NCPs (Davey et al., 2002; Luger et al., 1997; Richmond et al., 1984) (Figure I.3a) permit de mieux comprendre l'organisation et la formation de la chromatine tout en confirmant des observations et conclusions préalables: via des interactions entre leurs domaines globulaires, les histones H3 et H4 s'associent sous forme de tétramères (Kornberg & Thomas, 1974; Roark et al., 1974) alors que H2A et H2B forment des dimères (D'Anna & Isenberg, 1974; Kelley, 1973). Les dimères H2A-H2B composent également une région du nucléosome fortement électronégative, le 'patch acide' (acidic patch), qui constitue une plateforme d'interaction préférentielle pour de nombreux facteurs se liant à la chromatine (Gallego et al., 2016; He et al., 2020; Kalashnikova et al., 2013; McBride et al., 2020; McGinty & Tan, 2016; Xu et al., 2020; Ye et al., 2019; Zhou et al., 2007). Les extrémités (aussi appelées 'queues') N- et C-terminales des histones, peu

structurées, demeurent en général accessibles à l'extérieur du nucléosome. Elles sont impliquées dans la liaison à l'ADN (Lee & Hayes, 1997) et les interactions entre nucléosomes adjacents (Dorigo et al., 2003; Kan et al., 2007), et sont susceptibles d'être modifiées (cf. section I.2.4).

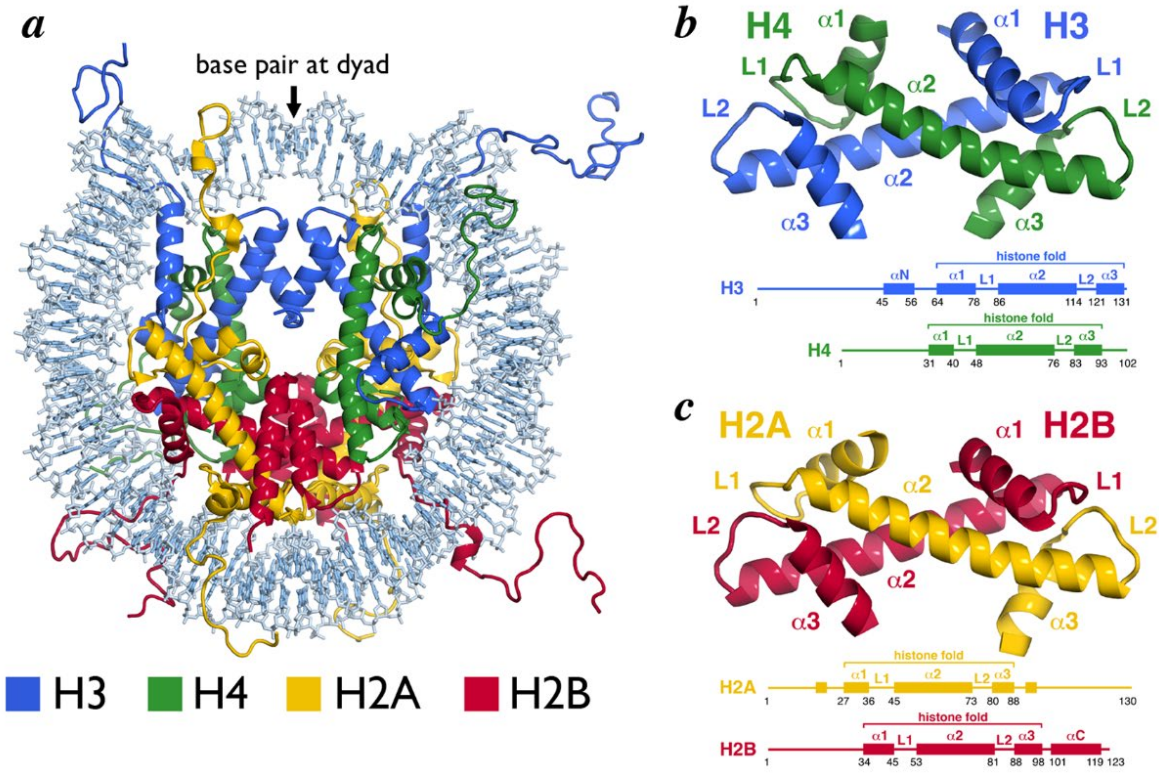


Figure I.3. Structure des histones canoniques et du cœur du nucléosome (NCP). (Adapté de McGinty & Tan, 2015)
a) Structure du NCP (PDB ID 1KX5). Les histones et l'ADN sont représentés respectivement sous forme de rubans et de bâtons.
b) Représentation en rubans des domaines globulaires d'un dimère des histones H3-H4 (haut), représentation schématique de la structure de H3 et H4 identifiant la position du domaine globulaire au sein de la protéine (bas).
c) Même chose qu'au panel b pour H2A etH2B.

Le nucléosome à proprement parler est complété par une portion d'ADN liant et une histone de liaison, H1 chez l'humain (il s'agit d'une famille d'histones moins conservées que les quatre histones canoniques (Hergeth & Schneider, 2015; Willcockson et al., 2021)). La chromatine est ainsi formée d'une succession de nucléosomes, qui s'organisent entre eux et dans l'espace au sein de structures plus

complexes incorporant d'autres facteurs et dont l'organisation précise est encore objet de débats (Ausió, 2015; Eltsov et al., 2008; Joti et al., 2012; Li & Reinberg, 2011; Maeshima et al., 2010; Maeshima et al., 2020) (Figure I.4).

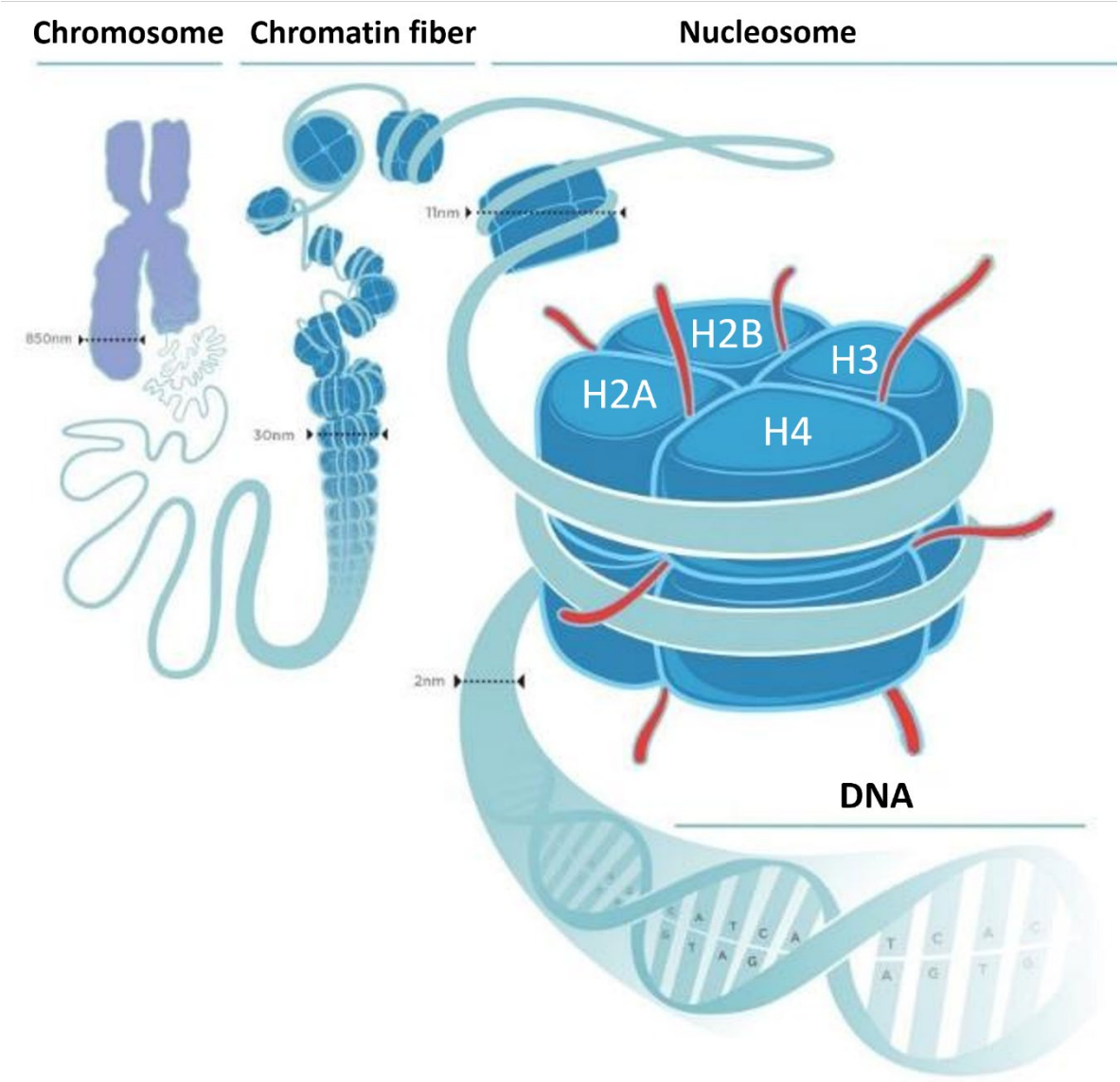


Figure I.4. Niveaux de compaction de l'ADN au sein de la chromatine. 147 pb d'ADN enroulées autour d'un octamère d'histones forment le nucléosome. Les nucléosomes s'organisent entre eux sous forme de structures chromatiniennes plus ou moins compactes, jusqu'au chromosome mitotique visible en microscopie. (Adapté de Richard E. Ballermann, ©2012)

Cette association étroite entre l'ADN et les protéines qui constituent la chromatine a une influence directe sur les processus cellulaires qui nécessitent un accès direct à l'ADN. Il a en effet été montré que les nucléosomes représentent un obstacle physique pour les machineries de transcription des gènes, de réplication et de réparation de l'ADN (Groth et al., 2007; Izban & Luse, 1992; Karl et al., 2022; Kurat et al., 2017; Lorch et al., 1987; Shaw et al., 1978; Teves et al., 2014).

Ainsi, la structure de la chromatine est l'un des éléments qui permettent de réguler l'expression du génome et de l'organiser en différentes régions, jouant un rôle clé dans le bon fonctionnement des cellules et l'établissement de types cellulaires spécialisés. Il est donc essentiel pour les cellules que cette organisation soit finement régulée, et de nombreuses pathologies sont associées à des perturbations de la structure chromatinienne et de ses mécanismes de régulation (Mirabella et al., 2016). La prochaine section présentera plus en détail ces différents mécanismes.

I.2 Mécanismes/facteurs de régulation de la chromatine

Le niveau de base de régulation de la structure chromatinienne repose sur ses différents degrés de compaction. Emil Heitz, en suivant visuellement le comportement des chromosomes lors de la mitose, fut le premier à suggérer l'existence de deux types de chromatine: l'hétérochromatine, dense et compacte, et l'euchromatine, plus lâche et accessible (Heitz, 1928). Nous savons aujourd'hui que ces deux structures sont en fait des états différents d'un élément de même nature, la chromatine, et que virtuellement l'ensemble de la chromatine est amené à transiter par ces deux états (Brown, 1966), mais les termes d'euchromatine et d'hétérochromatine restent pertinents pour décrire l'organisation de base de la chromatine au sein du noyau.

De façon schématique, le degré de compaction de la chromatine affecte directement son accessibilité pour les différentes machineries cellulaires (Michael & Thomä, 2021; Poirier et al., 2008). L'euchromatine correspond aux régions du génome les plus accessibles, principalement composées de gènes activement transcrits et de leurs séquences régulatrices au sein d'une cellule donnée (Lorzadeh et al., 2016).

Pour la plupart des cellules, la plus grande partie du génome est présente sous forme d'hétérochromatine, qui peut être subdivisée en deux catégories. La première est l'hétérochromatine 'facultative', composée principalement de gènes pouvant être transcrits mais qui ne sont pas exprimés à un temps donné dans une cellule donnée. Ces régions du génome peuvent alterner entre les états euchromatinien et hétérochromatinien en fonction du contexte et des besoins et fonctions spécifiques de la cellule. À l'inverse, l'hétérochromatine constitutive est compactée pratiquement en permanence et correspond principalement à des régions du génome aux rôles plus structurels, telles que les centromères et les télomères, ou à des séquences transposables ou pseudogéniques définitivement inactivées au cours de l'évolution (Flamm et al., 1969; Yunis & Yasmineh, 1970).

Les différents états de compaction de la chromatine sont également associés à l'organisation dans l'espace des chromosomes au sein du noyau et au 'timing' de réplication lors de la phase S du cycle cellulaire (Gibcus & Dekker, 2013; Rhind & Gilbert, 2013). Au niveau plus local, les facteurs de régulation de l'organisation chromatinienne peuvent être regroupés en quatre catégories principales: les chaperons d'histones qui accompagnent et régulent la formation et le désassemblage des nucléosomes, les remodeleurs de la chromatine qui déplacent ou retirent les nucléosomes, l'incorporation de variants d'histones qui peuvent conférer des propriétés spécifiques aux nucléosomes, et enfin les modifications post-traductionnelles des histones qui modifient les propriétés de la chromatine et les interactions avec de nombreux facteurs.

I.2.1 Les chaperons d'histones

Le terme de 'protéine chaperon' désigne usuellement des protéines dont la fonction est de se lier à d'autres protéines, soit pour faciliter leur adoption de la bonne conformation et structure (cas de la famille des Heat-shock proteins, HSPs (Kappé et al., 2003)), soit pour aider à la formation de structures multiprotéiques complexes. Par extension, les chaperons d'histones sont une classe de protéines qui 'accompagnent' les histones libres, aident à leur incorporation au sein de la chromatine (Laskey et al., 1978) et permettent leur 'recyclage' après leur éviction

par différentes machineries cellulaires. Il existe un certain nombre de ces facteurs et seuls les principaux et les plus pertinents pour la suite de cette thèse seront décrits ici.

FACT (FAcilitates Chromatin Transcription) est le chaperon d'histone le plus généraliste, puisqu'il est capable de se lier aussi bien aux dimères H2A-H2B qu'aux tétramères H3-H4, aux queues d'histones et à l'ADN nucléosomal (Jeronimo & Robert, 2022; T. Wang et al., 2018). Composé de deux sous-unités, SPT16 et SSRP1, FACT est impliqué dans un grand nombre de processus associés à la chromatine, que ce soit en facilitant la transcription (Orphanides et al., 1998), la réplication (Okuhara et al., 1999) ou la réparation de l'ADN (Bhakat & Ray, 2022). Principalement exprimé par des cellules hautement prolifératives (cellules souches ou cancéreuses), FACT fonctionne ainsi comme un 'gardien' de l'intégrité de la structure chromatinienne en participant au désassemblage et au réassemblage des nucléosomes lors des processus nécessaires à une telle activité cellulaire.

Les autres chaperons d'histones présentent généralement une plus grande spécificité de liaison, tels la famille des NAPs (Nucleosome Assembly Proteins) qui semble favoriser les dimères H2A-H2B (Gill et al., 2022), ASF1, CAF1 ou Rtt106 qui prennent en charge H3-H4 (English et al., 2006; Li et al., 2008; Natsume et al., 2007; Tyler et al., 1999). Il existe également des chaperons d'histones spécialisés associés aux variants d'histones (cf. partie I.2.3), comme ANP32E pour H2A.Z et DDAX pour H3.3. Ces différents chaperons participent à l'incorporation et au recyclage des histones au sein des différents types de régions chromatiniennes (Figure I.5), et la répartition des rôles, la collaboration et la redondance entre ces nombreux facteurs constituent un domaine d'étude encore en expansion (Hammond et al., 2017). Enfin, les chaperons d'histones participent au paysage épigénétique global en régulant et en maintenant les marques d'histones (cf partie I.2.3) au long du cycle cellulaire (Avvakumov et al., 2011; Escobar et al., 2021; Escobar et al., 2019; Reverón-Gómez et al., 2018; Stewart-Morgan et al., 2020).

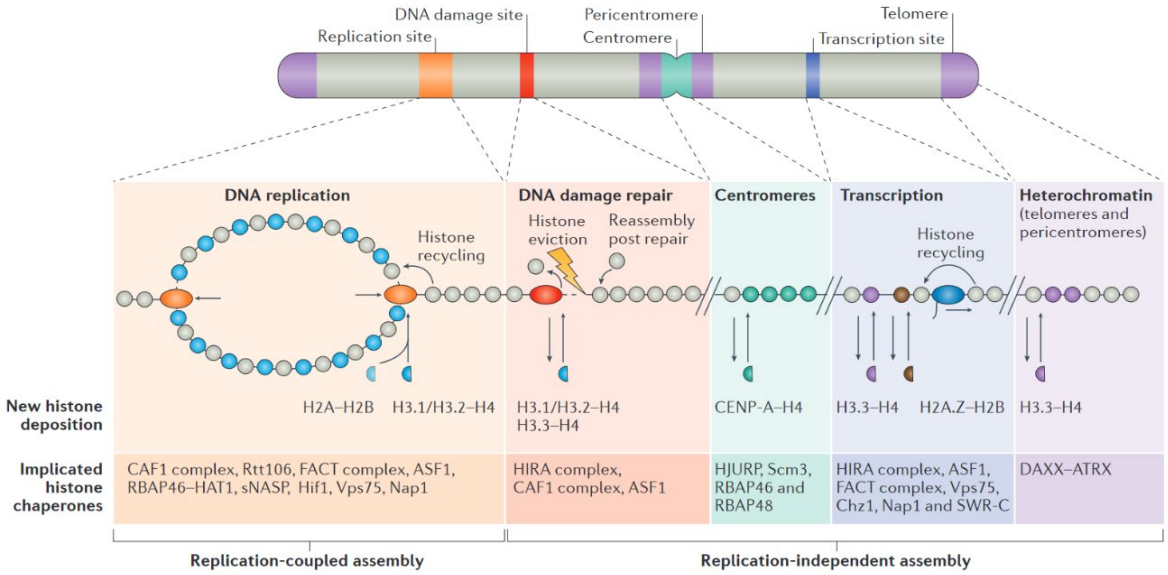


Figure I.5. Implication des chaperons d'histones dans l'assemblage de la chromatine. (Adapté de Hammond et al., 2017)

I.2.2 Les complexes de remodelage de la chromatine ATP-dépendants

Comme on l'a vu, l'organisation de base de la chromatine consiste en une répétition de nucléosomes autour desquels sont enroulées 147 pb d'ADN. Cet espacement régulier des nucléosomes, ainsi que d'autres fonctions comme l'incorporation de variants d'histones (cf partie I.2.3) ou la régulation de l'accessibilité de certaines portions de la molécule d'ADN, est régulé principalement par l'action de facteurs appelés complexes de remodelage de la chromatine ATP-dépendants (par la suite, 'Remodeleurs'). Comme leur nom l'indique, ces facteurs ont en commun le fait d'être généralement des complexes de plusieurs protéines, et d'utiliser l'énergie libérée par l'hydrolyse de l'ATP (Adenosine Tri-Phosphate ; via la sous-unité ATPase présente dans chaque complexe remodeleur) pour accomplir leurs actions sur la chromatine. Les remodeleurs peuvent être classés en quatre familles en fonction de la similarité de leurs sous-unités ATPases (Clapier et al., 2017; Flaus, 2006) (Figure I.6A), accomplissant trois types d'actions spécifiques sur la chromatine (Figure I.6B).

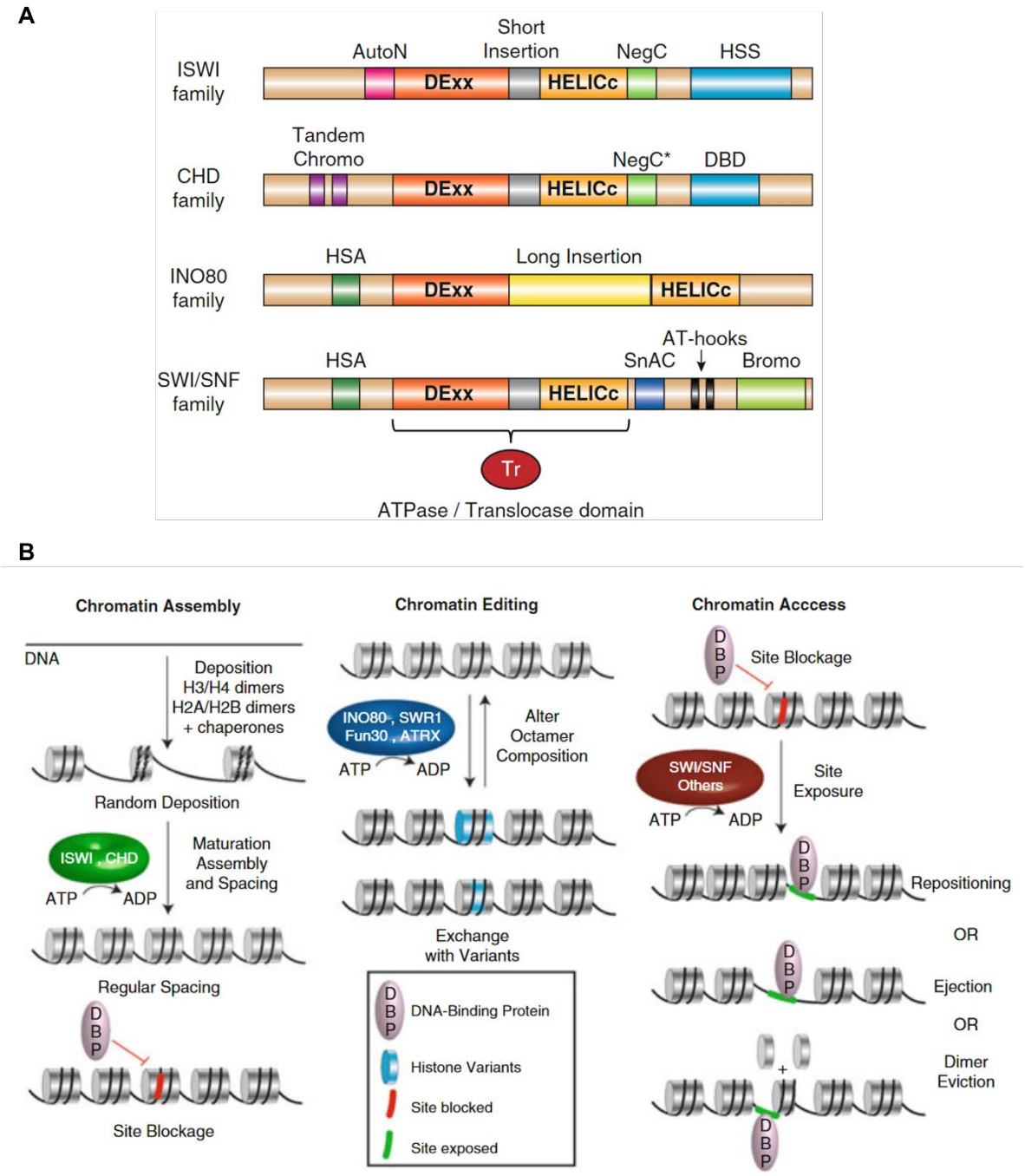


Figure I.6. Les différentes familles (A) et modes d'action (B) des complexes de remodelage de la chromatine. (Adapté de Clapier & Cairns, 2014)

La première catégorie de remodeleurs est impliquée directement dans le bon positionnement des nucléosomes, c'est-à-dire à leur espacement régulier (spacing) de façon similaire au sein de chaque cellule (phasing). Ces mécanismes font

intervenir deux familles de remodeleurs, ISWI/ACF/CHRAC/NURF (Längst & Becker, 2001) et CHD (Robinson & Schultz, 2003).

La deuxième catégorie de remodeleurs affecte la structure de la chromatine en déplaçant ou en retirant tout ou une partie des nucléosomes, permettant d'exposer ou non un site d'ADN spécifique, que cela soit une séquence régulatrice d'un gène pour assurer sa transcription, ou encore une séquence endommagée devant être rendue accessible à la machinerie de réparation. La famille SWI/SNF/BAF/PBAF est le principal exemple de cette catégorie, et joue un rôle important dans l'activation de la transcription (Angus-Hill et al., 2001; Laurent et al., 1991; Neely et al., 1999).

Enfin, les remodeleurs peuvent affecter la structure chromatinienne de façon plus subtile en remplaçant les histones canoniques par des variants d'histones au sein de nucléosomes particuliers. Ces variants sont décrits à la section suivante et confèrent des propriétés différentes aux nucléosomes qui les contiennent, permettant un niveau de régulation supplémentaire des processus prenant place à proximité. Les remodeleurs de cette catégorie appartiennent à la famille INO80 (Morrison & Shen, 2009; Shen et al., 2000). Si l'implication du complexe INO80 lui-même dans l'échange de variants d'histones reste controversée (Wang et al., 2016; Watanabe & Peterson, 2016; Watanabe et al., 2013), d'autres membres de cette famille seront évoqués plus en longueur au long de cette thèse: les complexes SRCAP et NuA4/TIP60, ainsi que leur équivalent chez la levure SWR1, qui sont impliqués dans l'incorporation du variant d'histone H2A.Z au sein de la chromatine (Auger et al., 2008; Doyon et al., 2004) (cf. parties I.2.3, I.3, et Chapitre 4).

I.2.3 Les variants d'histones

En parallèle des histones canoniques H2A, H2B, H3 et H4, produites et incorporées lors de la phase S du cycle cellulaire, il existe des histones généralement légèrement différentes de leurs contreparties canoniques, et pour la plupart codées par des gènes exprimés tout au long du cycle cellulaire. Ces variants d'histones sont le plus souvent retrouvés au sein de régions spécifiques du génome, auxquelles ils confèrent certaines spécificités en modifiant les caractéristiques des nucléosomes

qui les contiennent. Depuis la découverte du premier variant de l'histone H4 (Long et al., 2019), nous savons qu'il existe des variants pour chaque histone canonique (Figure I.7), mais les variants de H2A et H3 sont les plus nombreux et les mieux caractérisés à ce jour.

I.2.3.1 Variants de l'histone H3

Parmi les variants de l'histone H3, deux sont d'une importance particulière. Les centromères, régions de chromatine compactes qui servent de 'centre' aux chromosomes et de point d'attachement pour le fuseau mitotique, sont enrichis en nucléosomes contenant le variant CenH3/CENP-A, qui ne présente qu'environ 30% de similarité avec H3 (Mattiroli et al., 2015; Palmer et al., 1987), et est essentiel à la bonne ségrégation des chromosomes (Goshima et al., 2003; Stoler et al., 1995).

À l'inverse, le variant H3.3 ne diffère de H3 canonique (H3.1) que par quelques acides aminés, ce qui semble suffisant pour lui conférer des fonctions spécifiques : H3.3 est en effet enrichi au sein de régions de chromatine dynamiques, typiquement activement transcrites (Deaton et al., 2016; Ha et al., 2014), où son incorporation assistée de son chaperon spécifique HIRA permettrait de maintenir les marques épigénétiques locales au cours des cycles de transcription (Torné et al., 2020). Mais ce variant illustre la grande versatilité de nombreux éléments épigénétiques, étant également déposé au niveau de séquences hétérochromatiniennes, telles que la périphérie des centromères et les télomères, par une autre protéine chaperon, DAXX (Drané et al., 2010; Goldberg et al., 2010).

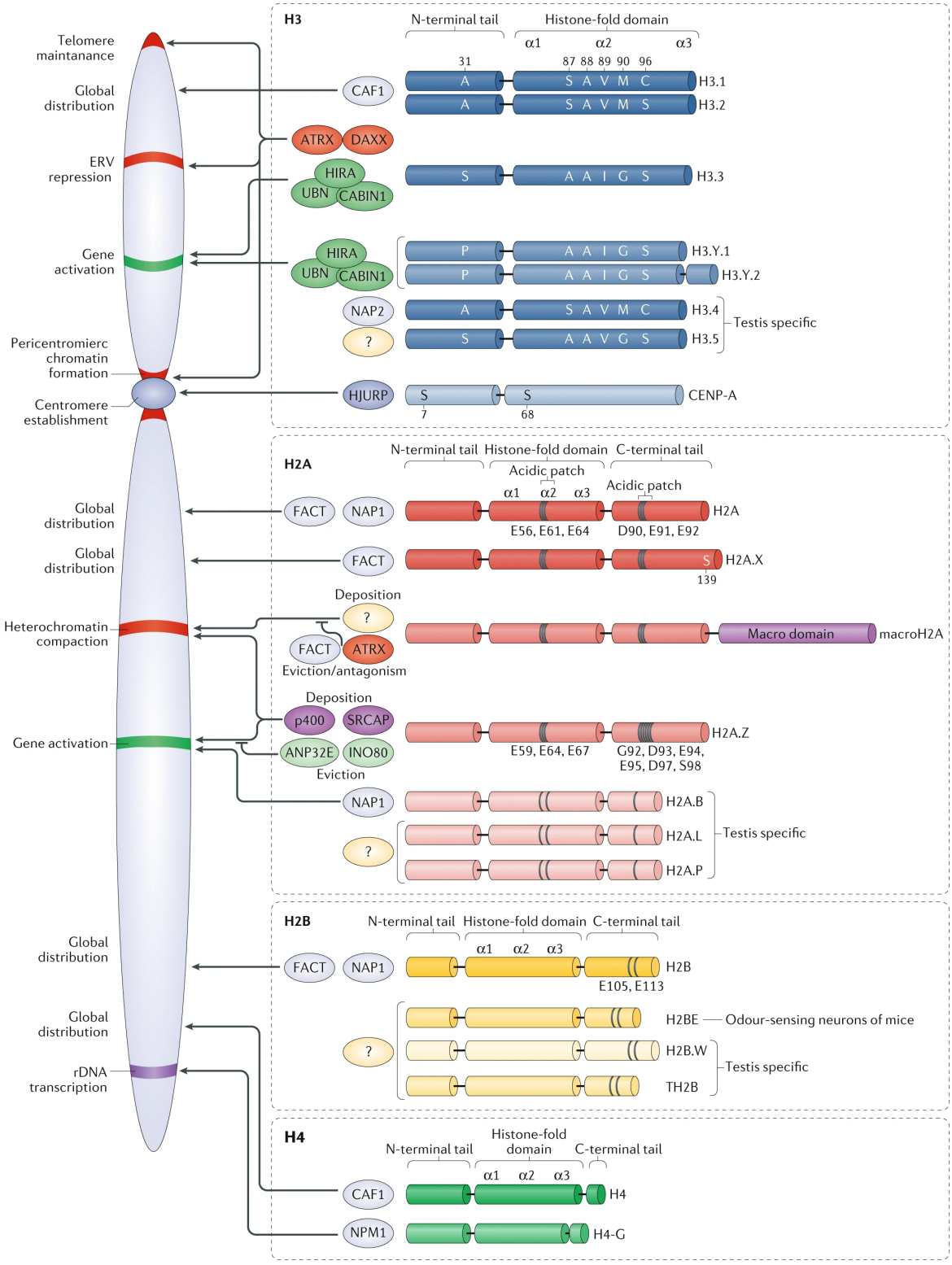


Figure I.7. Catalogue des variants associés à chaque histone canonique.
Sont également figurés les régions chromatiniennes concernées, les chaperons d'histones responsables de leur incorporation, et certaines différences avec les histones canoniques au sein de la séquence peptidique. (Adapté de Martire & Banaszynski, 2020)

I.2.3.2 Variants de l'histone H2A

H2A est l'histone pour laquelle le plus grand nombre et la plus grande diversité de variants ont été décrits à ce jour; de par l'importance du 'patch acide' formé par les dimères H2A-H2B dans les interactions entre nucléosomes et la liaison de nombreux facteurs à la chromatine, ces variants sont les plus à même d'influencer la structure locale de la chromatine et le recrutement de facteurs spécifiques au niveau des nucléosomes qui les contiennent (Paul, 2021).

H2A.Z fait partie de cette catégorie de variants de H2A ayant le potentiel de modifier sensiblement le patch acide du nucléosome. Il est particulièrement bien conservé dans l'évolution de la levure aux mammifères, présente environ 60% d'homologie de séquence avec H2A (Jackson et al., 1996), et est essentiel au développement de nombreux organismes (Clarkson et al., 1999; Faast et al., 2001; Liu et al., 1996; Ridgway et al., 2004). En partie du fait des différences au sein de son patch acide, la présence de H2A.Z affecte sensiblement les interactions entre nucléosomes (Fan et al., 2002), et plus subtilement la structure globale du nucléosome, généralement en le rendant moins stable et en facilitant son désassemblage lors de la transcription (Bonisch & Hake, 2012; Bönisch et al., 2012; Rudnizky et al., 2016; Suto et al., 2000; Wen et al., 2020), même si certaines observations suggèrent un rôle inverse (Park et al., 2004). Les fonctions précises de H2A.Z semblent différer d'un organisme à l'autre et d'un contexte chromatinien à l'autre; les nucléosomes contenant H2A.Z sont souvent enrichis au niveau des promoteurs et des enhancers des gènes (Barski et al., 2007; Guillemette et al., 2005), où ils peuvent jouer les rôles contradictoires d'activateurs ou de répresseurs de la transcription (Guillemette & Gaudreau, 2006; Marques et al., 2010).

En plus des complexes remodeleurs SRCAP et NuA4/TIP60, qui l'incorporent au sein de la chromatine (Couture et al., 2012; Gévry et al., 2007; Ruhl et al., 2006), et de son chaperon ANP32E qui contribue à l'en retirer (Gursoy-Yuzugullu et al., 2015; Obri et al., 2014), H2A.Z interagit avec de nombreux partenaires spécifiques

(Kreienbaum et al., 2022; Ng et al., 2019; Pünzeler et al., 2017) pouvant expliquer cette grande diversité de fonctions (Figure I.8), que ce soit dans la régulation de la transcription, de la réplication (Long et al., 2020) ou de la réparation de l'ADN (Billon & Côté, 2012; Xu et al., 2012). Il est intéressant de noter qu'il existe chez les vertébrés deux isoformes de H2A.Z, H2A.Z.1 et H2A.Z.2, codés respectivement par les gènes paralogues H2AFZ et H2AFV et qui diffèrent par seulement trois acides aminés (Dryhurst et al., 2009; Matsuda et al., 2010). Cette très forte similarité a longtemps conduit les efforts de recherche à se concentrer sur l'isoforme le plus abondant, H2A.Z.1, mais il apparaît de plus en plus clair que ces deux protéines jouent des rôles spécifiques (Greenberg et al., 2019; Kreienbaum et al., 2022; Sales-Gil et al., 2021). La caractérisation de ces deux isoformes, de leur influence sur l'expression des gènes et de leurs interactions avec des partenaires protéiques spécifiques a fait l'objet de l'article présenté au chapitre 3 de cette thèse.

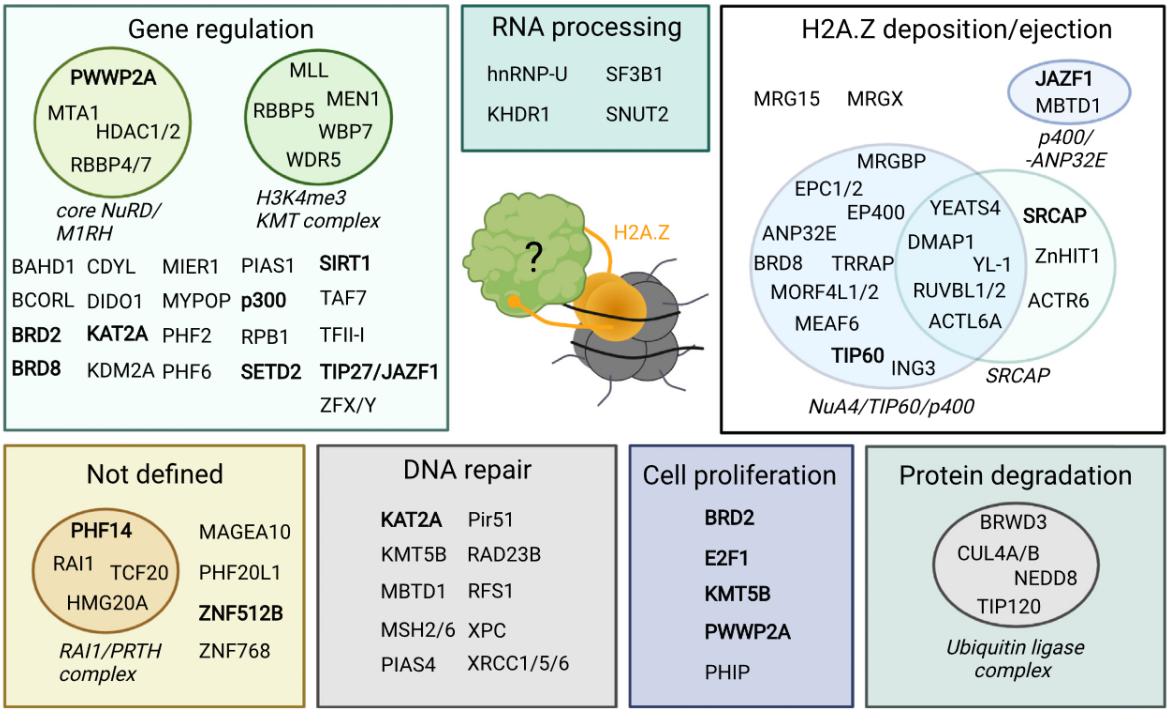


Figure I.8. Protéines identifiées à ce jour comme interagissant avec H2A.Z, organisées selon les processus cellulaires impliqués. (Adapté de Kreienbaum et al., 2022)

Probablement le variant d'histone le plus abondant chez les mammifères (entre 2,5% et 25% des protéines H2A totales) (Rogakou et al., 1998), H2A.X se distingue principalement par sa partie C-terminale étendue (West & Bonner, 1980), contenant un motif SQ[E/D]Φ qui est une cible spécifique pour les kinases de la famille PIKK. Lorsqu'elle est phosphorylée au niveau de cette sérine 139 par ATM, ATR ou DNA-PK, H2A.X est communément dénommée γH2A.X et constitue l'une des premières étapes de la signalisation des cassures double brin de l'ADN (Burma et al., 2001; Rogakou et al., 1998). γH2A.X participe au recrutement, à la propagation et à la rétention des facteurs de réparation au niveau des sites endommagés (Lou et al., 2006).

Parmi les variants notables de H2A, signalons également macroH2A, caractérisé par un large domaine supplémentaire (le 'macrodomaine') par rapport à H2A qui en fait l'histone la plus grande connue à ce jour (Pehrson & Fried, 1992). MacroH2A est généralement associé à des régions silencieuses du génome, notamment au niveau du chromosome X inactivé (Costanzi & Pehrson, 1998; Douet et al., 2017; Gamble et al., 2010), et joue un rôle important dans le maintien de l'intégrité génomique (Hurtado-Bagès et al., 2018; Sun & Bernstein, 2019). Enfin, des variants de H2A plus courts, regroupés sous le nom de sH2A (short H2A), normalement exprimés au cours de la spermatogénèse chez les mammifères, ont été récemment identifiés comme de potentielles oncohistones, dont l'expression inappropriée peut contribuer à l'apparition de cancers (Chew et al., 2021; Clyde, 2021).

I.2.4 Les modifications post-traductionnelles des histones

La quasi-totalité des protéines existantes sont susceptibles de voir leurs fonctions affectées par des modifications 'déposées' sur certains acides aminés après leur traduction, d'où le nom de modifications post-traductionnelles (PTMs). Les histones n'échappent pas à la règle, et leurs PTMs (aussi appelées marques d'histones) constituent l'un des principaux mécanismes épigénétiques régulant l'organisation de la chromatine et l'expression des gènes (Kouzarides, 2007; Lawrence et al., 2016). Comme évoqué à la section I.1.3, les queues N- et C-terminales des histones sont peu structurées et exposées à l'extérieur du nucléosome, ce qui en fait des cibles

privilégiées pour ces modifications, mais il existe également des PTMs au niveau du domaine globulaire des histones (Tropberger & Schneider, 2013). Les modifications sont effectuées par des enzymes appelées ‘écrivains’ (writers), sont réversibles grâce à l’action d’autres enzymes, les ‘effaceurs’ (erasers), et peuvent être reconnues par une grande diversité de ‘lecteurs’ (readers) possédant des domaines de reconnaissance spécifique aux différentes marques (Figure I.9). Ces différents types de facteurs sont souvent retrouvés associés au sein de larges complexes multi-protéiques (Musselman et al., 2012).

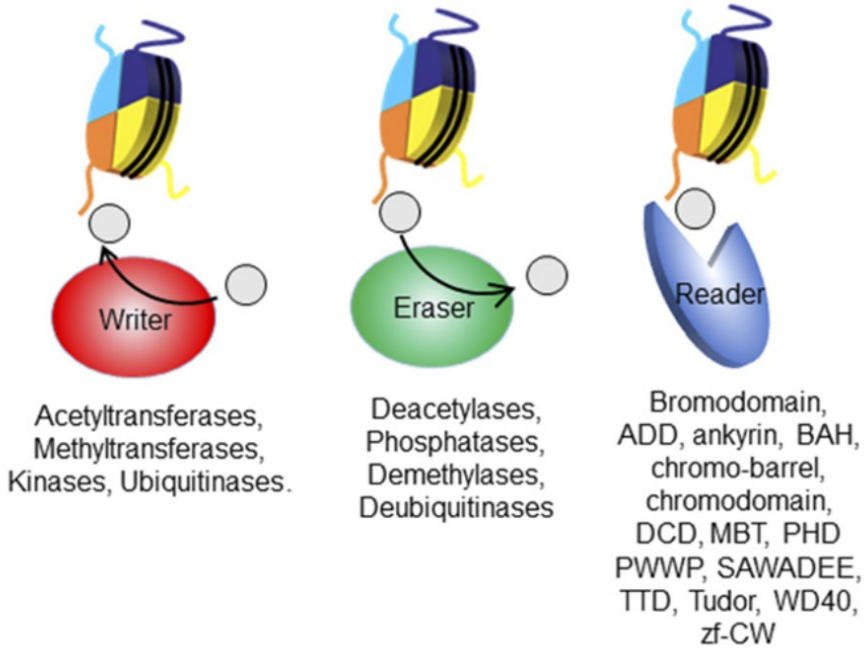


Figure I.9. Les trois classes de facteurs impliqués dans les modifications post-traductionnelles des histones. Des exemples sont mentionnés pour chaque classe. (Adapté de Ueda & Seki, 2020)

Si certaines PTMs ont un effet direct sur la structure du nucléosome et l’organisation de la chromatine, la plupart de leurs fonctions sont liées à celles des lecteurs spécifiques qu’elles contribuent à recruter et à retenir sur la chromatine (Musselman et al., 2012; Ruthenburg et al., 2007). Une grande diversité de fonctions est permise par la combinaison de différents types de modifications (les principaux étant l’acétylation, la méthylation, la phosphorylation et l’ubiquitination) et du grand

nombre de résidus différents pouvant être modifiés sur chaque histone (Figure I.10). Ceci a conduit à formuler l'hypothèse du 'code des histones' (Jenuwein & Allis, 2001; Strahl & Allis, 2000) selon laquelle les combinaisons quasi-infinies en théorie des différentes marques entre elles permettraient de 'coder' pour une gamme de fonctions tout aussi large, de façon analogue aux gènes codant pour des protéines. S'il semble clair aujourd'hui que les PTMs des histones ne forment pas un véritable code au sens strict du terme, les fonctions de chaque marque étant éminemment dépendantes du contexte, et leurs combinaisons étant globalement moins 'variées' qu'anticipées, ce modèle garde une valeur conceptuelle certaine pour appréhender l'importance des marques d'histones dans la régulation de nombreux phénomènes cellulaires (Farrelly & Maze, 2019; Oftedal, 2022). Les sections suivantes présenteront plus en détails deux types de PTMs pertinents dans le cadre de mes travaux de thèse, l'acétylation et la méthylation, ainsi que certains des lecteurs chromatiniens qui leur sont associés (Figure I.11).

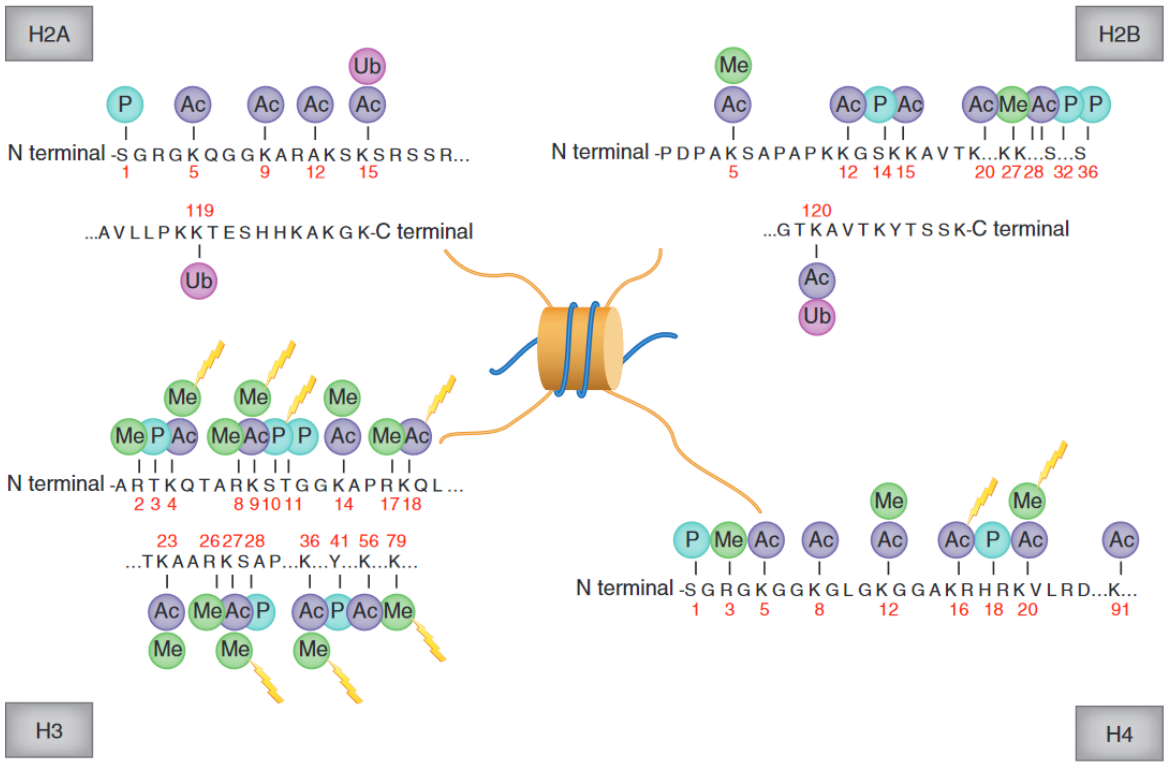


Figure I.10. Principales modifications post-traductionnelles identifiées sur les quatre histones canoniques. Ac : acétylation, Me : méthylation, P : phosphorylation, Ub : ubiquitination (Adapté de Rodríguez-Paredes & Esteller, 2011)

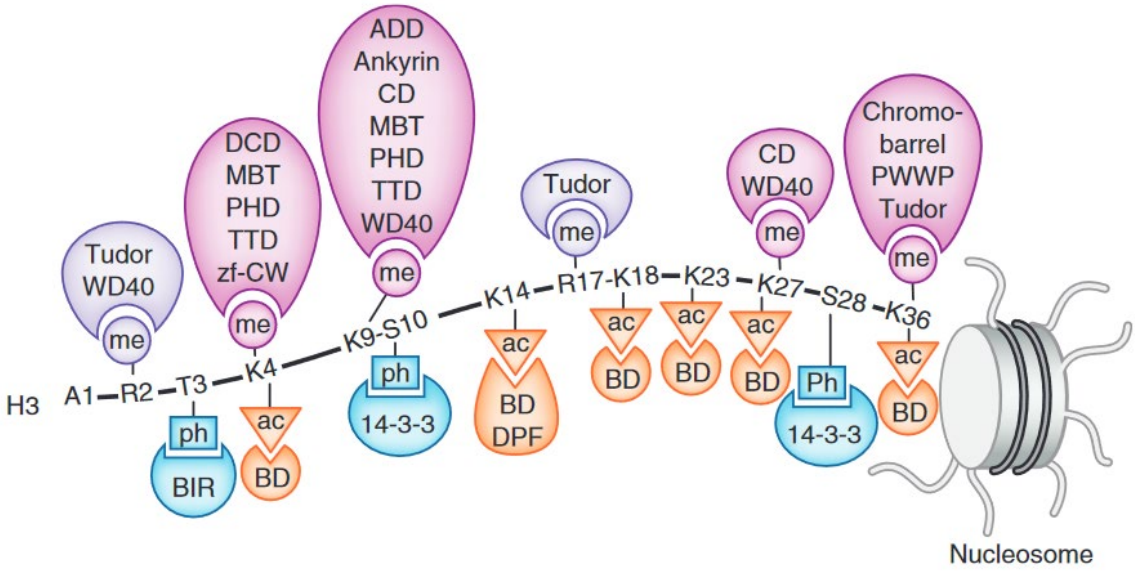


Figure I.11. Principales classes de domaines lecteurs de la chromatine. La queue N-terminale de l'histone H3 a été choisie comme exemple représentatif de la diversité de PTMs et de résidus modifiés. (Adapté de Musselman et al., 2012)

I.2.4.1 L'acétylation

L'acétylation est (avec la méthylation) le premier type de modification des histones à avoir été découvert en 1964 par Vincent Allfrey (Allfrey et al., 1964), qui avait dès cette époque avancé l'hypothèse visionnaire selon laquelle la modification de la charge des histones affecterait leur interaction avec l'ADN et ainsi la dynamique de l'expression des gènes. En effet, l'acétylation est l'une des rares marques d'histones à influer directement sur les propriétés physiques des nucléosomes. Il s'agit de l'ajout d'un groupement acétyle sur le groupement ε-amine de la chaîne latérale d'une lysine, un processus opéré à partir d'une molécule d'acétyl-CoA par des enzymes (writers) appelées KAT ou HAT (pour Lysine ou Histone AcetylTransferase). Ce faisant, la charge positive de la lysine est neutralisée, ce qui affaiblit l'interaction entre les histones et l'ADN pour aboutir à un état plus ouvert de la chromatine (Ausio et al., 1989; Chen et al., 2022a; Li & Reinberg, 2011; Shogren-Knaak et al., 2006; Steunou et al., 2014; Wang & Hayes, 2008) (Figure I.12) et réguler l'organisation spatiale de la chromatine à plus large échelle (Ulianov et al.,

2016). La réaction est rapidement réversible, par l'intervention d'enzymes (erasers) de la famille des KDAC ou HDAC (Lysine ou Histone De-ACetylase).

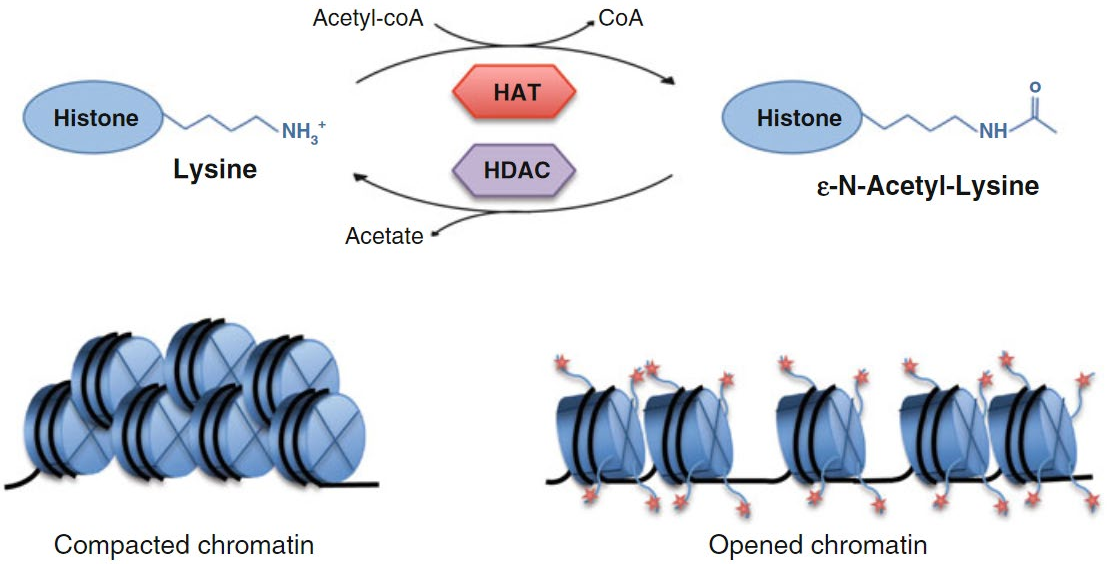


Figure I.12. Représentation schématique de la réaction d'acétylation des lysines (en haut) et de son effet sur la structure de la chromatine (en bas). (Adapté de Steunou et al., 2014)

Si l'action de certaines KATs est localisée dans le cytoplasme pour acétyler les histones nouvellement synthétisées avant leur incorporation à la chromatine (Campos et al., 2010), la majorité des KATs connues à ce jour sont nucléaires et sont capables d'acétyler les histones directement dans la chromatine. Parmi les caractéristiques généralement partagées par ces enzymes, on peut noter la présence d'un site de liaison fortement conservé à l'acétyl-CoA (Wang et al., 2008), le recours à des évènements d'auto-acétylation de certains résidus nécessaires à leur activité (Rossetto et al., unpublished; Wang & Chen, 2010; Yuan et al., 2012), et l'appartenance à de larges complexes multiprotéiques essentiels pour permettre à une grande partie des KATs d'acétyler leur substrat 'naturel', les nucléosomes (Steunou et al., 2014). En dehors de ces caractéristiques, il existe une grande diversité de KATs qui se caractérisent par leurs propriétés biochimiques et leurs spécificités de substrats. Plusieurs grandes familles de KATs ont été déterminées sur la base de leur homologie structurale (Tableau I.1).

Famille	Nom standardisé	*S. cerevisiae*	*H. sapiens*	Complexes
GNAT	KAT1	Hat1	HAT1	HATB
	KAT2	Gcn5		HAT-A2/ADA, SAGA
	KAT2A		GCN5	STAGA, ATAC
	KAT2B		PCAF	PCAF, ATAC
	KAT9	Elp3	ELP3	Elongator
	KAT10	Hpa2		
	KAT14		CSRP2BP	ATAC
	NATF		HAT4/NAA60	
MYST	KAT5	Esa1	Tip60	NuA4/TIP60
	KAT6	Sas3		NuA3
	KAT6A		MOZ/MYST3	MOZ
	KAT6B		MORF/MYST4	MORF
	KAT7		HBO1/MYST2	HBO1-JADE, HBO1-BRPF
	KAT8	Sas2	MOF/MYST1	MSL, NSL
P300/CBP	KAT3A		CBP	
	KAT3B		P300	
	KAT11	Rtt109		RTT109/VPS75
SRC/p160	KAT13A		SRC/NCOA1	
	KAT13B		ACTR/NCOA3	
	KAT13C		GRIP1/NCOA2	
	KAT13D		CLOCK	CLOCK-BMAL1
Autres	KAT4	Taf1	TAF1	TFIID
	KAT12		TFIIIC90	TFIIIC
		Nut1		Mediator

Tableau I.1. Liste des principales KATs chez la levure et l'homme, classées par familles, et des complexes qui les incluent. (Adapté de Steunou et al., 2014)

Beaucoup de KATs acétylent également des protéines non-histones dont la liste s'agrandit régulièrement, certaines de ces modifications étant essentielles au bon fonctionnement de ces protéines (Shvedunova & Akhtar, 2022). Quelques exemples seront évoqués dans une prochaine section consacrée au complexe NuA4/TIP60.

Les KDACs présentent elles aussi une certaine diversité, même si elles ont tendance (à l'exception de certaines sirtuines) à être moins spécifiques que les KATs quant aux résidus qu'elles ciblent. Elles sont réparties en deux grandes familles, selon qu'elles dépendent du zinc (famille 'classique', classes I, II et IV) ou du NAD+ (sirtuines, classe III) pour leur activité (Tableau I.2).

Classe	Sous-classe	*S. cerevisiae*	*H. sapiens*	Complexes
I		Rpd3	HDAC1	Rpd3L, Rpd3S Sin3L, Sin3S, Mi2-NURD, CoRest
		Hos1	HDAC2	Sin3L, Sin3S, Mi2-NURD, CoRest
		Hos2	HDAC3	N-CoR
			HDAC8	
II	A	Hda1	HDAC4	Hda1-Hda2-Hda3
		Hos3	HDAC5	
			HDAC7	
			HDAC9	
	B		HDAC6	
			HDAC10	
III (Sirtuines)	I	Sir2	SIRT1	
		Hst1	SIRT2	
		Hst2	SIRT3	
		Hst3		
		Hst4		
	II		SIRT4	
	III		SIRT5	
	IV		SIRT6	
			SIRT7	
IV			HDAC11	

Tableau I.2. Liste des principales KDACs chez la levure et l'homme, classées par classes, et des complexes qui les incluent. (Adapté de Steunou et al., 2014)

Comme les KATs, les KDACs ne limitent pas leur activité aux histones (Shvedunova & Akhtar, 2022; Yao & Yang, 2011). De par leur activité peu spécifique et leur implication dans de nombreuses pathologies, les KDACs sont depuis plusieurs années une cible thérapeutique prometteuse, notamment pour le traitement du cancer, et de nombreux inhibiteurs spécifiques ont été développés (Ganai et al., 2016; Khan & La Thangue, 2012; Ramaiah et al., 2021).

Au-delà de son effet direct sur la structure de la chromatine qui en fait un élément essentiel dans la régulation entre autres de la transcription (Kurdistani et al., 2004) ou de la réparation des dommages à l'ADN (Peterson & Côté, 2004) en facilitant l'accès à la chromatine, l'acétylation des histones fonctionne aussi, comme la plupart des autres marques, via l'interaction spécifiques avec des facteurs possédant des domaines 'lecteurs'. On distingue plusieurs familles de domaines reconnaissant spécifiquement les histones acétylées.

La première, de loin la plus nombreuse à l'heure actuelle, est celle des bromodomaines. La liaison à la lysine acétylée se fait au sein d'une cavité hydrophobe formée par quatre hélices α et plusieurs résidus aromatiques accompagnés d'une Asparagine (Taverna et al., 2007). Environ une cinquantaine de protéines contenant des bromodomaines ont été décrites, la plupart d'entre elles appartenant à des complexes régulateurs de la chromatine (Fujisawa & Filippakopoulos, 2017). La présence de bromodomaines dans plusieurs complexes KATs contribue vraisemblablement à l'amplification et à la propagation du signal d'acétylation.

Plus récemment, les domaines de la famille YEATS ont été identifiés comme lecteurs de l'acétylation (Andrews et al., 2016; Klein et al., 2017; Li et al., 2014; Schulze et al., 2009; Zacharaki et al., 2012; Zhang et al., 2021). La lysine acétylée est ici reconnue par plusieurs acides aminés aromatiques disposés de façon à former une 'cage aromatique' (Li et al., 2014), un mécanisme partagé par d'autres domaines lecteurs de la chromatine, notamment en lien avec la méthylation des histones (cf. section suivante). YEATS4/GAS41 (Yaf9 chez la levure) est un exemple de protéine à domaine YEATS, qui joue un rôle essentiel dans l'incorporation du

variant H2A.Z au sein de la chromatine, et dont la capacité à se lier à la chromatine acétylée contribue au lien étroit existant entre H2A.Z et l'acétylation de la chromatine dans la régulation de l'expression des gènes (Cho et al., 2018; Doyon et al., 2004; Hsu, Shi, et al., 2018; Hsu, Zhao, et al., 2018; Klein et al., 2017; Munnia et al., 2001; Zhang et al., 2021).

Il est à noter que l'acétylation appartient à une classe de PTMs appelée 'acylations'. En présence de composés apparentés à l'acétyl-CoA où le groupement acétyle est remplacé par un groupement crotonyle, succinyle, butyryle, propionyle, etc., ce groupement peut être transféré au niveau d'une lysine pour former les marques d'histones associées (respectivement crotonylation, succinylation, butyrylation, propionylation, etc.). Les mécanismes par lesquels ces marques sont déposées, retirées et lues sont très largement les mêmes que pour l'acétylation; au vu de leur très faible abondance, leur rôle apparaît relativement marginal, même si certaines fonctions spécifiques, notamment en lien avec la régulation du métabolisme, commencent à apparaître (Barnes et al., 2019; Choudhary et al., 2014; Dutta et al., 2016; Kollenstart et al., 2021; Sabari et al., 2017).

I.2.4.2. La méthylation

Les histones peuvent être méthylées sur leurs résidus lysines (au niveau du groupement ε-amine, comme pour l'acétylation, ce qui en fait des modifications exclusives) et arginines (au niveau du groupement guanidine) (Figure I.13). La réaction utilise la S-Adénosyl-Méthionine (SAM) comme source de groupement méthyles, et est catalysée par des enzymes appelées KMTs/HMTs (Lysine/Histone MethylTransferases) ou PRMTs (Protein Arginine MethylTransferases) selon qu'elles ciblent respectivement les lysines ou les arginines. Si plusieurs KDMs/HDMs (Lysine/Histone Demethylases) ont été identifiées au cours des dernières années, la réversibilité de la méthylation des arginines est encore mal caractérisée et seulement deux déméthylases ont été identifiées à ce jour, dont JMJD6 (Chang et al., 2007; Zhang et al., 2019). Contrairement à l'acétylation, la méthylation des histones n'affecte pas la structure des nucléosomes, et agit donc essentiellement via les effecteurs qui s'y lient. La méthylation des histones est ainsi

une marque épigénétique très versatile, pouvant avoir des effets opposés sur l'accessibilité de la chromatine ou l'expression des gènes en fonction de ces effecteurs. Cette versatilité est renforcée par le fait qu'il existe différents niveaux de méthylation, les lysines pouvant accueillir un, deux ou trois résidus méthyle, les arginines deux (Figure I.13), chaque état pouvant être reconnu différemment par les domaines lecteurs associés. Pour la suite de cette section, nous nous concentrerons sur la méthylation des résidus lysines.

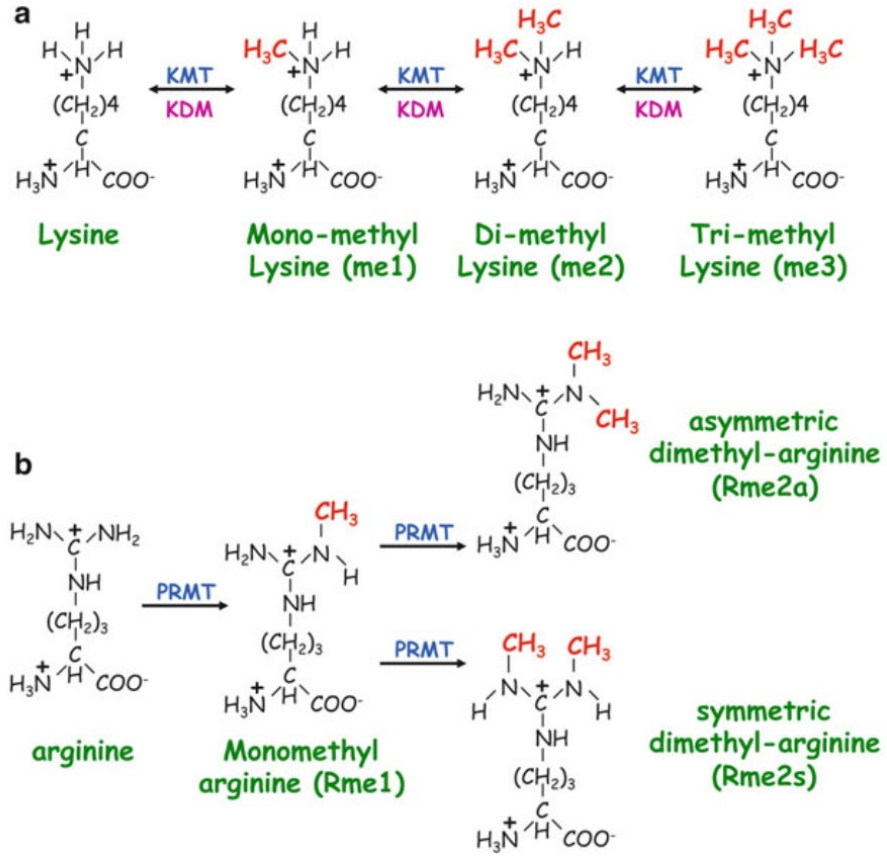


Figure I.13. Réaction de méthylation des lysines (a) et des arginines (b), illustrant les différents niveaux de méthylation possibles. (Adapté de Gozani & Shi, 2014)

La plupart des KMTs appartiennent à la famille des protéines à domaine SET (Dillon et al., 2005), mais il existe une classe structurellement très différente dont l'unique représentant connu à ce jour est DOT1L (Nguyen & Zhang, 2011). Les KMTs et KDMs sont extrêmement spécifiques en termes de résidus ciblés et de niveaux de

méthylation/déméthylation qu'elles catalysent, et la majorité des lysines méthylées sont situées sur les histones H3 et H4 (Figure I.14).

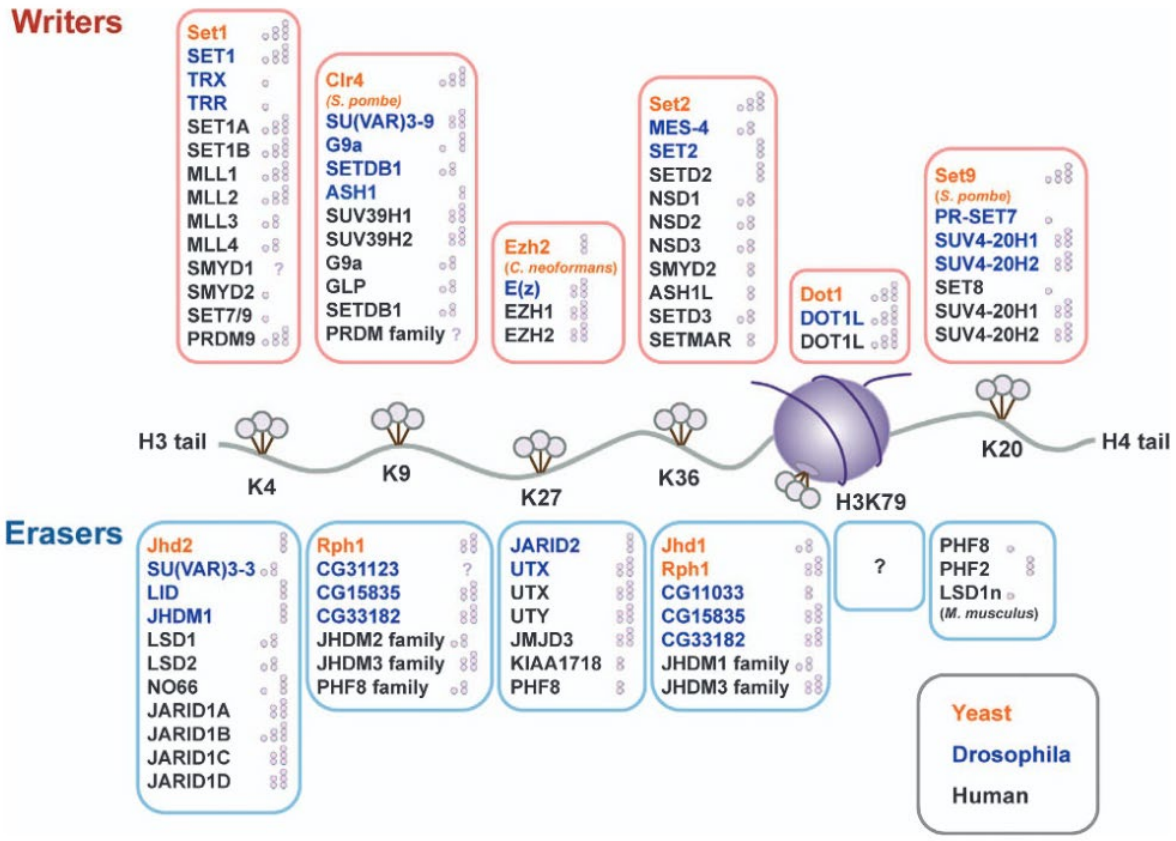


Figure I.14. Principaux sites de méthylation des lysines sur les histones H3 et H4 et leurs KMTs et KDMs spécifiques chez la levure (en orange), la Drosophile (en bleu) et l'humain (en noir). Le nombre de cercles représente le niveau de méthylation concerné. (Adapté de Hyun et al., 2017)

On peut classer grossièrement les principales marques de méthylation selon qu'elles sont plutôt caractéristiques de l'euchromatine (c'est le cas de H3K4, H3K36 (Wagner & Carpenter, 2012) et H3K79) ou de l'hétérochromatine (H3K9 (Ninova et al., 2019), H3K27 (Margueron & Reinberg, 2011) et dans une certaine mesure H4K20), mais les nuances sont nombreuses : H3K4me1 est par exemple enrichie au niveau des séquences enhancers (Heintzman et al., 2009; Heintzman et al., 2007), alors que H3K4me3 est associée aux promoteurs des gènes actifs (Guenther et al., 2007).

Comme évoqué précédemment, les effets de la méthylation des histones dépendent entièrement des facteurs qui les reconnaissent (Kouzarides, 2007; Musselman et al., 2012). Ceux-ci possèdent des domaines de reconnaissance variés appartenant à plus d'une dizaine de familles (Figure I.11), mais dont la plupart fonctionnent au moyen d'une cage aromatique formée de deux à quatre résidus (Taverna et al., 2007). Les domaines appartenant aux familles des chromodomaines (CHD), des domaines Tudor, des domaines PWWP et des domaines MBT forment la 'famille royale' des lecteurs de la chromatine, et partagent certaines similarités de structure et de fonction (Taverna et al., 2007) (Figure I.15).

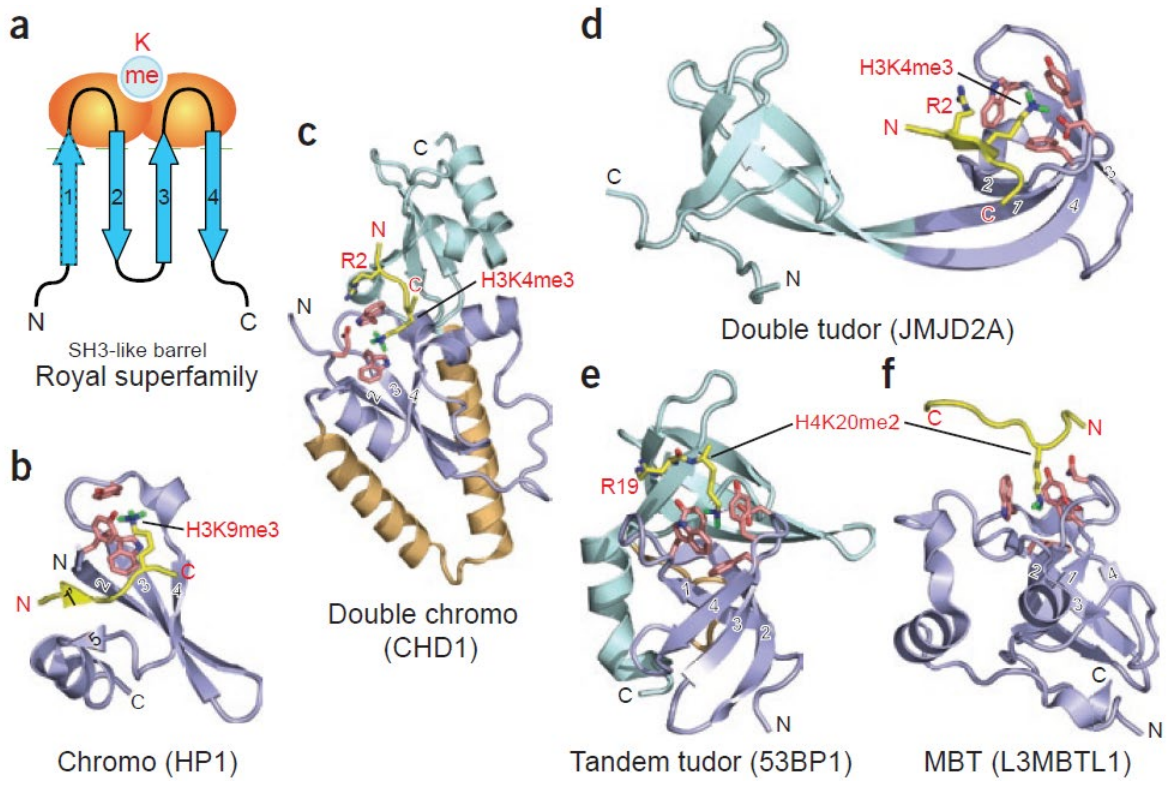


Figure I.15. La 'Famille Royale' des lecteurs de la méthylation.
a) Représentation schématique du domaine commun aux protéines de la 'famille royale'. b-f) Exemples de structures connues de domaines membres de la 'famille royale'. Les résidus formant les cages aromatiques sont représentés en rose. (Adapté de Taverna et al., 2007)

Le chromodomaine de la protéine hétérochromatinienne HP1, spécifique de H3K9me3, a été le premier domaine lecteur de la chromatine caractérisé en détail (Bannister et al., 2001; Jacobs & Khorasanizadeh, 2002; Jacobs et al., 2001;

Lachner et al., 2001; Nielsen et al., 2002) et a servi de modèle pour l'étude des nombreux autres lecteurs identifiés depuis.

Enfin, il est à noter que, contrairement à l'acétylation beaucoup plus labile, la méthylation des histones est 'héritée' très rapidement par la descendance au cours de la division cellulaire par un recyclage localisé des histones parentales (Escobar et al., 2019; Reverón-Gómez et al., 2018), ce qui en fait une 'véritable' marque épigénétique au sens strict du terme (Reinberg & Vales, 2018).

I.3 Le complexe NuA4/TIP60

L'objet d'étude principal de notre laboratoire est le complexe NuA4/TIP60, qui constitue un bon exemple des larges complexes multiprotéiques formés par la majorité des facteurs de régulation de la chromatine. Un complexe est formé de protéines liées fortement entre elles par des interactions hydrophobes (plus solides que les interactions électrostatiques qui représentent la majorité des interactions transitoires entre protéines et peuvent être facilement perturbées en jouant sur la concentration de sels). NuA4 a ainsi été identifié par des étapes de fractionnement successives chez la levure en tant que complexe d'environ 1.3 MDa possédant une activité acétyltransférase sur les histones H4 et H2A au sein des nucléosomes, activité qui lui donne son nom (Nucleosome Acetyltransferase of histone H4) (Allard et al., 1999; Grant et al., 1997; Steger et al., 1998). NuA4 ainsi que toutes ses sous-unités sont extrêmement bien conservés de la levure à l'homme, où il est aussi connu sous le nom de TIP60 (Doyon et al., 2004). Sa sous-unité acétyltransférase est la seule KAT essentielle à la viabilité cellulaire chez la levure (Allard et al., 1999; Smith et al., 1998) et sa déplétion est létale très tôt lors du développement embryonnaire chez les mammifères (Hu et al., 2009). NuA4/TIP60 joue un rôle important dans un grand nombre de processus biologiques, en particulier la transcription de nombreux gènes et le maintien de l'intégrité génomique.

Chez l'humain, TIP60 est un complexe de plus d'1,5 MDa formé de 17 sous-unités. Il est particulièrement intéressant de noter que TIP60 semble formé de la fusion de deux complexes de *S. cerevisiae*, NuA4 à proprement parler et SWR1, qui est un remodeleur ATP-dépendant responsable de l'incorporation du variant Htz1 (H2A.Z) dans la chromatine (Auger et al., 2008). Ainsi, en plus de l'activité acétyltransférase de NuA4, TIP60 est également l'un des deux complexes chez l'humain à incorporer H2A.Z, l'autre, SRCAP, étant plus proche d'un équivalent direct de SWR1 (Figure I.16). En se basant sur les travaux effectués chez la levure (Auger et al., 2008; Rossetto et al., 2014; Setiaputra et al., 2018; X. Wang et al., 2018) et plus récemment chez l'humain (Devoucoux, Roques, et al., 2022), il est possible de répartir les sous-unités du complexe en plusieurs modules fonctionnels (Tableau I.3, Figure I.16), qui feront l'objet d'une brève présentation avant d'évoquer plus en profondeur les principaux rôles biologiques du complexe.

Module	Nom	Taille (kDa)	Domaines lecteurs	Autres domaines	Paralogues/ Isoformes	Équivalents chez S.cerevisiae	Autres complexes
Recrutement	TRRAP	400		PI3K, FAT, FATC		Tra1	SAGA PCAF TFTC
Acétylation	Tip60/KAT5	55	CHD	MYST	4 isoformes dont PLIP	Esa1	
	EPC1	93,4		EpcA/B	p: EPC2	Epl1	
	ING3	46	PHD: H3K4me3			Yng2	
	MEAF6	22				Eaf6	HBO1, MOZ/MORF
	MBTD1	70,5	MBT: H4K20me1/2	Znf	p: L3MBTL1,2,3	-	
Incorporation de H2A.Z	EP400	344		SANT, HSA, Q rich	p: EP400NL	Swr1 + Eaf1	
	DMAP1	53		SANT		Eaf2/Swc4	SRCAP
	BAF53a/ACTL6A	53		Actin related		ARP4	INO80 SRCAP BAF; PBAF
	Actin	41,7				Act1	SRCAP INO80 BAF; PBAF; …
	YEATS4/GAS41	26	YEATS: H3K14ac, H3K27ac			Yaf9	SRCAP
	VPS72/YL1	40,5				Swc2	SRCAP
TINTIN	BRD8	150	BrD: H4ac		iso: double BrD	Bdf1 Eaf5 (dans TINTIN)	
	MRG15/MORF4L1	41	CHD: H3K36me2/3	MRG: H2BK119ub	p: MRGX/ MORF4L2 iso: long CHD	Eaf3	Sin3B/Rpd3S PALB2 Ash1L
	MRGBP	30				Eaf7	
Hélicase	RUVBL1/2	49					INO80 SRCAP; …

Tableau I.3. Modules et sous-unités du complexe NuA4/TIP60 humain. (Adapté de Jacquet, 2016)

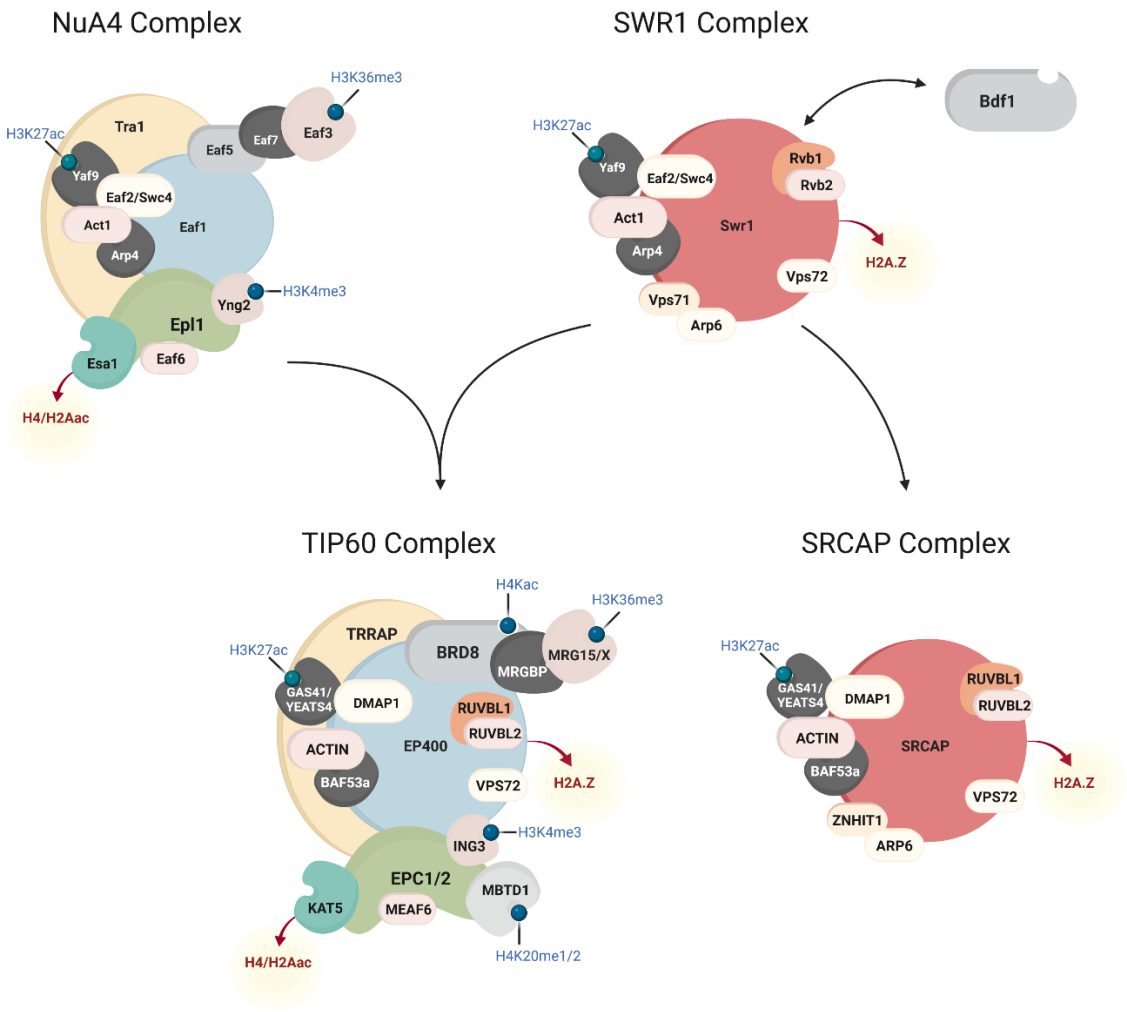


Figure I.16. Modèle de l'évolution et de la composition des complexes NuA4, SWR1, TIP60 et SRCAP de la levure à l'homme. (Créé avec Biorender.com, adapté de Auger et al., 2008)

I.3.1 Le module acétyltransférase

Il existe chez la levure un sous-complexe, Piccolo NuA4, capable d'acétyler la chromatine de manière non ciblée (Boudreault et al., 2003). Un tel complexe indépendant n'existe pas chez l'humain (possiblement remplacé par une autre KAT, HBO1 (Doyon et al., 2006)), mais pour simplifier la description nous considèrerons que les orthologues chez la levure des sous-unités de Piccolo NuA4 (Esa1, Epl1,

Yng2 et Eaf6), ainsi que MBTD1 qui y est directement rattachée, forment le ‘cœur’ catalytique du complexe.

KAT5/Tip60 (Tat interacting protein 60kDa) est la sous-unité catalytique responsable de l’activité acétyltransférase du complexe TIP60 auquel elle donne son nom. Initialement décrite comme interagissant avec la protéine Tat du VIH (Kamine et al., 1996), il s’agit d’une KAT de la famille des MYST (Sapountzi & Côté, 2010; Utley & Côté, 2003; Yamamoto & Horikoshi, 1997), caractérisée par la présence d’un domaine du même nom dans sa partie C-terminale contenant un site de liaison à l’acétyl-CoA et un domaine à doigts de Zinc (ZnF) (Yan et al., 2000). La partie C-terminale contient des domaines d’interaction avec différents facteurs ainsi qu’un chromodomaine (Selleck et al., 2005; Yan et al., 2000). Les chromodomaines sont classiquement des lecteurs des histones méthylées, mais la fonction et la spécificité de liaison du chromodomaine de KAT5/Tip60 est encore relativement obscure et discutée. Il a ainsi été proposé par différents groupes qu’il reconnaîtrait H3K4me1 (Jeong et al., 2011), H3K4me3 (Kim et al., 2015) ou H3K9me3 (Sun et al., 2009), alors que le chromodomaine de Esa1 (son orthologue chez la levure) semblait plutôt favoriser les queues d’histones non modifiées (Huang & Tan, 2013; Selleck et al., 2005). Le chapitre 1 de cette thèse représente notre proposition pour régler cette question. KAT5/Tip60 possède une activité acétyltransférase sur la chromatine uniquement en présence du reste du cœur catalytique NuA4/TIP60, avec une activité spécifique sur les lysines K5, K8, K12 et K16 de H4, K5, K9, K13 et K15 de H2A/H2A.X, et K4, K7, K11, K13 et K15 de H2A.Z. (Allard et al., 1999; Doyon et al., 2004; Ikura et al., 2015; Ikura et al., 2000; Ishibashi et al., 2009; Jacquet et al., 2016; Sapountzi et al., 2006; Sevilla & Binda, 2014). Plusieurs substrats non-histones ont également été décrits, et seront évoqués dans la section I.3.5.

EPC1 (Enhancer of Polycomb 1) relie le module catalytique au reste du complexe par son interaction avec EP400 (X. Wang et al., 2018). Cette protéine interagit avec la queue N-terminale de l’histone H2A (Chittuluru et al., 2011; Huang & Tan, 2013), et confère sa spécificité à NuA4/TIP60 de façon similaire à ses équivalents dans d’autres complexes formés par des KATs de la famille des MYST comme les

protéines JADE et BRPF (Lalonde et al., 2013). Cette interaction semble critique pour l'acétylation de H4, suggérant un mécanisme différent pour l'acétylation de H2A et H4 par NuA4/TIP60 (Lalonde et al., 2013; Lalonde et al., 2014).

ING3 (Inhibitor of Growth) appartient à la famille de suppresseurs de tumeurs ING (Nourani et al., 2003), caractérisée par la présence d'un domaine PHD. Le domaine PHD d'ING3 reconnaît H3K4me2/me3 au niveau des promoteurs des gènes, et bien que facultatif pour le recrutement du complexe à la chromatine, ING3 est essentiel pour l'activité acétyltransférase de NuA4/TIP60 sur la chromatine (Doyon et al., 2006; Steunou et al., 2016).

MEAF6 (Mammalian Esa1 Associated Factor 6) est une petite protéine commune à différents complexes KATs de la famille des MYST à l'architecture similaire, à savoir NuA4/TIP60, HBO1 et MOZ/MORF (Lalonde et al., 2013). Sa fonction demeure mystérieuse à ce jour, les études chez la levure suggérant qu'elle est dispensable pour l'activité acétyltransférase du complexe et la viabilité cellulaire (Boudreault et al., 2003; Rossetto et al., 2014; Selleck et al., 2005) mais participe à la liaison d'ING3 à EPC1 (Avvakumov et al., 2012; P. Xu et al., 2016).

MBTD1 n'a été identifiée que récemment comme une sous-unité à part entière de NuA4/TIP60 (Jacquet et al., 2016). MBTD1 interagit directement avec EPC1 (Heng Zhang et al., 2020), et est capable grâce à ses domaines MBT de reconnaître H4K20me1/2 (Jacquet et al., 2016). MBTD1 semble également faciliter l'acétylation de H2AK15 par NuA4/TIP60, et la combinaison de ces deux propriétés permet d'influencer le choix de la voie de réparation des cassures double brin de l'ADN ainsi que de réguler la transcription de gènes spécifiques (Jacquet et al., 2016; Heng Zhang et al., 2020).

I.3.2 Le module d'incorporation de H2A.Z

EP400 (E1A-binding Protein p400) est l'équivalent des deux protéines Eaf1 et Swr1 de levure, ce qui lui confère la double fonction de protéine d'échafaudage centrale pour le complexe NuA4/TIP60 (Auger et al., 2008; X. Wang et al., 2018) et de remodeleur ATP-dépendant capable d'incorporer H2A.Z au sein de la chromatine

(Gévry et al., 2007). La fusion évolutive des activités acétyltransférase et d'incorporation de H2A.Z au sein d'un même complexe est cohérente au vu des rôles synergiques de ces deux mécanismes dans la régulation de l'expression de nombreux gènes (Altaf et al., 2010; Couture et al., 2012; Venkatesh & Workman, 2015). La répartition exacte de l'activité d'incorporation de H2A.Z entre NuA4/TIP60 et SRCAP (dont la sous-unité SRCAP est similaire à EP400 (Fuchs et al., 2001; Ruhl et al., 2006)) est encore inconnue, mais il est probable que ces deux complexes agissent dans des contextes chromatiniens différents pour réguler des évènements spécifiques. Le fait que des mutations de SRCAP seulement soient directement impliquées dans une pathologie très spécifique, le syndrome de Floating-Harbor (Hood et al., 2012), va dans ce sens. Enfin, il a été montré très récemment par notre laboratoire que EP400NL, un paralogue de EP400 que l'on croyait non exprimé, codait en fait pour une protéine EP400 tronquée impliquée dans la formation de sous-complexes indépendants de NuA4/TIP60 (Devoucoux, Roques, et al., 2022).

En plus de EP400, d'autres sous-unités de NuA4/TIP60 sont des équivalents directs de sous-unités du complexe SWR1 de levure, et sont également présentes dans le complexe SRCAP, suggérant un rôle potentiel dans l'incorporation de H2A.Z. Deux d'entre elles sont une molécule d'actine monomérique et une protéine semblable à l'actine ('actin-like'), BAF53a (53 Kda BRG1-Associated Factor). L'actine et les protéines actin-like sont retrouvées dans de nombreux complexes régulateurs de la chromatine (Viita et al., 2019), au sein desquels leurs fonctions sont restées longtemps méconnues; des études récentes suggèrent un rôle structural dans la régulation allostérique de l'activité ATPase des remodeleurs (Jungblut et al., 2020; Ulferts et al., 2021). YEATS4/GAS41 (Glioma-amplified sequence 41) et son orthologue Yaf9 ont vu leur importance démontrée pour l'incorporation de H2A.Z (Hsu, Zhao, et al., 2018; A. Y. Wang et al., 2009; Zhang et al., 2004) et possèdent un domaine YEATS leur permettant de reconnaître les histones acétylées (Cho et al., 2018; Hsu, Zhao, et al., 2018; Klein et al., 2017), contribuant à lier encore davantage ces deux mécanismes. VPS72/YL-1 a quant à lui été parfois décrit comme un chaperon spécifique du variant H2A.Z, et est également important pour son incorporation (Dai et al., 2021; Moreno-Andrés et al., 2020; Wu et al., 2005).

Enfin, DMAP1 (DNA Methyltransferase 1 Associated Protein) est probablement la sous-unité la plus mystérieuse de ce module. Découverte initialement comme un partenaire de DNMT1 pour la méthylation de l'ADN post-réplication (Rountree et al., 2000), DMAP1 a ensuite été clairement identifiée comme étant principalement une sous-unité de NuA4/TIP60 et SRCAP (Cai et al., 2003; Doyon et al., 2004). En dehors de permettre la liaison de GAS41 au reste du complexe (Auger et al., 2008), le rôle joué par DMAP1 au sein de NuA4/TIP60 et SRCAP est encore inconnu, mais le fait qu'il s'agisse d'une protéine essentielle (Auger et al., 2008; Mohan et al., 2011), contrairement à GAS41, implique une fonction importante. Une partie de mes travaux de doctorat a porté sur la caractérisation de DMAP1 et sur sa potentielle utilisation pour déterminer les contributions respectives de NuA4/TIP60 et SRCAP à l'incorporation de H2A.Z, et est présentée en annexe.

I.3.3 Le sous-module TINTIN

Il a été montré à la fois chez la levure (Rossetto et al., 2014) et l'humain (Devoucoux, Roques, et al., 2022) que trois sous-unités de NuA4/TIP60 sont également présentes sous la forme d'un trimère indépendant du complexe 'principal' et nommé TINTIN (Trimer Independant of NuA4 for Transcription Interactions with Nucleosomes). Les fonctions distinctes de NuA4/TIP60 de ce complexe restent méconnues, mais le fait qu'il soit enrichi au niveau du corps des gènes suggère des rôles potentiels dans la phase d'élongation de la transcription et/ou la maturation de l'ARN en cours de transcription.

Ce sous-module dispose de deux domaines lecteurs de la chromatine: le chromodomaine de MRG15 se lie spécifiquement à H3K36me3 alors que le bromodomaine de BRD8 semble reconnaître l'acétylation sur l'histone H4. Ces deux marques contribuent ainsi probablement au recrutement de NuA4/TIP60 au niveau des régions de chromatine actives transcriptionnellement.

I.3.4 Autres sous-unités

Représentant quasiment un quart de la masse moléculaire de NuA4/TIP60, TRRAP en est la plus grande sous-unité. En plus d'un rôle architectural important pour

l'intégrité du complexe, la fonction principale de TRRAP est de permettre le recrutement de NuA4/TIP60 au niveau de séquences spécifiques via son interaction avec des facteurs de transcription comme Myc et E2F ou de réparation comme MRN (McMahon et al., 1998; Park et al., 2001; Robert et al., 2006). Curiosité intéressante, TRRAP possède un domaine PIKK (ainsi que les domaines FAT et FATC adjacents) qui l'apparente aux kinases de la famille d'ATM/ATR; cependant, dans le cas de TRRAP ce domaine ne présente aucune activité kinase (McMahon et al., 1998; Saleh et al., 1998).

RUVBL1/2 sont des protéines à activité ATPase et hélicase (Gorynia et al., 2011; Kanemaki et al., 1999) présentes dans de nombreux complexes remodeleurs de la chromatine (Doyon & Côté, 2004; Watanabe et al., 2015), mais leurs fonctions sont encore peu comprises (Jha et al., 2008; Zhou et al., 2017).

Enfin, des travaux récents de notre laboratoire et d'autres (Procida et al., 2021; Sudarshan et al., 2022) indiquent que JAZF1 serait également une sous-unité stable du complexe NuA4/TIP60, jouant un rôle important pour l'incorporation et l'acétylation de H2A.Z. La présence de domaines Znf suggère un rôle dans le recrutement du complexe au niveau de régions spécifiques, possiblement en lien avec le métabolisme et la transcription des gènes ribosomiques au vu des similarités entre JAZF1 et la protéine Sfp1 de *S. cerevisiae* (Kobiita et al., 2020).

I.3.5 Aperçu des fonctions principales de NuA4/TIP60

I.3.5.1 Régulation de l'expression des gènes

Comme évoqué à la section I.2.4.1, l'acétylation des histones est connue depuis longtemps pour son association avec des régions d'euchromatine activement transcrites (Allfrey et al., 1964; Brownell & Allis, 1996). En toute logique, en vertu de son activité acétyltransférase sur H2A et H4, NuA4/TIP60 joue un rôle essentiel de co-activateur de la transcription au niveau des promoteurs et des enhancers des gènes actifs, fonction conservée de la levure (Allard et al., 1999; Galarneau et al., 2000) à l'humain (Jeong et al., 2011; Z. Wang et al., 2009). NuA4/TIP60 contribue ainsi à l'action de nombreux facteurs de transcription (voir sections suivantes).

Chez *S. cerevisiae*, l'acétylation de la chromatine par NuA4 agit en synergie avec l'incorporation de H2A.Z par SWR1 au niveau des promoteurs pour permettre l'activation de la transcription (Altaf et al., 2010), et ces deux activités au sein du même complexe chez l'homme (Auger et al., 2008). H2A.Z joue un rôle important dans la régulation de la transcription de nombreux gènes (Adam et al., 2001; Barski et al., 2007; Ibarra-Morales et al., 2021; Mylonas et al., 2021), et NuA4/TIP60 dispose donc à ce titre de deux activités principales lui permettant de réguler l'expression génique. Un bon exemple se trouve chez la drosophile, où H3K4me3 stimule l'acétylation de la chromatine et l'incorporation de H2Av (hybride de H2A.Z et H2A.X spécifique à cet organisme) par dTIP60 pour activer la transcription (Kusch et al., 2004; Kusch et al., 2014). Pour ajouter à la complexité de ces mécanismes, la façon dont NuA4/TIP60 et SRCAP se répartissent l'incorporation de H2A.Z et l'impact que cette répartition a sur l'expression des gènes reste à préciser, de même que les effets spécifiques des deux paralogues H2A.Z.1 et H2A.Z.2 et leur comportement vis-à-vis de ces deux complexes (voir Chapitre 3).

Enfin, certains éléments indiquent que NuA4/TIP60, en particulier le sous-module TINTIN, enrichi au niveau du corps des gènes, pourrait jouer un rôle durant la phase d'élongation de la transcription, assistant au recyclage des histones et influant possiblement sur l'épissage de l'ARN (Devoucoux, Roques, et al., 2022; Edmond et al., 2011; Luco et al., 2010; Rossetto et al., 2014; Schwartz et al., 2009).

I.3.5.2 Régulation du cycle cellulaire, la survie et la différenciation

En tant que coactivateur de la transcription de nombreux gènes dépendants de facteurs tels E2F (McMahon et al., 1998) ou p53 (Berns et al., 2004; Doyon et al., 2004), NuA4/TIP60 joue un rôle important dans la régulation du cycle cellulaire et la réponse au stress, mais il est également impliqué plus directement via l'acétylation de cibles non-histones. L'acétylation de p53 sur la lysine K120 favorise l'induction de la réponse pro-apoptotique (Sykes et al., 2006; Tang et al., 2006), un processus qui peut être contrecarré par l'interaction de NuA4/TIP60 avec l'ubiquitine-ligase UHRF1 (Dai et al., 2013). NuA4/TIP60 semble également capable de stabiliser p21 en l'acétylant (Lee et al., 2013). Ces mécanismes contribuent au rôle essentiel de

NuA4/TIP60 dans la régulation de l'apoptose dans différents contextes (Chevillard-Briet et al., 2014; Mattera et al., 2009), faisant du complexe un suppresseur de tumeur haplo-insuffisant s'opposant généralement à la prolifération excessive des cellules (Gorrini et al., 2007). NuA4/TIP60 est également essentiel au maintien de l'identité des cellules souches embryonnaires (Fazzio et al., 2008).

I.3.5.3 Maintien de l'intégrité génomique

NuA4/TIP60 est connu depuis longtemps comme un facteur important dans la réponse aux dommages à l'ADN, en particulier aux cassures double brin (Adamson et al., 2012; Ahmad et al., 2021; Bassi et al., 2016; Cheng et al., 2021; Downs et al., 2004; Ikura et al., 2000; Jacquet et al., 2016; Kusch et al., 2004; Murr et al., 2006; Rossetto et al., 2010) qui représentent un risque important d'instabilité génomique si elles ne sont pas réparées correctement. NuA4/TIP60 est rapidement recruté au niveau des cassures, où il contribue à la résection de l'ADN (Ahmad et al., 2021; Cheng et al., 2021; Cheng et al., 2018) et à l'ouverture et au remodelage de la chromatine pour permettre l'accès à la machinerie de réparation (Bird et al., 2002; Chailleux et al., 2010; Cheng et al., 2018; Courilleau et al., 2012; Downs et al., 2004; Ikura et al., 2007; Murr et al., 2006; Robert et al., 2006; Xu et al., 2010). NuA4/TIP60 favorise également la dynamique de la chromatine et de γH2A.X en particulier, contribuant au rétablissement de la structure chromatinienne suite à la réparation (Ikura et al., 2015; Jha et al., 2008; Kusch et al., 2004; Sharma et al., 2010).

NuA4/TIP60 est impliqué dans le 'choix' de la voie de réparation des cassures double brin, favorisant la recombinaison homologue (HR, processus de réparation plus fidèle) par rapport à la jonction d'extrémités non-homologues (NHEJ, plus 'immédiate' mais plus susceptible d'introduire des erreurs et donc des mutations) (Jacquet et al., 2016; Tang et al., 2013; Taty-Taty et al., 2016). Pour cela, NuA4/TIP60 s'oppose au recrutement de 53BP1 (facteur favorisant la NHEJ) au niveau de la chromatine ; 53BP1 reconnaissant à la fois H4K20me et H2AK15ub, NuA4/TIP60 concurrence 53BP1 via la liaison de MBTD1 sur H4K20me1/2 et via l'acétylation de H2AK15 qui empêche son ubiquitination.

Certaines études ont suggéré que NuA4/TIP60 pourrait acétyler ATM, kinase essentielle pour la réponse aux dommages à l'ADN, pour favoriser son activation (Sun et al., 2005; Sun et al., 2009). Cette question est abordée au cours du chapitre 1.

I.3.5.4 Fonctions émergentes

Comme la transcription et la réparation de l'ADN, la réplication de l'ADN est théoriquement fortement dépendante de l'accessibilité de la chromatine pour la machinerie réplicative. Pourtant, peu de liens ont été décrits à ce jour entre l'acétylation de la chromatine, qui régule cette accessibilité, et la réplication (Iizuka et al., 2006; Iizuka & Stillman, 1999; Kim et al., 2020; Miotto & Struhl, 2010). Deux sous-unités de NuA4/TIP60, KAT5 et DMAP1, ont cependant été identifiées comme de potentiels régulateurs de la réplication (Feng et al., 2015), bien qu'il puisse s'agir d'influence indirecte via leur rôle transcriptionnel. NuA4 pourrait également jouer un rôle dans la réparation de fourches de réplication endommagées et permettre la reprise de la réplication (Noguchi et al., 2019).

Plusieurs éléments suggèrent un rôle de NuA4/TIP60 dans la mitose. TRRAP est impliqué dans la régulation du point de passage mitotique (Li et al., 2004). L'acétylation de H4K12 affecte directement la ségrégation des chromosomes (Grézy et al., 2016), et la kinase Aurora B, essentielle pour cette ségrégation, fait partie des substrats non-histones à pouvoir être acétylés par NuA4/TIP60, ce qui favorise son activation (Mo et al., 2016).

Une autre des fonctions émergentes de NuA4/TIP60 au cours des dernières années concerne son importance potentielle pour la régulation du métabolisme. L'acétyl-CoA utilisé par les KATs pour la réaction d'acétylation est un intermédiaire de plusieurs processus métaboliques, en particulier du cycle de Krebs (Pietrocola et al., 2015), et leur activité est ainsi affectée par les voies métaboliques et les conditions environnementales qui dictent la quantité d'acétyl-CoA disponible (Donohoe & Bultman, 2012; Donohoe et al., 2012; Etchegaray & Mostoslavsky, 2016; Hsieh et al., 2022; Wellen et al., 2009). Comme évoqué à la section I.2.4.1, des variations

dans la disponibilité des différents groups acyles peut conduire au remplacement de l'acétylation par d'autres modifications aux fonctions très proches mais dont il n'est pas exclu qu'elles puissent entraîner des conséquences plus spécifiques (Barnes et al., 2019; Choudhary et al., 2014; Dutta et al., 2016; Kollenstart et al., 2021; Sabari et al., 2017). Réciproquement, l'action des KATs a un effet sur le stock d'acétyl-CoA et les voies métaboliques qui l'utilisent (Zhao et al., 2010). Parmi les exemples précis de fonctions de NuA4/TIP60 dans la régulation du métabolisme, on peut citer l'acétylation de Pck1p permettant sa fonction dans la néoglucogenèse (Lin et al., 2009), et l'acétylation de la kinase ULK1, essentielle pour l'autophagie (Lin et al., 2012). Enfin, comme évoqué un peu plus tôt, la nouvelle sous-unité JAZF1 est un candidat prometteur pour lier NuA4/TIP60 à la réponse aux signaux de croissance via la voie mTOR et la régulation de l'expression des gènes ribosomiques (Kobiita et al., 2020; Procida et al., 2021; Sudarshan et al., 2022).

I.3.5.5 Pathologies liées à NuA4/TIP60

De par sa position centrale dans la régulation de nombreux processus cellulaires clés, la dérégulation de NuA4/TIP60 et de ses sous-unités est logiquement liée à de multiples pathologies, dont il ne s'agit pas de faire ici la liste exhaustive. En particulier, son rôle de cofacteur pour plusieurs oncogènes ou suppresseurs de tumeurs comme p53, Rb, Twist, PRAK, NFkB, Notch and Myc (Avvakumov & Côté, 2007; Gorrini et al., 2007; Kim et al., 2012; Kim et al., 2007; Shi et al., 2014; Zheng et al., 2013) fait de NuA4/TIP60 un facteur impliqué dans de nombreux cancers (Avvakumov & Côté, 2007; Yamada, 2012), où son expression est le plus souvent réduite en vertu de son rôle de suppresseur de tumeur (Gorrini et al., 2007), même si des cas inverses existent (Awasthi et al., 2005).

Au cours des dernières années, de nombreuses translocations chromosomiques ont été identifiées, dont la particularité est d'aboutir à la fusion de différentes protéines appartenant à divers complexes régulateurs de la chromatine, ces protéines de fusion ayant généralement des propriétés oncogéniques au sein de tumeurs épithéliales (Tuna et al., 2019). Plusieurs sous-unités de NuA4/TIP60 sont impliquées dans des fusions de ce type (Brunetti et al., 2018; Devoucoux, Fort, et

al., 2022; Hofvander et al., 2020; J. Li et al., 2021; Micci et al., 2017), où elles semblent contribuer à activer la transcription d'oncogènes en acétylant des régions normalement réprimées (Sudarshan et al., 2022).

NuA4/TIP60 semble également essentiel pour le bon fonctionnement et développement du cerveau, à en juger par les différentes pathologies neuronales qui l'impliquent (Beaver et al., 2020). On peut citer en particulier la maladie d'Alzheimer contre laquelle NuA4/TIP60 jouerait un rôle neuroprotecteur (Baek et al., 2002; Beaver et al., 2021; Cao & Südhof, 2001; Marks et al., 2021; Schmidt & Sheeley, 2015; Xu & Elefant, 2015; S. Xu et al., 2016; Xu et al., 2014; Haolin Zhang et al., 2020). Ceci s'inscrit dans un rôle plus général de l'acétylation dans l'établissement et la consolidation de la mémoire (Fischer, 2014). Le chapitre 2 présente nos résultats identifiant des mutations au sein de la protéine KAT5 liées à un syndrome neurodéveloppemental rare, un point commun avec plusieurs autres facteurs de régulation de la chromatine (voir discussion).

Enfin, il a été montré que des protéines de virus, notamment dans le cas du VIH (Virus de l'Immunodéficience Humaine) et du VPH (Virus du Papillome Humain), pouvaient interagir avec NuA4/TIP60 et moduler ses fonctions pour faciliter la prolifération virale (Jha et al., 2010; Subbaiah et al., 2016).

I.4 Buts du projet de doctorat

Mon doctorat s'est concentré sur deux objectifs distincts, visant à mieux caractériser la biochimie de certaines sous-unités du complexe NuA4/TIP60 et à améliorer notre compréhension de la façon dont il contribue à l'action du variant d'histone H2A.Z.

Le premier objectif consistait à caractériser le chromodomaine de la sous-unité catalytique KAT5/Tip60. Comme indiqué dans cette introduction, les chromodomaines sont classiquement des lecteurs de la chromatine méthylée. Cependant, dans le cas du chromodomaine de KAT5/Tip60, les choses sont plus compliquées puisque des rapports contradictoires existent dans la littérature concernant sa fonction et sa spécificité de liaison. Le modèle qui semble s'être imposé dans la littérature (voir (Savani et al., 2020) pour exemple) stipule que, de

façon analogue à HP1, le chromodomaine de KAT5/Tip60 ciblerait spécifiquement H3K9me3 pour favoriser la réparation des régions d'hétérochromatine en acétylant et activant ATM. Ce modèle pose plusieurs problèmes d'ordre théorique, notamment du point de vue structural puisque si les chromodomaines de HP1 et KAT5/Tip60 présentent bien une certaine homologie de séquences, leurs structures et en particulier leurs cages aromatiques diffèrent fortement. De plus, le chromodomaine de KAT5/Tip60 est extrêmement bien conservé de la levure à l'homme, or la marque H3K9me3 est absente chez la levure, ce qui suggère également une fonction différente pour ce domaine. Nous avons donc cherché à répondre à cette question en analysant l'interaction du chromodomaine avec différentes marques d'histone au sein de la chromatine, tout en utilisant des approches de mutagenèse et d'essais *in vitro* pour déterminer l'importance du chromodomaine pour la fonction du complexe NuA4/TIP60 (Chapitre 1). Les mêmes approches de mutagenèse nous ont également permis de caractériser, en collaboration avec le groupe de Philippe Campeau (CHU Sainte-Justine, Montréal) des mutations au sein de KAT5/Tip60 retrouvées chez des patients souffrant d'un syndrome neurodéveloppemental nouvellement identifié (Chapitre 2).

Le second objectif de mon doctorat s'intéressait aux liens entre NuA4/TIP60 et H2A.Z, ainsi qu'à la sous-unité DMAP1 qui appartient au module d'incorporation de H2A.Z dans NuA4/TIP60 et à SRCAP, l'autre complexe responsable de cette incorporation. Les résultats spécifiques à DMAP1 et à la façon dont cette protéine pourrait nous servir à interroger les contributions spécifiques de NuA4/TIP60 et SRCAP sont présentés en annexe. Ces travaux nous ont également conduit à travailler, en collaboration avec le groupe de Didier Trouche (CNRS, Toulouse, France), sur la caractérisation des deux paralogues de H2A.Z, H2A.Z.1 et H2A.Z.2, pour lesquels nous avons cherché à déterminer leur implication respective dans la régulation de la transcription et à identifier leurs partenaires spécifiques (Chapitre 3).

Chapitre 1: KAT5/Tip60 chromodomain functions independently of histone marks to target chromatin for acetylation

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1.1 Résumé

L'acétyltransférase KAT5/Tip60, en tant que sous-unité du complexe NuA4/TIP60, acétyle les histones H2A et H4 au niveau de la chromatine pour réguler de nombreux processus cellulaires. Les chromodomaines sont généralement des domaines de liaison aux histones méthylées, mais nous montrons ici que le chromodomaine de KAT5/Tip60 n'est pas capable de reconnaître des marques d'histones spécifiques au sein de la chromatine. Nos résultats indiquent que le chromodomaine serait plutôt requis pour l'activité acétyltransférase de NuA4/TIP60 sur la chromatine, particulièrement sur H4, de façon indépendante des modifications préexistantes sur les histones. Ainsi, l'activité de NuA4/TIP60 pourrait être modulée par des modifications du chromodomaine, comme l'illustrent nos résultats montrant que l'acétylation de la lysine 52 de KAT5/Tip60 au sein du chromodomaine affecte l'activité acétyltransférase du complexe et peut être régulée en réponse aux dommages à l'ADN. Nous montrons également que KAT5/Tip60 est dispensable pour l'activation d'ATM en réponse aux cassures double-brin de l'ADN, en contraste avec le modèle proposé dans la littérature liant le chromodomaine à l'acétylation d'ATM. Nos travaux contribuent à clarifier la fonction et les propriétés de liaison à la chromatine du chromodomaine de KAT5/Tip60, et le rôle essentiel qu'il joue dans le fonctionnement du complexe NuA4/TIP60.

1.2 Abstract

The acetyltransferase KAT5/Tip60, as a part of the NuA4/TIP60 complex, acetylates histones H2A and H4 in chromatin to regulate many cellular processes. Chromodomains usually act as readers of methylated histones, but here we show that KAT5/Tip60 chromodomain is unable to recognize specific histone marks in chromatin. Instead, our results indicate that KAT5/Tip60 chromodomain is required for the acetyltransferase activity of NuA4/TIP60 on chromatin, especially on H4, indifferently to pre-existing histone modifications. The acetyltransferase activity of NuA4/TIP60 could, therefore, be affected by modifications in the chromodomain, as illustrated by our results showing that acetylation of lysine 52 in the chromodomain impairs acetyltransferase activity and can be regulated by DNA damage. Furthermore, we show that KAT5/Tip60 is dispensable for ATM activation in response to DNA double-strand breaks, in contrast with what has been suggested in the literature linking the chromodomain to ATM acetylation. Our findings contribute to the clarification of the function and chromatin reader properties of KAT5/Tip60 chromodomain and how it plays a critical role in NuA4/TIP60 activity.

1.3 Introduction

In eukaryotic cells, the chromatin organization and its regulation play major roles in regulating all DNA-based processes such as transcription, replication and repair. One of the main ways this structure is regulated is through post-translational modifications (PTMs, or histone ‘marks’) on nucleosomal histones, the most frequent including phosphorylation, methylation, ubiquitylation, and acetylation. Since the original proposal of the idea of a ‘histone code’ by Strahl and Allis (Strahl & Allis, 2000), it has become increasingly clear that most PTMs act as docking sites for chromatin-binding factors through binding to the reader domains they contain (Musselman et al., 2012; Ruthenburg et al., 2007). Reader domains are present in a large quantity of chromatin regulators, and they display a specificity both for types of PTMs and histone residues.

Acetylation also acts more directly on chromatin structure by destabilizing histone-DNA and nucleosome-nucleosome interactions, creating a more relaxed chromatin

structure associated with active regions of the genome (Chen et al., 2022b; Steunou et al., 2014). KAT5/Tip60 is the catalytical subunit of the large (at least 17 subunits) NuA4/TIP60 complex, a major acetyltransferase in mammals. In the context of chromatin, KAT5 acetylates H4 on lysines 5, 8, 12 and 16, H2A on lysines 5, 9, 13 and 15, as well as histone variants such as H2A.Z and numerous non histone proteins (Doyon et al., 2004; Sapountzi & Côté, 2010). TIP60 is an essential coactivator for the transcription of numerous genes including targets of p53, Myc or Rb (Leduc et al., 2006; Martinato et al., 2008; Sykes et al., 2006; Tang et al., 2006; Taubert et al., 2004). KAT5 has also been reported as a critical effector of stem cell maintenance and renewal (Fazzio et al., 2008) and response to stress including DNA double-strand breaks (Chailleux et al., 2010; Eymin et al., 2006; Ikura et al., 2015; Ikura et al., 2000; Jacquet et al., 2016). It has been identified as a haplo-insufficient tumor suppressor often deregulated in cancers (Brown et al., 2016; Gorrini et al., 2007).

Along with other chromatin reader domains present in the TIP60 complex, KAT5 itself has been described as possessing a chromodomain (Selleck et al., 2005). Chromodomains are a family of histone readers generally associated with the recognition of methylated histone residues (Blus et al., 2011; Musselman et al., 2012; Taverna et al., 2007; Yap & Zhou, 2011). However, despite its clear conservation from yeast to human, and its proposed importance for KAT5 and TIP60 functions (Huang & Tan, 2013; Selleck et al., 2005; Shimojo et al., 2008; Sun et al., 2009), the precise function and binding specificity of KAT5 chromodomain are still unclear. Reports have suggested a binding specificity for diverse residues, including H3K4me1 (Jeong et al., 2011), H3K4me3 (Kim et al., 2015), and H3K9me3 (Sun et al., 2009). The latter seems to be the most accepted view in current literature and has been proposed to be critical for proper DNA damage response in heterochromatin by facilitating the recruitment of TIP60 to acetylate and activate ATM (Sun et al., 2005; Sun et al., 2009). However, the recently published crystal structure of KAT5 chromodomain argues against the idea of the chromodomain recognizing methylated histone residues and especially H3K9me3 (Zhang et al., 2018). Chromodomains usually bind to methylated residues through aromatic cages,

as illustrated by the case of HP1 binding to H3K9me3 (Fig 1.S1). In contrast to HP1, both yeast and human KAT5 chromodomains display only two aromatic residues forming an incomplete cage (Fig 1.1A and B), which, along with the observation of an arginine residue occupying the central part (Zhang et al., 2018), likely indicates significantly different binding specificity compared to HP1 despite significant sequence conservation (Fig 1.1C).

Previous studies of KAT5 chromodomain have used mostly histone peptides to study binding specificity. Work from our lab and others, however, has shown that it is important to study chromatin regulating factors in their native form and in the 'natural' context of chromatin. Here, we provide evidence that KAT5 chromodomain doesn't display binding specificity for any specific histone PTMs in nucleosomes, but that it is required for proper acetylation by the NuA4/TIP60 complex of its chromatin targets, independently of pre-existing histone marks. We further investigated the function of a specific residue inside KAT5 chromodomain, K52, the acetylation of which being linked to DNA damage response regulation. We show that this acetylation likely functions by affecting the acetyltransferase activity of the complex on its nucleosomal targets, and that in turns affects the DNA damage response. Furthermore, we observed no measurable effect of KAT5 depletion on ATM acetylation and activation, arguing that the roles it plays in DNA damage response are independent of this previously proposed function through its chromodomain (Sun et al., 2009). Taken together, our results provide mechanistic insights into the regulation of TIP60 acetyltransferase activity, and how it affects its subsequent functions in regulating cellular processes.

1.4 Results

KAT5 chromodomain does not display binding preference on peptides or nucleosomes

To test the binding specificity of KAT5 chromodomain in an unbiased fashion, we generated and purified a recombinant protein corresponding to KAT5 aa 2-81 containing the chromodomain (Fig 1.S2) and used it in a large-scale screening assay looking at binding specificity on a large array of histone peptides (Fig 1.1D) and

recombinant nucleosomes (Fig 1.1E) harboring different histone modifications. In both cases, KAT5 chromodomain did not display significant binding to any specific chromatin feature, in stark contrast with HP1 used as a positive control and showing very specific binding to H3K9me3 peptides and nucleosomes. Accordingly, ChIP experiments with KAT5 mutants for the chromodomain did not suggest an involvement from the chromodomain in recruiting NuA4/Tip60 to its genomic targets (Fig 1.3D and data not shown). Taken together, and along with the observations made by others (Zhang et al., 2018) related to the chromodomain structure, these results suggest that KAT5 chromodomain is in fact not involved in the recognition of specific histone marks on chromatin, as opposed to models previously suggested (Jeong et al., 2011; Kim et al., 2015; Sun et al., 2009).

KAT5 chromodomain is essential for cell viability

As shown by sequence alignments (Fig 1.1C) and structural data (Fig 1.1A and B), KAT5 chromodomain is highly conserved between yeast and mammals, strongly suggesting an important role for cell viability. To assess this importance in yeast cells, we focused on two conserved aromatics residues, Y53 (human Y44) and Y56 (human Y47), the latter being part of the partial aromatic cage of the domain (Fig1.1A and B) and having been previously suggested to be involved in KAT5 function (Selleck et al., 2005; Sun et al., 2009). We expressed WT and mutant Esa1 (budding yeast KAT5) from plasmids covered by the conditional expression of a WT protein. As shown in Fig1.2A, cells expressing only an Esa1 protein where either of these tyrosine residues is mutated in alanine or glutamate are not viable, illustrating the essential nature of KAT5 chromodomain for cell viability.

KAT5 chromodomain is required for proper acetyltransferase activity of NuA4/TIP60 *in vitro*

In order to assess the importance of KAT5 chromodomain in the cellular functions of the NuA4/TIP60 complex that could explain its requirement for cell viability, we generated cell lines expressing, from the *AAVS1* safe harbour locus, near physiological levels of tagged KAT5, either wild-type or with Y44 or Y47 mutated (Fig1.S3). This allowed us to perform tandem affinity purification (TAP) of native

NuA4/TIP60 complexes. Importantly, the full set of NuA4 subunits was purified along both WT and mutant KAT5, indicating that mutations in the chromodomain did not affect the structural integrity of the full complex (Fig1.2B). We then used these native purified complexes in *in vitro* histone acetyltransferase assays, using either purified (Fig1.2C) or recombinant (Fig1.2D) nucleosomes as substrates. In both cases, select mutations in the chromodomain dramatically decreased acetyltransferase activity of NuA4, specifically on chromatin substrates as opposed to free histones (Fig1.S4). The fact that a similar effect is observed on purified chromatin and on recombinant nucleosomes, devoid of any previously established histone PTM, indicates that the effect is independent of histone marks, which is consistent with the idea that the chromodomain does not bind to specific PTMs in chromatin. H4 acetylation being affected more dramatically than H2A is indicative of different structural requirements for proper presentation of the lysine residue to KAT5 catalytic site within the NuA4/TIP60 complex (see Discussion).

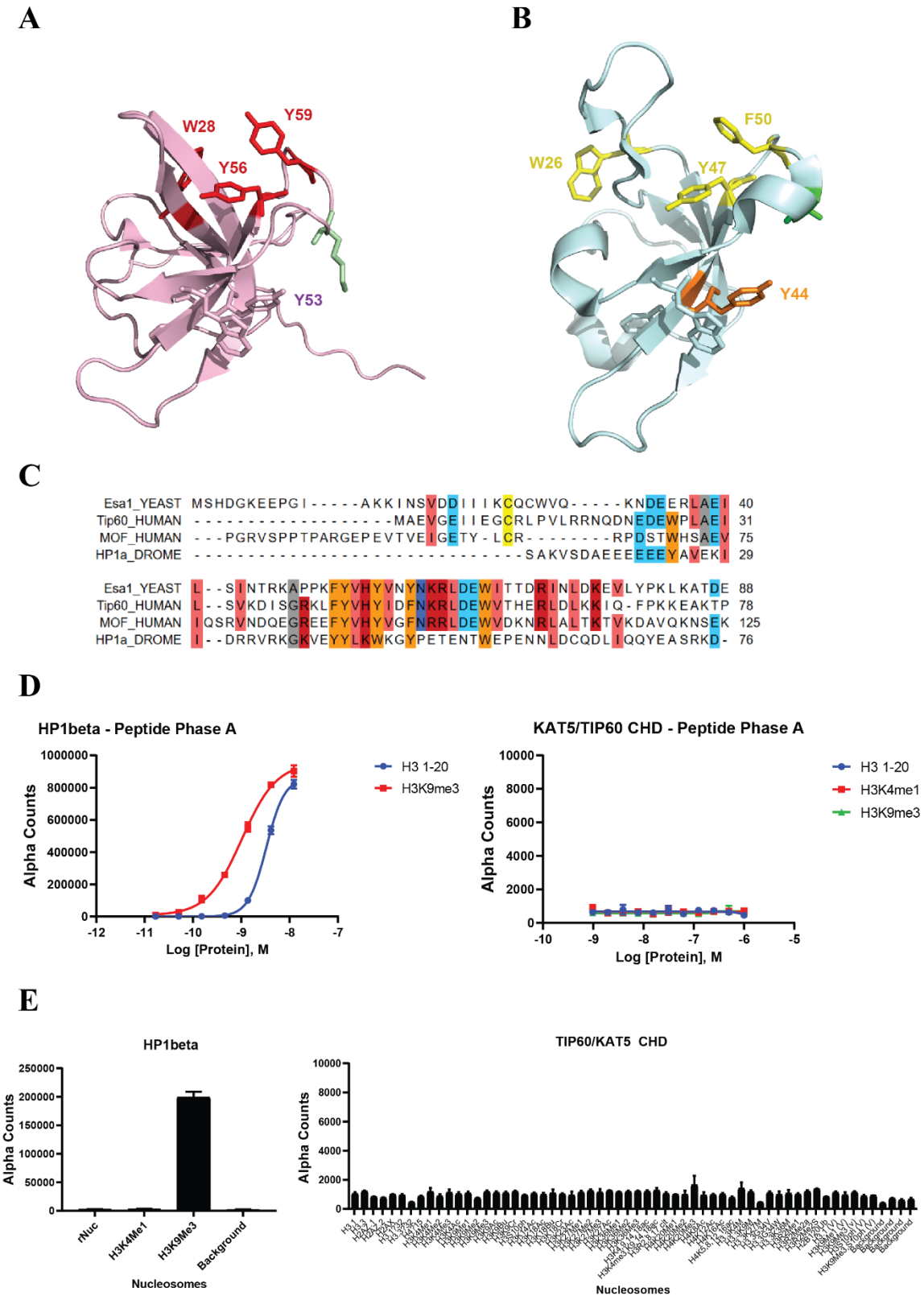

Figure 1.1: KAT5 CHD displays no binding specificity to specific histone PTMs

A. Structure of yeast Esa1 chromodomain (PDB 2RO0). B. Structure of human KAT5 chromodomain (PDB 4QQG). C. Sequence alignment of the HAT Esa 1 Tip 60 and MOF chromodomains with HP1a classical chromodomain. D. Alpha counts for the interaction of recombinant HP1 (left panel) or KAT5 chromodomain (right panel) with indicated peptides. E. Alpha counts for the interaction of recombinant HP1 (left panel) or KAT5 chromodomain with nucleosomes harboring indicated modifications.

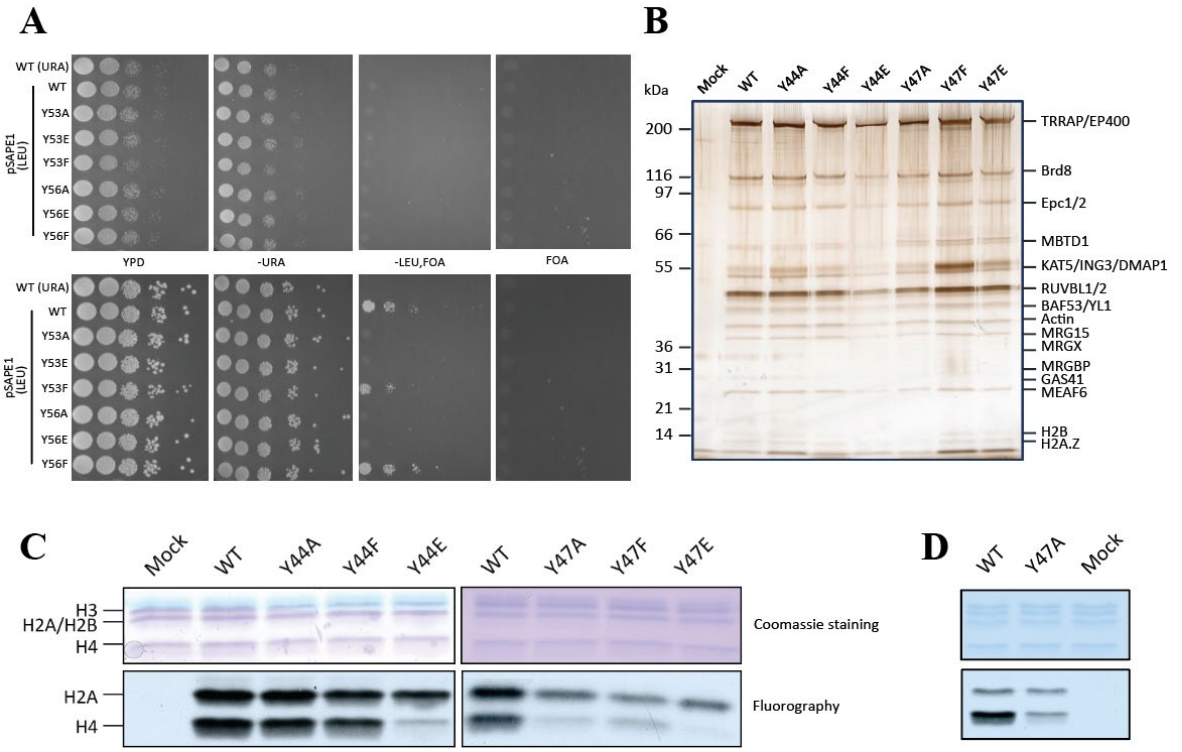

Figure 1.2. KAT5 chromodomain is essential for cell viability and acetyltransferase activity on chromatin, independently of pre-existing histone marks.
A. Yeast strains deleted for Esa 1 and containing a wild type Esa1 gene on a URA plasmid were transformed with a LEU plasmid expressing wild type Esa1 or Esa1 containing the indicated point mutations. Left panels show growth of 10-fold serial dilutions on YPD rich medium, right panels show similar growth on 0.1% fluoroorotic acid plates. Top panel: 2 days growth; bottom panel: 3 days growth. B. Silver-stained SDS-PAGE gel of native NuA4/TIP60 complexes purified through the indicated tagged KAT5 constructs.
C. Autoradiograph of in vitro histone acetylation assays using native WT and mutant complexes shown in B with purified chromatin (short oligonucleosomes). Samples were loaded on SDS-PAGE gels, treated with En3Hance, dried and exposed on film in order to visualize the effect on specific histones (lower panels). Coomassie-stained gels are shown to control relative substrate amounts in the reactions (upper panels). n=3, a representative result is shown. D. Autoradiograph as in C using indicated purified NuA4/TIP60 complexes on recombinant nucleosomes.

Acetylation of lysine 52 in KAT5 chromodomain modulates NuA4/TIP60 activity

Interestingly, one residue in KAT5 chromodomain, lysine 52 (K52), has been reported as being acetylated in vivo (Choudhary et al., 2009; Peng et al., 2012). Modulating the acetylation state of subunits within KAT complexes has been suggested as a way to regulate their activity, for instance in response to signals such as DNA damage (Peng et al., 2012; Yamagata & Kitabayashi, 2009). We first sought to detect K52 acetylation in presence or not of DNA damage, by performing AP-MS on native NuA4/TIP60 complexes after treating the cells with etoposide. Levels of K52 acetylation appeared to decrease following DNA damage (Fig1.3A), suggesting a potential regulatory mechanism in line with what had been suggested previously (Peng et al., 2012; Yamagata & Kitabayashi, 2009). Then, to determine how KAT5 K52 acetylation affects NuA4/TIP60 function, we generated cell-lines expressing either WT KAT5 or acetyl-mimic (K52Q) or non-acetylable (K52R) mutants. We then purified native complexes from those cell lines and performed acetyltransferase assays as previously described. Similarly to other mutations in KAT5 chromodomain, we observed a decrease in acetyltransferase activity on chromatin in the K52 acetyl-mimic mutant (K52Q) (Fig1.3B). The acetyl-mimic mutation of the equivalent residue (K61) in yeast KAT5/Esa1 greatly impairs cell viability and survival to the DNA damaging agent MMS (Fig1.3C). ChIP-seq experiments showed that mutant KAT5/Tip60 is still bound to its genomic targets in human cells (Fig1.3D), supporting the idea that the chromodomain is not required for the recruitment of the complex to specific loci, playing instead a role in the proper recognition of chromatin substrates for acetylation by NuA4/TIP60.

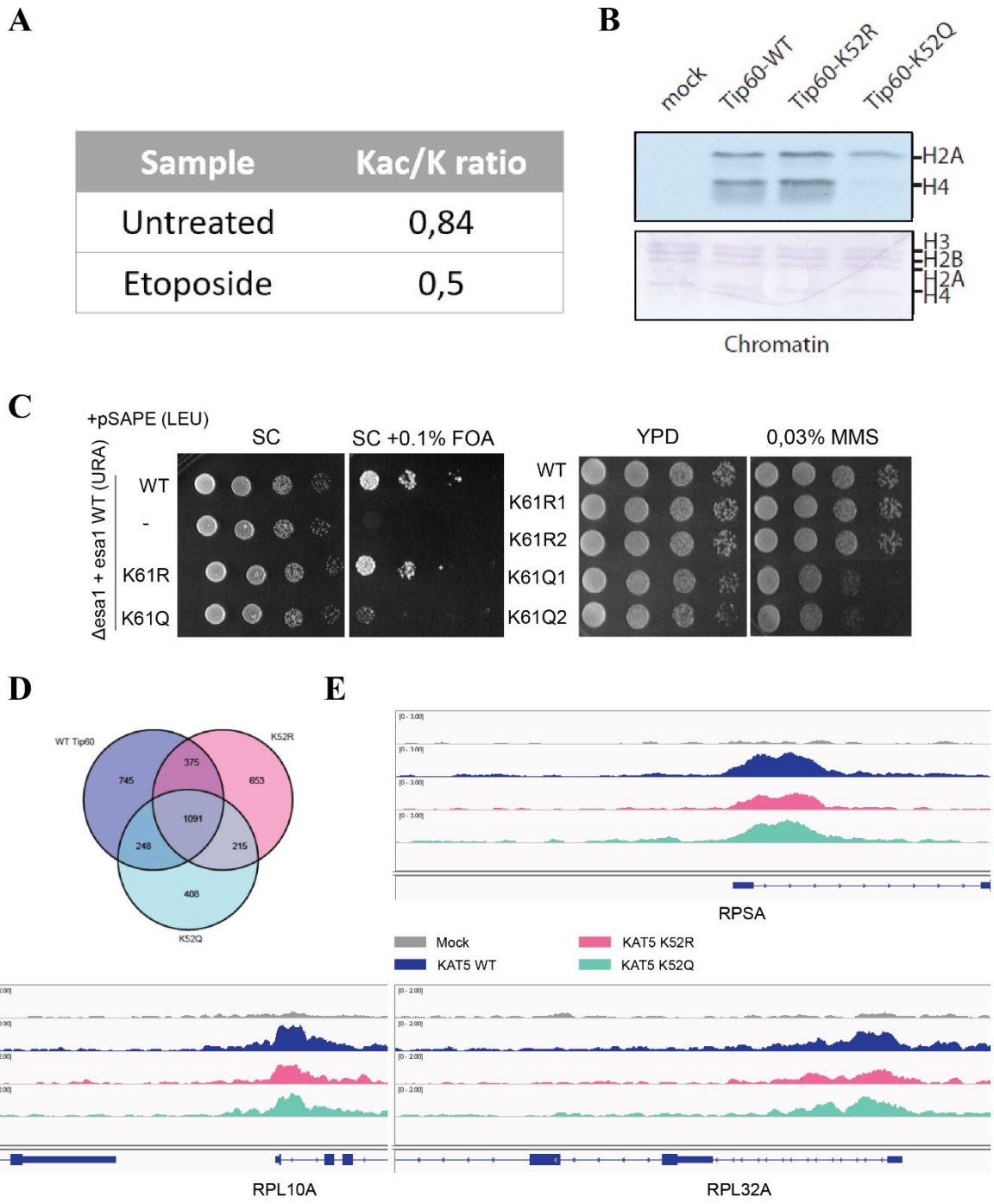

Figure 1.3. KAT5 K52 acetylation regulates NuA4/TIP60 activity.
A. Mass-spectrometry peptide counts of acetylated KAT5 K52 after purification of native NuA4/TIP60 complexes and anti-acetyl IP in presence or not of etoposide. B. Autoradiograph of in vitro histone acetylation assays using native WT and mutant NuA4/TIP60 complexes with purified chromatin (short oligonucleosomes). Samples were loaded on SDS-PAGE gels, treated with En3Hance, dried and exposed on film in order to visualize the effect on specific histones (upper panel). Coomassie-stained gels are shown to control relative substrate amounts in the reactions (lower panel). n=3, a representative result is shown. C. Yeast survival assays of esa1 depleted cells expressing the indicated

constructs, in normal conditions (left panel) or after induction of DNA damage by treating cells with 0,03% MMS (right panel). D. ChIP-seq of Flag-tagged WT or mutant KAT5/TIP60 in K562 cells. Overlap of KAT5/TIP60 peaks show most peaks are conserved in K52 mutants. E. ChIP-seq profiles of three representative genes (RPSA, RPL32A and RPL10A) bound by WT, K52R and K52Q KAT5. y-axis: reads per million.

KAT5 is not required for ATM activation upon DNA damage

It has been suggested that KAT5 acetylates ATM, a modification specifically required for its activation in response to DNA damage. Furthermore, KAT5 chromodomain was argued to be required for this modification/activation by recruiting NuA4/TIP60 to H3K9me3-marked nucleosomes (Sun et al., 2005; Sun et al., 2009). Having shown that the KAT5 chromodomain is not able to recruit the complex to specific chromatin marks, we wanted to reassess the acetyltransferase activity of NuA4/TIP60 on ATM and its importance for ATM activation, using our approach with native purified complexes and reagents.

We first we expressed 3xFlag-2xStrep-tagged ATM from the AAVS1 locus as previously described (Dalvai et al., 2015). Cells expressing tagged ATM were treated with siRNAs to knockdown KAT5/Tip60, and DNA damage was induced with doxorubicin. Tagged ATM was then immunoprecipitated with anti-Flag antibodies (Figure 1.4A). We observed normal levels of phosphorylation by Western-Blot for both ATM and KAP1 after treatment with doxorubicin, whether KAT5/Tip60 was depleted or not, indicating that KAT5/Tip60 is dispensable for ATM activation in response to DNA damage. Furthermore, we were not able to observe any change in ATM acetylation after inducing DNA damage, in presence or not of normal levels of KAT5/Tip60. We then tested directly if purified native ATM could be acetylated *in vitro* by native NuA4/TIP60 complexes purified from cells treated or not with DNA damaging agents (Figure 1.4B) but were not able to observe detectable levels of acetylation, in contrast to other substrates tested like nucleosomes or even autoacetylation events in NuA4/TIP60 itself (mainly TRRAP subunit).

Altogether these results suggest that KAT5/Tip60 does not acetylate ATM and does not play a significant role in its activation in response to DNA damage.

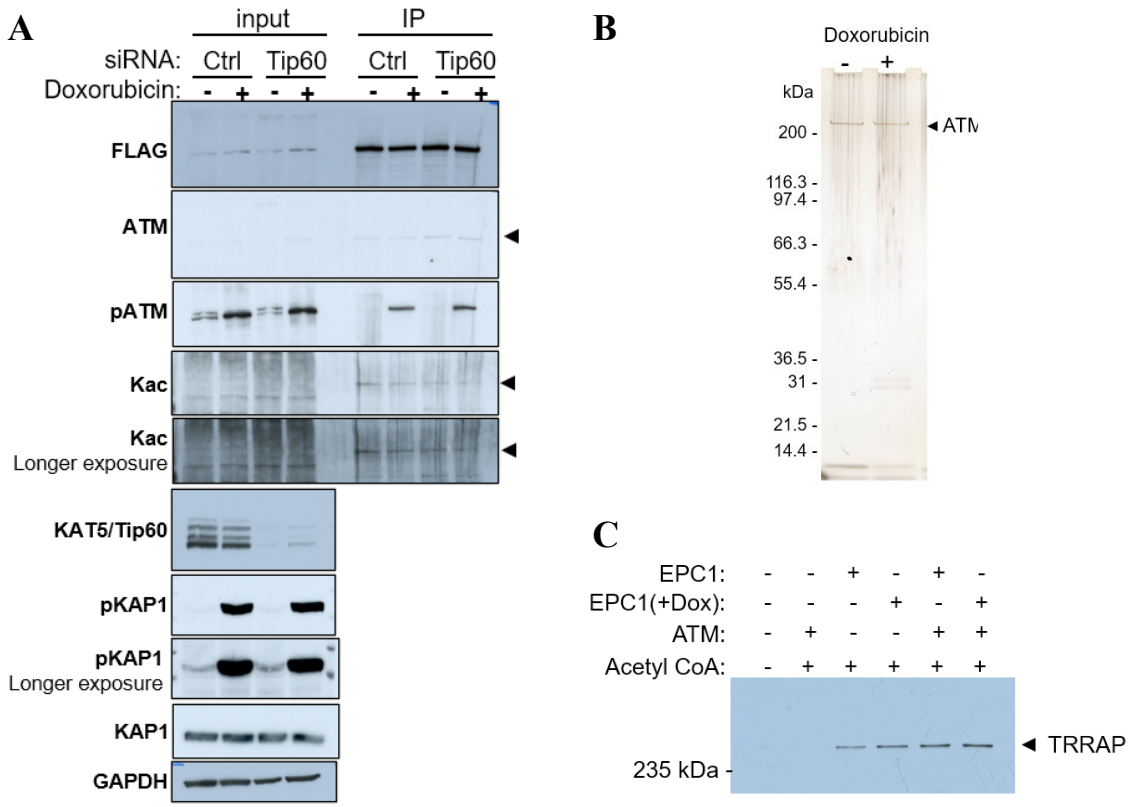

Figure 1.4. KAT5 is not required for ATM activation in response to DNA damage. A. Cells were treated with indicated siRNAs, treated or not with doxorubicin, and purification of tagged ATM was performed. Phosphorylation states of ATM and KAP1, as well as acetylation state of ATM, were observed by Western-Blot. Arrows indicate ATM. B. Silver stained SDS-PAGE gel of purified ATM from K562 cells treated or not with Doxorubicin. C. In vitro acetylation assay using purified NuA4/TIP60 (from EPC1 subunit) and ATM.

1.5 Discussion

KAT5 chromodomain has been the subject of contradictory reports regarding its ability to recognize histone marks and direct the functions of NuA4/TIP60 to specific genomic regions. Our results show that KAT5 chromodomain does not recognize specific histone marks in the context of chromatin, an observation in line with structural considerations previously reported (Zhang et al., 2018) and our own observations that mutations in the chromodomain do not prevent recruitment of NuA4/TIP60 to chromatin. However, we found that, consistently with previous works in yeast (Selleck et al., 2005) KAT5 chromodomain is still essential for the acetyltransferase activity of NuA4/TIP60, specifically on chromatin and with a specific impact on H4 acetylation. This effect is independent of pre-existing histone

marks on the chromatin, once again arguing against the ability of the chromodomain to recognize specific marks.

The structure of the yeast NuA4 core complex bound to a nucleosome has been reported (P. Xu et al., 2016), showing that in absence of Esa1 chromodomain NuA4 is still able to bind nucleosomes, but the overall strength of the interaction is altered. This provides an interesting potential explanation of the importance of KAT5 chromodomain for NuA4/TIP60 activity: the chromodomain would be required for NuA4/TIP60 to adopt a proper conformation once bound to chromatin that would allow the catalytic site to be properly oriented to acetylate histone tails, especially H4. This process might be modulated by autoacetylation processes, as suggested by our observation that acetylation of K52 inside the chromodomain could recapitulate acetylation defects observed in other mutants. The same report shows that KAT5/Esa1 catalytic site within NuA4 is oriented towards the H4 tail, suggesting a whole different mechanism for the recognition of H2A compared to H4, explaining how mutations can affect more specifically the acetylation of one or the other (P. Xu et al., 2016). More work on the structure of the human NuA4/TIP60 complex and its binding to nucleosomes will be required to really determine how the chromodomain and potential mutations in it can affect the functions of the complex.

We recently identified mutations in KAT5 linked to a rare neurodevelopmental syndrome (Humbert et al., 2020). Interestingly, one of these mutations is located inside the chromodomain, and seems to affect the acetyltransferase activity of NuA4/TIP60 in the same way as mutants described in the current report, illustrating that KAT5 chromodomain should be regarded as potentially as important as the catalytic MYST domain when it comes to adverse effects and therapeutic avenues.

Finally, our result failed to show any significant involvement of NuA4/TIP60 in ATM acetylation and activation in response to DNA damage. This is in sharp contrast to some reports in the literature arguing for a signaling cascade from H3K9me3 recognition by KAT5 chromodomain leading to acetylation of ATM and its activation (Sun et al., 2009). We do not have a clear explanation for these discrepancies, but feel that our experimental approach using native purified complexes, reagents and

substrates provide more physiological conditions to draw conclusions in comparison to using among other things over-expression of KAT5 by itself and histone peptides. We could also not replicate the claim that a large portion of cellular ATM is associated with KAT5/Tip60 in vivo within the NuA4/TIP60 complex. In parallel, it was shown by different groups that KAT5 and H3K9me3 are not required for ATM function *in vivo* (Goodarzi et al., 2008; Sharma et al., 2010).

Altogether our results identify a non-canonical role of KAT5 chromodomain in mediating acetylation of chromatin, independent of pre-existing histone marks, and repositioning NuA4/TIP60 in the response to DNA damage, downstream of ATM activation, as described in lower eukaryotes and other studies in mammalian systems.

1.6 Experimental Procedures

Recombinant protein purification

Recombinant KAT5 chromodomain (aa 2-81) was purified as described previously (Setiaputra et al., 2018). In short, bacterial cells were grown overnight at 18°C with IPTG induction. Next day cells were lysed with lysozyme followed by sonication. The soluble portion was incubated with glutathione-sepharose (GE healthcare) beads for 3-4 hours at 4°C and eluted with glutathione. The eluted protein concentration was quantified by running on SDS-PAGE with BSA standards followed by coomassie staining.

Large scale peptide and nucleosome-binding assays

Assays using purified recombinant KAT5 chromodomain were performed by Epicypher® Inc., Durham NC USA, as a part of their dCypher™ commercial service.

Yeast Strains and Culture

Esa1 mutants have been created by PCR-mediated mutagenesis on pSAPE1 plasmid containing wild-type ESA1 open reading frame tagged with N-terminal hemagglutinin (HA) epitopes, under the control of the endogenous promoter, with

the LEU2 selection marker. Plasmids carrying the mutated forms of ESA1 were verified by sequencing and transformed into QY118 MATa his3Δ1 leu2Δ0 ura3Δ0 esa1Δ::KanMX pLP795 (ESA1 ARS/CEN URA3) by standard protocol. This generated strains QY1850 to 1853 and QY2283 to 2289. Protein expression of each mutant was verified by Western-blot on whole-cell-extracts prepared in RIPA buffer (50 mM HEPES [pH 7.9], 2 mM EDTA, 0.25 M NaCl, 0.1% SDS, 0.1% DOC, 1% Triton X-100), separated on 10% acrylamide gels, transferred on nitrocellulose membranes and blotted with anti-HA HRP antibody (Roche; 1/1000).

Viability test, plasmid shuffling

Esa1 mutants viability test have been performed following standard plasmid shuffling method. Exponentially growing yeast containing the plasmid form of both wild type (URA3) and the mutant versions of ESA1 (LEU2) in synthetic complete medium lacking leucine and uracil (SC-LEU-URA) were used in serial tenfold dilution and spotted on synthetic complete SC control plates and SC containing 0.1% of 5'-fluoroorotic acid (FOA) plates. Cells were grown at 30°C for 2 to 4 days. Viable esa1 mutants were isolated from 0.1%FOA-containing plates and verified on SC-LEU and SC-URA plates. Serial tenfold dilution performed from exponentially growing cells in SC-LEU were then spotted on YPD plates containing 0.03%MMS and grown at 30°C. Photos were taken after 2 to 5 days.

Human Cell Culture and Transfection

K562 cells were obtained from the American Type Culture Collection (ATCC) and maintained at 37°C under 5% CO2 in RPMI medium supplemented with 10% newborn calf serum (Wisent) and GlutaMAX (Thermo Fisher). When cultivated in spinner flasks, 25 mM HEPES-NaOH (pH 7.4) was added. Cells were transfected using Lipofectamine 2000 (Thermo Fisher) per the manufacturer's instructions.

Generation of Stable Cell Lines Producing Tagged KAT5 Variants and Affinity Purification of NuA4/TIP60 and ATM Complexes

KAT5 (461aa isoform) was cloned into the AAVS1_Puro_PGK1_3xFLAG_Twin_Strep plasmid (addgene #68375), and the constructs carrying desired mutations in the chromodomain were designed via site-

directed mutagenesis. Generation of K562 cells expressing tagged ATM, EPC1 and either wild-type (WT) or variant tagged KAT5 was performed through break-induced recombination and/or insertion at the *AAVS1* locus (MIM: 102699) as described (Dalvai et al., 2015). Where indicated, cells were treated with 250 µM doxorubicin for 17 h. Purification of native NuA4/TIP60 complexes as well as tagged ATM was performed as described (Doyon & Côté, 2016; Lashgari et al., 2019). Typically, soluble nuclear extracts (Abmayr et al., 2006) were prepared from 3E9 cells (3 L cultures at 0.6–1.0 million cells per ml), adjusted to 0.1% Tween-20, and centrifuged at 100,000 g for 45 min. Extracts were precleared with 300 ul Sepharose CL-6B (Sigma), then 250 ul anti-FLAG M2 affinity resin (Sigma) was added for 2 hr at 4℃. The beads were then washed in Poly-Prep columns (Bio-Rad) with 40 column volumes (CV) of buffer #1 (20 mM HEPES-KOH). [pH 7.9], 10% glycerol, 300 mM KCl, 0.1% Tween 20, 1 mM DTT, 1 mM PMSF, 2 mg/mL Leupeptin, 5 mg Aprotinin, 2 mg/mL Pepstatin, 10 mM Na-butyrate, 10 mM β-glycerophosphate, 100 mM Na-orthovanadate, 5 mM N-Ethylmaleimide, 2 mM Ortho-Phenanthroline) followed by 40 CV of buffer #2 (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 150 mM KCl, 0.1% Tween 20, 1mMDTT, 1 mM PMSF, 2 mg/mL Leupeptin, 5 mg Aprotinin, 2 mg/mL Pepstatin, 10 mM Na-butyrate, 10 mM β-glycerophosphate, 100 mM Na-orthovanadate, 5 mM N-Ethylmaleimide, 2 mM Ortho-Phenanthroline). Complexes were eluted in two fractions with 2.5 CV of buffer #2 supplemented with 200 ug/ml 3xFLAG peptide (Sigma) for 1 hr at 4℃. Next, fractions were mixed with 125 ul Strep-Tactin Sepharose (IBA) affinity matrix for 2 hr at 4°C, and the beads were washed with 20 CV of buffer #2. Complexes were eluted in two fractions with 2 CV of buffer #2 supplemented with 4 mM D-biotin, flash frozen in liquid nitrogen, and stored at -80°C.

In Vitro HAT Assays

500 ng of core histones (CH) or short oligonucleosomes (SON), or 6 µL of purified ATM fraction, were incubated with affinity-purified NuA4/TIP60 complexes harboring the different KAT5 variants and 0,125 µCi of 3H-labeled Acetyl-CoA (4,7 Ci/mmol, Perkin-Elmer) in HAT buffer (50 mM Tris-HCl ph8.0, 50 mM KCl, 5% glycerol, 0,1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 mM Sodium Butyrate) for 30 min at 30°C.

Half of the reaction was spotted on P81 filter paper, washed, and analyzed via liquid scintillation. The other half was loaded on SDS-PAGE 15% gels. Gels were Coomassie-stained to ensure homogeneous loading, then destained, fluorographed using EN3HANCE (Perkin-Elmer), dried, and exposed at -80°C. The amounts of purified TIP60/NuA4 complex used in the reactions were normalized between samples based on 3H counts on CH and Flag-KAT5 signal measured via immunoblot. All reactions were done in triplicates, and the assay was performed two times.

Detection of K52 acetylation by Acetyl-lysine IP and Mass-spectrometry analysis

Sample preparation

Affinity purification of FLAG-tagged KAT5 was performed essentially as described for the first part of KAT5 variants purification in the previous paragraph. Cells were treated with HDAC inhibitors (40ng/mL TSA, 10μM Nicotinamide) for 24h and with 50μM Etoposide for 1h before extracting nuclei. After incubation of the extracts with anti-FLAG M2 resin, beads were washed three times with 50mM Ammonium Bicarbonate [pH 8], then resuspended in 200μL of 50mM Ammonium Bicarbonate [ph8] with 2μg of Trypsin and incubated overnight at 37°C. Digestion was boosted by adding 2μg of Trypsin and incubating for three more hours at 37°C. Beads were pelleted by spinning for 3min at 1000g and the supernatant was collected. Beads were washed two times with 200μL of HPLC-grade H2O, the supernatants were pooled with the one previously collected. Digestion was stopped by adding 2% Formic acid, samples were dried by 3h of vacuum at 30°C, flash-frozen and stored at -80°C.

The dry samples were resuspended in 400 μL of Kac IP buffer (50mM MOPS pH7.4, 10mM NaPO4, 50mM NaCl) by vortexing for 5-10 seconds and keeping the samples on ice. For each sample, 50 μL of anti-Kac beads (ImmuneChem, product #ICP0388) were aliquoted into a 1.7 mL tube and washed three times with Kac IP buffer. The peptides were then added to the beads and incubated overnight at 4°C on a nutator. The next morning, the samples were gently centrifuged and the unbound fraction was transferred to a fresh tube (Flowthrough fraction). The beads were then washed

with 1 mL of Kac IP buffer then with 1mL of 20 mM Tris-HCl pH8 2 mM $CaCl_2$. The peptides were then eluted with 1 mL of 0.5 %TFA by incubating the beads at room temperature for 20 minutes on a nutator. The samples were then gently centrifuged, the supernatant transferred to a fresh tube and speedvac to dryness (Bound fraction). Samples were desalted prior to mass spec analysis.

Mass-spectrometry analysis

Dry bound and flowthrough samples were resuspended in 5 % formic acid in water and half of each sample directly loaded at 400 nL/min onto an equilibrated HPLC column. The peptides were eluted from the column by a gradient generated by a NanoLC-Ultra 1D plus (Eksigent, Dublin CA) nano-pump and analyzed on a LTQ-Orbitrap Velos (Thermo Electron) equipped with a nanoelectrospray ion source (Proxeon, Thermo Scientific). The LTQ-Orbitrap Elite instrument under Xcalibur 2.0 was operated in the data dependent mode to automatically switch between MS and up to 10 subsequent MS/MS acquisitions. Buffer A was 99.9% H2O, 0.1% formic acid; buffer B was 99.9% ACN, 0.1% formic acid. The HPLC gradient program delivered an acetonitrile gradient over 125 min. For the first 20 min, the flow rate was 400 mL/min with 2% B. The flow rate was then reduced to 200 mL/min and the fraction of solvent B increased in a linear fashion to 35% until 95.5 min. Solvent B was then increased to 80% over 5 min and maintained at that level until 107 min. The mobile phase was then reduced to 2% B until the end of the run (125 min). The parameters for DDA on the mass spectrometer were: 1 centroid MS (mass range 400–2000) followed by MS/MS on the 10 most abundant ions. General parameters were: activation type = CID, isolation width = 1 m/z, normalized collision energy = 35, activation Q = 0.25, activation time = 10 ms. The minimum threshold was 500, repeat count = 1, repeat duration = 30 s, exclusion size list = 500, exclusion duration = 30 s, exclusion mass width (by mass) = low 0.03, high 0.03.

Mass spectrometry data were stored, searched, and analyzed using the ProHits laboratory information management system (LIMS) platform (G. Liu et al., 2016). Within ProHits, AB SCIEX WIFF files were first converted to an MGF format using WIFF2MGF converter and to an mzML format using ProteoWizard (v3.0.4468) and

the AB SCIEX MS Data Converter (v.1.3 beta). Thermo Fisher scientific RAW mass spectrometry files were converted to mzML and mzXML using ProteoWizard (version 3.0.4468 - http://proteowizard.sourceforge.net/). The mzML and mzXML files were then searched using Mascot (version 2.3.02) and Comet (version 2012.02 rev.0). The spectra were searched with the RefSeq database (version 57, January 30th, 2013) acquired from NCBI against a total of 72,482 human and adenovirus sequences supplemented with common contaminants from the Max Planck Institute (http://141.61.102.106:8080/share.cgi?ssid=0f2gfuB) and the Global Proteome Machine (GPM; https://www.thegpm.org/crap/index.html). Charges of +2, +3 and +4 were allowed and the parent mass tolerance was set at 12 ppm while the fragment bin tolerance was set at 0.6 amu. Lysine acetylation, N-terminal acetylation, deamidated asparagine and glutamine and oxidized methionine were allowed as variable modifications. The results from each search engine were analyzed through the Trans-Proteomic Pipeline (version 4.6 OCCUPY rev 3) via the iProphet pipeline (Shteynberg et al., 2011).

ChIP-Seq

ChIP-seq in K562 cells expressing Flag-KAT5 were performed and analyzed as previously described (Jacquet et al., 2016; Lalonde et al., 2013). Briefly, 1 mg of cross-linked chromatin from K562 cells was incubated with 10 µg of anti-FLAG antibody (Sigma, M2) pre-bound to 300 µl of Dynabeads Prot-G (Invitrogen) overnight at 4°C. The beads were washed extensively and eluted in 0.1 % SDS, 0.1 M $NaHCO_3$. Crosslink was reversed with 0.2 M NaCl and incubation overnight at 65°C. Samples were treated with RNase and Proteinase K for 2 h and recovered by phenol chloroform and ethanol precipitation. Quantitative real-time PCRs were performed on a LightCycler 480 (Roche) with SYBR Green I (Roche) to confirm the specific enrichment at defined loci compared to intergenic regions. Librairies for sequencing were prepared as described (Avvakumov et al., 2012). Samples were sequenced by 50 bp single reads on HiSeq 2000 plateform (Illumina) and analyzed as described (Jacquet et al., 2016).

ATM activation assay

48h post-transfection with siRNAs, ATM-tagged K562 cells were treated or not with 250μM doxorubicin for 17h as indicated. Cells were then harvested, washed twice in 1× PBS (phosphate-buffered saline), and lysed for 30 min in lysis buffer (450 mM NaCl, 10% glycerol, 50 mM Tris-HCl at pH 8, 1% Triton X-100, 2 mM MgCl2, 0.1 mM ZnCl2, 2 mM EDTA, 1 mM DTT, protease inhibitors), and the same volume of lysis buffer without salt was added to a final salt concentration of 225 mM. The whole-cell extracts were incubated with FLAG-M2 agarose resin (Sigma) overnight at 4°C. The resin was centrifuged, washed in lysis buffer with 225 mM salt, and eluted with 3xFLAG peptide (Sigma). The eluted fraction was loaded onto 4%–15% gradient gels with input and immunoblotted with the appropriate antibodies.

Antibodies and immunoblotting

Standard Western blotting protocol was used, with antibodies against Flag-HRP (Sigma, A8592), total ATM (Upstate, 05-513), ATM-S1981p (Abcam, ab81292), acetyl-lysine (Cell Signalling, 9441), KAT5/Tip60 (Santa Cruz, sc166323), total KAP1 (Bethyl, A300-274A), KAP1-S824p (Bethyl, A300-767A), GAPDH (ThermoFisher, 39-8600).

1.7 Acknowledgements

We thank all members of the Côté lab for technical and conceptual support. We thank McGill Genome Center for sequencing and the Centre Hospitalier Universitaire de Québec-Université Laval proteomic platform for mass-spectrometry. This work was supported by grants from the Canadian Institutes of Health Research (CIHR; FDN-143314) to J.C., the Government of Québec, Ministry of Economy and Innovation to B.C., and the Natural Sciences and Engineering Research Council (NSERC) of Canada to J.-P.L. (RGPIN-2017-06124). K.J. was supported by a PhD studentship from Fonds de la Recherche Québec-Santé (FRQS). J.-P.L. is a Junior 2 FRQS scholars. J.C. holds the Canada Research Chair in Chromatin Biology and Molecular Epigenetics.

1.8 Supplemental Information

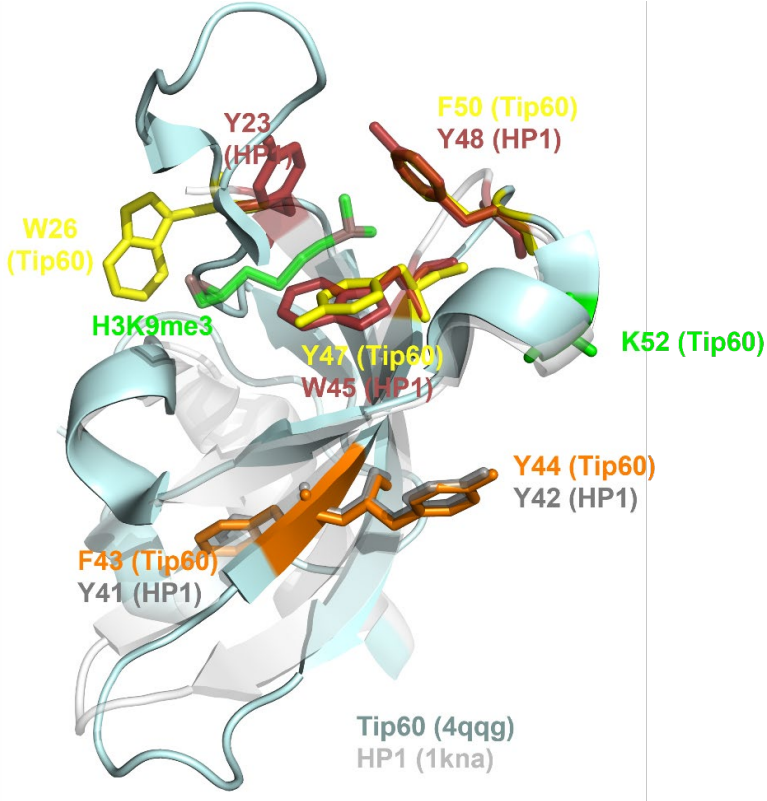

Figure 1.S1. Structure of human KAT5/Tip60 chromodomain (PDB ID 4QQG) superposed with Drosophila HP1 chromodomain (1KNA) illustrating the divergence in the disposition of the aromatic cage.

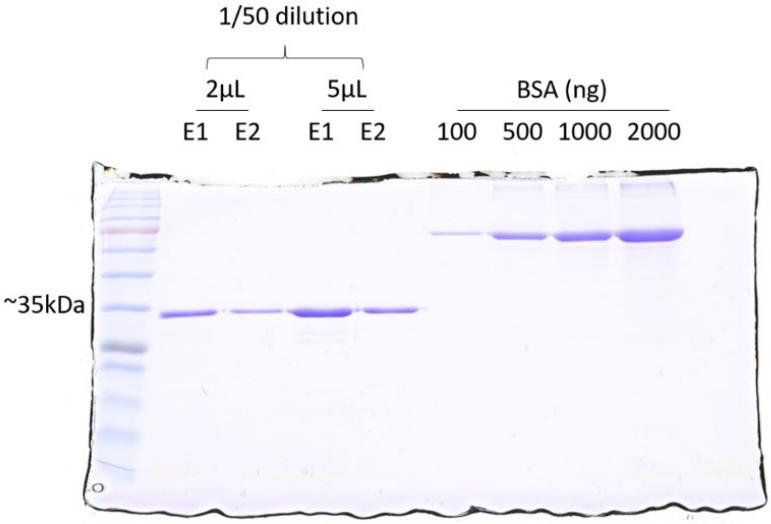

Figure 1.S2. Coomassie-stained gel of purified recombinant GST-KAT5-2-81 (lanes 1-4).

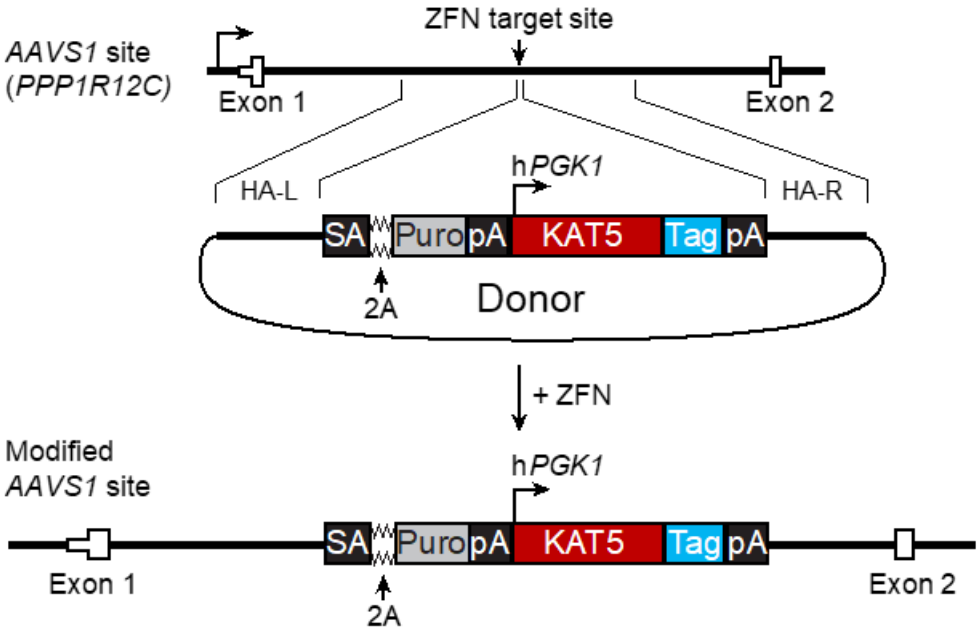

Figure 1.S3. Generation of K562 cell lines stably expressing KAT5 mutants. Schematic of the strategy used to integrate KAT5 cDNA at the AAVS1 locus. The donor construct and the AAVS1 locus following KAT5 cDNA addition are represented. The first two exons of the PPP1R12C gene are shown as open boxes. Also annotated are the locations of the splice acceptor site (SA), 2A self-cleaving peptide sequence (2A), puromycin resistance gene (Puro), polyadenylation sequence (pA), human phosphoglycerate kinase 1 promoter (hPGK1), and 3xFLAG-2xSTREP tandem affinity tag (Tag); homology arms left and right (HA-L, HA-R) are respectively 800 and 840 bp.

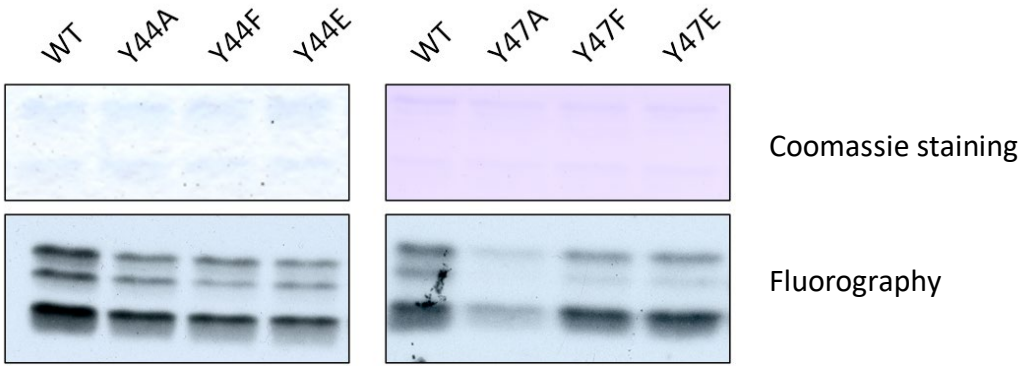

Figure 1.S4. In vitro HAT assays using indicated NuA4/TIP60 purified complexes and core histones as a substrate.

Chapitre 2: De Novo KAT5 Variants Cause a Syndrome with Recognizable Facial Dysmorphisms, Cerebellar Atrophy, Sleep Disturbance, and Epilepsy

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2.1 Résumé

KAT5, également appelée Tip60, code pour une lysine acétyltransférase essentielle impliquée dans la régulation de l'expression génique, la réparation de l'ADN, le remodelage de la chromatine, l'apoptose, et la prolifération cellulaire; mais un lien entre des variants de ce gène et des maladies génétiques reste à établir. Nous étudions ici trois individus porteurs de mutations hétérozygotes faux-sens dans KAT5 affectant des résidus normalement invariants, dont l'un se situe dans le chromodomaine (p.Arg53His) et deux proches du site de liaison de l'acétyl-CoA (p.Cys369Ser et p.Ser413Ala). Les trois individus présentent des malformations cérébrales, des convulsions, un retard développemental global ou une déficience intellectuelle, et des troubles sévères du sommeil. Une atrophie progressive du

cervelet a également été observée. Des essais d'acétylation des histones en utilisant les variants de KAT5 purifiés démontrent que ces mutations diminuent ou éliminent la capacité du complexe NuA4/TIP60 à acétyler l'histone H4 dans la chromatine. L'analyse du transcriptome de fibroblastes issus des individus affectés montrent une dérégulation de nombreux gènes contrôlant le développement. De plus, l'expression de PER1 (un gène clé pour le contrôle du cycle circadien) est augmentée, en accord avec les troubles du sommeil observée chez tous les individus. En conclusion, des variants faux-sens dominants de KAT5 causent une déficience dans l'acétylation des histones et la dérégulation de nombreux gènes, menant à un syndrome neurodéveloppemental reconnaissable avec troubles du sommeil, atrophie du cervelet, et dysmorphismes faciaux.

2.2 Abstract

KAT5 encodes an essential lysine acetyltransferase, previously called TIP60, which is involved in regulating gene expression, DNA repair, chromatin remodeling, apoptosis, and cell proliferation; but it remains unclear whether variants in this gene cause a genetic disease. Here, we study three individuals with heterozygous de novo missense variants in KAT5 that affect normally invariant residues, with one at the chromodomain (p.Arg53His) and two at or near the acetyl-CoA binding site (p.Cys369Ser and p.Ser413Ala). All three individuals have cerebral malformations, seizures, global developmental delay or intellectual disability, and severe sleep disturbance. Progressive cerebellar atrophy was also noted. Histone acetylation assays with purified variant KAT5 demonstrated that the variants decrease or abolish the ability of the resulting NuA4/TIP60 multi-subunit complexes to acetylate the histone H4 tail in chromatin. Transcriptomic analysis in affected individual fibroblasts showed deregulation of multiple genes that control development. Moreover, there was also upregulated expression of PER1 (a key gene involved in circadian control) in agreement with sleep anomalies in all of the individuals. In conclusion, dominant missense KAT5 variants cause histone acetylation deficiency with transcriptional dysregulation of multiples genes, thereby leading to a neurodevelopmental

syndrome with sleep disturbance, cerebellar atrophy, and facial dysmorphisms, and suggesting a recognizable syndrome.

2.3 Introduction

Epigenetic regulation by histone acetylation is essential for proper development, and its role in human genetic diseases is increasingly being recognized. Notably, variants in lysine acetyltransferase genes, such as KAT6A (MIM: 601408) and KAT6B (MIM: 605880), have been identified in individuals with neurodevelopmental disorders characterized by intellectual disability and malformations (Arboleda et al., 2015; P. M. Campeau et al., 2012). KAT5 (MIM: 601409) (a.k.a. TIP60) variants have not yet been associated with a syndrome. KAT5 can act as a haploinsufficient tumor suppressor gene, and it encodes an essential lysine acetyltransferase involved in gene expression, DNA repair, chromatin remodeling, apoptosis, and cell proliferation (Avvakumov & Côté, 2007; Gorrini et al., 2007). It is part of a large, multi-protein complex named NuA4 (also known as the TIP60/p400 complex), which includes TRRAP, EP400, and ING3 among other proteins (Doyon & Côté, 2004). Local recruitment of the NuA4 complex and KAT5-mediated acetylation of conserved lysine residues on histones H4 and H2A(.Z/.X) are linked to transcription activation as well as repair of DNA double-strand breaks, in part through chromatin relaxation but also through signaling and/or crosstalk with other chromatin-binding factors (Jacquet et al., 2016; Steunou et al., 2014). KAT5 can also directly acetylate non-histone proteins such as ATM in DNA damage response, p53 at lysine 120 in apoptosis, and other mitotic regulators that impact cell cycle control. KAT5-dependent acetylation of specific transcription factors can also lead to transcription activation or repression (Bararia et al., 2008; Huang et al., 2014; Xiao et al., 2003). The NuA4/TIP60 complex is essential for stem cell maintenance and renewal (Fazzio et al., 2008), and recent work revealed that KAT5 may play a role in epithelial-mesenchymal transition induction (Fukagawa et al., 2015); all of these are key processes in the developing embryo. Finally, KAT5 contributes to genome integrity by maintaining accurate chromosome alignment and segregation (Mo et al., 2016). KAT5 depletion was shown to impair the chromosomal segregation during

mitosis and to result in polyploidy (Grézy et al., 2016). We studied three individuals with de novo heterozygous missense variants in KAT5 that affect normally invariant residues. All three individuals have short stature, cerebral malformations, seizures, and global developmental delay or intellectual disability along with a significant speech disorder and a severe sleep disorder. In order to understand the molecular mechanisms underlying the phenotype in these individuals with KAT5 variants, we engineered cell lines to purify native NuA4 complexes that contain the variant catalytic subunit so that we could determine the possible effects on complex assembly/protein interactions and acetylation of chromatin substrates, and we performed transcriptomic analyses in primary cells to determine possible target genes implicated in the pathology.

2.4 Material and Methods

Recruitment and Sequencing

Apart from the published individual, other individuals were recruited through GeneMatcher.15 Information was obtained from each clinical team. Exome sequencing (ES) was performed by the National Institutes of Health (NIH) Intra-mural Sequencing Center (NISC) for individual 1 (complete method described by (Berger et al., 2017)). ES was performed on a research basis for individual 2 at University of Geneva Medical School and Geneva University Hospitals. ES was performed in a commercial laboratory for individual 3. Informed consent to publish clinical information and photographs was obtained from the parents of the individuals reported in this article. For each individual, the procedures followed were in accordance with the ethical standards of the responsible committees on human experimentation.

Cell Culture and Transfection

K562 cells were obtained from the American Type Culture Collection (ATCC) and maintained at 37°C under 5% CO2 in RPMI medium supplemented with 10% newborn calf serum (Wisent) and GlutaMAX (Thermo Fisher). When cultivated in spinner flasks, 25 mM HEPES-NaOH (pH 7.4) was added. Cells were transfected using Lipofectamine 2000 (Thermo Fisher) per the manufacturer’s instructions.

Generation of Stable Cell Lines Producing Tagged KAT5 Variants and Affinity Purification of NuA4/TIP60 Complexes

KAT5 (461aa isoform) was cloned into the AAVS1_Puro_PGK1_3xFLAG_Twin_Strep plasmid (addgene #68375), and the variants found in each individual were introduced via site-directed mutagenesis. Generation of K562 cells that expressed either wild-type (WT) or variant-tagged KAT5 was performed through break-induced recombination and/or insertion at the AAVS1 locus (MIM: 102699) as described (Dalvai et al., 2015). Nuclear cell extracts were prepared from 3.10^9 cells and used to perform tandem affinity purification as described (Doyon & Côté, 2016). In brief, nuclear extracts were adjusted to 0.1% Tween-20, and ultracentrifuged at 100,000 g for 1 h. Extracts were precleared with 250 ml Sepharose CL-6B (Sigma), then 250 ml anti-FLAG M2 affinity resin (Sigma) was added for 2 h at 4°C. The beads were then washed in Poly-Prep columns (Bio-Rad) with 40 column volumes (CV) of buffer #1 (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 300 mM KCl, 0.1% Tween 20, 1 mM DTT, 1 mM PMSF, 2 mg/mL Leupeptin, 5 mg Aprotinin, 2 mg/mL Pepstatin, 10 mM Na-butyrate, 10 mM b-glycerophosphate, 100 mM Sodium Orthovanadate, 5 mM NEthylmaleimide, 2 mM Ortho-Phenanthroline) followed by 40 CV of buffer #2 (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 150 mM KCl, 0.1% Tween 20, 1mMDTT, 1 mM PMSF, 2 mg/mL Leupeptin, 5 mg Aprotinin, 2 mg/mL Pepstatin, 10 mM Na-butyrate, 10 mM b-glycerophosphate, 100 mM Sodium Orthovanadate, 5 mM N-Ethylmaleimide, 2 mM Ortho-Phenanthroline). Complexes were eluted in two fractions with 2.5 CV of buffer #2 supplemented with 200 ug/mL 3xFLAG peptide (Sigma) for 1 h at 4°C. Typically, 15 ul of the first elution (3% of total) was loaded on NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and analyzed via silver staining.

In Vitro HAT Assays

1 µg of core histones (CH) or short oligonucleosomes (SON) was incubated with affinity-purified NuA4/TIP60 complexes harboring the different KAT5 variants and 3H-labeled acetylCoA (0,1 µCi, Perkin-Elmer) in HAT buffer (50 mM Tris-HCl ph8.0, 50 mM KCl, 5% glycerol, 0,1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 mM Sodium Butyrate) for 30 min at 30°C. Half of the reaction was spotted on P81 filter paper,

washed, and analyzed via liquid scintillation. The other half was loaded on SDS-PAGE 15% gels. Gels were Coomassie-stained to ensure homogeneous loading, then destained, fluorographed using EN^{3}HANCE (Perkin-Elmer), dried, and exposed at -80°C. The amounts of purified TIP60/NuA4 complex used in the reactions were normalized between samples based on 3H counts on CH and Flag-KAT5 signal measured via immunoblot. All reactions were done in triplicates, and the assay was performed two times.

RNaseq Methods

RNA libraries were prepared from low-passage fibroblasts from individuals 2 and 3 through the use of the Illumina RNA Truseq V2 and Truseq mRNA stranded kits, respectively. The libraries were then sequenced on an Illumina HiSeq4000 sequencer at 2 3 100bp and 1 3 100bp read lengths, respectively. Two and four healthy control low-passage fibroblasts were also sequenced from Truseq V2 and Truseq mRNA stranded libraries at 2 3 100bp and 1 3 100bp read lengths, respectively. Transcriptomics analyses were performed as previously described (Cogné et al., 2019). Common differentially expressed genes were selected by using the DESeq2 R package to compare the affected individuals to the respective healthy controls with thresholds at $|Log2FC| > 0.5$, 5% FDR, and adjusted p value < 0.05.

RT-qPCR

Total RNA from affected individuals and three new controls was isolated from low-passage fibroblasts through the use of the PureLink RNA mini kit (Life Technologies). Controls were male children of White, African American, and Asian backgrounds. Equal amounts of RNA were used to synthesize cDNA through the use of the qScript cDNA synthesis kit (Quanta Biosciences). cDNA was quantified through the use of PowerUp SYBR green Master Mix (Applied Biosystems) on a LightCycler® 96 system (Roche) using primers listed in Table 2.S3. Amplicons were resolved by using agarose gel to determine the size. Relative gene expression levels were analyzed via $2^{-\Delta\Delta CT}$ method with b-actin used as the reference gene. Statistical significance was determined via two-way ANOVA with Dunnett's multiple comparisons test. Variation was reported as standard deviation (SD).

2.5 Results

Clinical Descriptions

Individual 1 is a 30-year-old female with intellectual disability who has been reported by Berger et al. in a Smith-Magenis Syndrome (SMS)-like cohort (Berger et al., 2017). She presented with behavioral problems with perseverative speech, poor language function, and sleep disorder. At age 10, she had disruptive behaviors and a diagnosis of attention deficit disorder. At age 29, she had an IQ of 40 with expressive language at the 8-year-old level and receptive language at 4-to-5-year-old skill level. Her head circumference is 55cm (73rd centile). She also presents with adult-onset seizures, severe myopia, hyperacusis, kyphoscoliosis, brachydactyly, and frequent urinary tract infections. Her facial dysmorphisms include a round face with a flat facial profile, prognathism, down-slanting corners of the mouth, low-set ears, depressed nasal bridge, and almond-shaped eyes. She has partial agenesis of the corpus callosum. She developed secondary amenorrhea at 29 years of age. Sleep problems present since early childhood included early sleep offset, nighttime awakenings (1–2), and increased daytime naps. In adulthood, sleep diaries document 24 h sleep cycle characterized by early morning awakening (between 05:30–06:30), bedtime at 20:30, two daytime naps (09:30–10:30 and 13:00–14:30), and nocturnal awakenings (~30 min long) after sleep onset, usually occurring at 23:00 and 01:00. Increased daytime salivary melatonin level was documented at 11:15 (mean 46 pg/mL for two samples), which is consistent with the inverted circadian melatonin profile observed in SMS (Chik et al., 2010). ES identified a de novo missense variant c.158G>A (p.Arg53His) in KAT5 (RefSeq accession number NM_006388.3). No other variants met the filtering exome criteria. Individual 2 is a 13-year-old male with intellectual disability and multiple malformations. He was born at 38 weeks with a unilateral cleft lip and palate. At 12 years of age, he is nonverbal. and a cognitive evaluation documented an IQ of 20–30. He has disruptive behavior with hyperactivity and multiple stereotypies. He suffers from generalized tonic-clonic seizures and has severe sleep disorder (with sleep onset delay and night waking). His head circumference was 50 cm (1st centile, -2.6 SD). Facial dysmorphisms include prognathism, lateral thinning of the eyebrows, macrostomia, thick lower lip,

and bulbous and asymmetric nose. He also has bilateral single palmar creases and fifth finger clinodactyly, as well as unilateral cryptorchidism. Horseshoe kidney and bilateral vesico-ureteral reflux were diagnosed during childhood. Brain MRI showed global progressive cerebellar atrophy (vermis more than hemispheres), dysgenesis of corpus callosum (short, thickened, and hypoplasia of rostrum and splenium), and a small anterior pituitary gland. He suffers from growth hormone (GH) deficiency diagnosed at the age of 2 years and for which he is treated with GH injections. ES identified a de novo missense variant c.1105T>A (p.Cys369Ser) in KAT5 (RefSeq NM_006388.3). Individual 3 is a 2-year-old male with developmental delay and multiple malformations. At 16 months of age, he presented with short stature and congenital microcephaly, height 71.1 cm (<1st centile, -2.9 SD), weight 11.2 kg (48th centile), and head circumference 44.5 cm (<1st centile, -2.2 SD). He has severe developmental delay with disruptive behaviors and an important sleep disorder (night waking and sleep onset delay which was improved by nighttime clonidine, which was prescribed because daytime clonidine caused daytime sleepiness). He suffers from generalized myoclonic seizures. He has a perimembranous ventricular septal defect and a dysplastic pulmonary valve with supravalvular and valvular stenosis. He also has a high-arched palate with a submucous cleft. His genitourinary anomalies consist of hypospadias and bilateral cryptorchidism. His facial dysmorphisms include a round face with a flat facial profile, epiblepharon, epicanthal folds, down-slanting corners of the mouth, and upturned nose with depressed nasal bridge. He also has bilateral fifth finger clinodactyly. His medication includes risperidone and clonidine. Brain MRI showed polymicrogyria of right sylvian fissure, cystic dilation of 4th ventricle, and inferior cerebellar vermis atrophy. ES identified a de novo missense variant c.1237T>G (p.Ser413Ala) in KAT5 (RefSeq NM_006388.3). Additional clinical information is available for these three individuals in Tables 2.S1 and 2.S2 (for comparison of sleep disorder characteristics).

Analysis of the Variants

We named the variants through the use of isoform NM_006388.3 (513 amino acids) because it is highly expressed and is the canonical isoform in Uniprot (Consortium, 2019). However, the longest isoform is NM_182710.2 (546 amino acids), and a

commonly studied isoform is NM_182709.2 (also known as PLIP, 461 amino acids). All three variants are absent from the Genome Aggregation Database (gnomAD) (Lek et al., 2016), and this absence indicates that these variants are not present in the more than 100,000 individuals from population genetic studies included in this database. As shown in Figure 2.1B, the p.Arg53His variant is in the chromodomain, following an acetylated lysine residue. In addition to potentially affecting KAT5's ability to interact with histones via its chromodomain, the p.Arg53His variant may disrupt the protein's structure and thus stability (as suggested by STRUM analysis (Quan et al., 2016), and by the protein yields in K562 cell extracts shown in Figure 2.S1). The p.Cys369Ser variant is near the Acetyl-CoA binding domain, and this residue has been shown to be critical for the catalysis of yeast Esa1 (KAT5 ortholog) and other MYST-family acetyltransferases (Yan et al., 2002), as well as for KAT5 autoacetylation (Yang et al., 2012). Finally, the p.Ser413Ala variant is in the Acetyl-CoA binding domain. All residues are invariant throughout evolution, and in fact, they are conserved down to yeast Esa1 (Figure 2.1C). An analysis of the 3D structure of KAT5 (Figure 2.2A) suggests that the p.Cys369Ser and p.Ser413Ala variants may alter the interaction of the protein with Acetyl-CoA. This was also suggested by 3D mutation impact analysis using HOPE and VarSite (Laskowski et al., 2020; Venselaar et al., 2010).

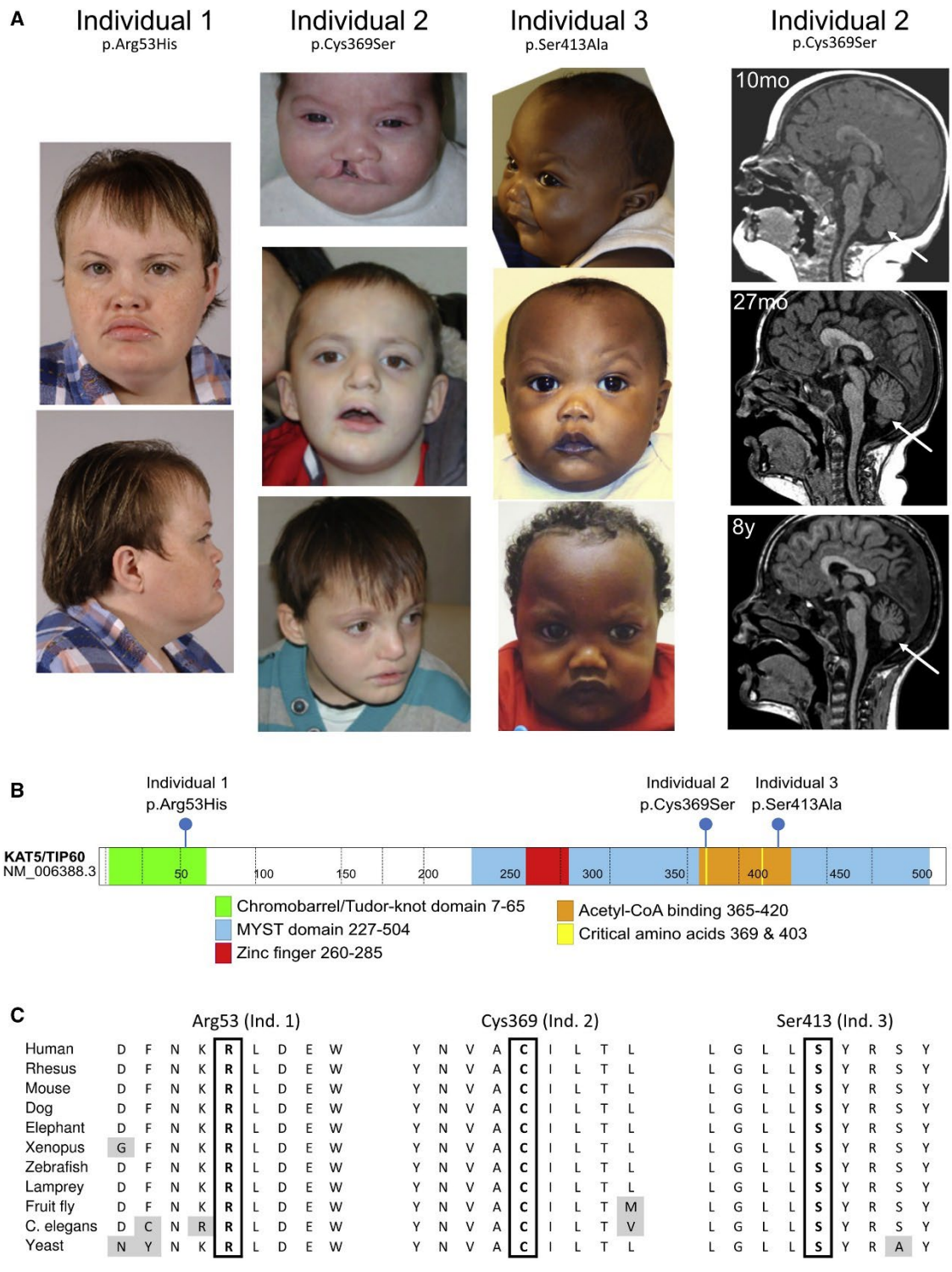

Figure 2.1. Clinical Images and Variant Details (A) Photographs of the three individuals, showing shared facial dysmorphisms. Individual 1 and individual 3 have round faces, flat facial profiles, down-slanting corners of their mouths, and depressed nasal bridges. Individual 1 and individual 2 have prognathism. The images on the right are sagittal MRI images for individual 2 at the indicated ages, showing progressive cerebellar atrophy

(arrow). (B) Variant location in functional domains of the KAT5 protein. (C) Affected amino acids are invariant between different species.

Expected versus observed counting of single-nucleotide changes in gnomAD show that KAT5 is only moderately intolerant to loss-of-function (LoF) variants (pLI score [probability of being loss of function intolerant] 0.09; observed/expected [o/e] ratio 0.26 [90% confidence interval (CI): 0.15–0.47]) (Lek et al., 2016). Moreover, fewer missense variants were observed than were expected (o/e ratio 0.44 [90% CI: 0.39-0.51] with a Z score of 3.61) (gnomAD v2.1.1). Regarding other assessments of KAT5 as a gene potentially associated with a dominant disease, the %HI score (haploinsufficiency score from DECIPHER) is 4.47%. %HI scores below 10% indicate that a gene is more likely to be deleterious if haploinsufficient (Huang et al., 2010). The KAT5 P(AD) score is 0.996 (probability for a gene to carry dominant mutations from the DOMINO website, accessed June 2, 2020). A P(AD) score of 0.95 is highly associated with autosomal dominant inheritance through haploinsufficiency, gain of function, or dominant-negative effects (Quinodoz et al., 2017). An analysis of the affected residues performed through the use of Metadome and the MTR Gene Viewer suggested that all affected residues are intolerant to variations (Traynelis et al., 2017; Wiel et al., 2019). Moreover, most pathogenicity prediction tools we used considered the variants to be likely pathogenic. That was the case for DANN(Quang et al., 2015), DEOGEN2, EIGEN, FATHMM-MKL, M-CAP, MutationAssessor, MutationTaster, and SIFT (scores from the dbNSFP (X. Liu et al., 2016) database except for DANN and analyzed through the Varsome website (Kopanos et al., 2019)). The variants had CADD scores of 32, 27, and 26 respectively (scores 20 or above indicate that they are among the 1% most likely pathogenic variants in the genome) (Rentzsch et al., 2019). The variants were also considered to be deleterious according to results from Rhapsody and MutPred2 (Pejaver et al., 2020; Ponzoni et al., 2020).

Table 2.1 Main Clinical Features

Individuals	1	2	3
KAT5 variants (RefSeq NM_006388.3)	c.158G>A (p.Arg53His)	c.1105T>A (p.Cys369Ser)	c.1237T>G (p.Ser413Ala)
Chromosomal positions (hg19)	Chr11:65480402G>A	Chr11:65484393T>A	Chr11:65486132T>G
Age and gender	29-year-old female	13-year-old male	18-month-old male
Microcephaly	-	+	+
Developmental delay or intellectual disability	+, IQ 40	+, IQ 20–30	+, severe
Behavioral issues	ADHD, sleep disorder, disruptive behavior	ADHD, severe sleep disorder, multiple stereotypies and disruptive behavior	behavioral difficulties with tantrums and head banging
Seizures	+	+	+
Cerebral malformations	partial agenesis of the corpus callosum	corpus callosum dysgenesis, cerebellar atrophy	focal polymicrogyria, cerebellar atrophy
Urogenital anomalies	recurrent urinary tract infections	horseshoe kidney, vesico-ureteral reflux, cryptorchidism	hypospadias, cryptorchidism
Congenital heart defect	-	-	VSD, dysplastic pulmonary valve
Orofacial malformations	-	unilateral cleft lip and palate	submucous cleft palate
Ocular anomaly	severe myopia	strabismus and hypermetropia	epiblepharon
Dysmorphisms	round face, flat facial profile; down-slanting corners of mouth; depressed nasal bridge; prognathism; low-set ears; almond-shaped eyes	lateral thinning of eyebrows, macrostomia, bulbous and asymmetric nose, thick lower lip, prominent chin	round face, flat facial profile; down-slanting corners of mouth

Purification and Biochemical Analysis of KAT5 Variants

In order to determine the effect of the variants on KAT5 protein interactome and enzymatic activity, we used genome editing to introduce WT and mutant KAT5 cDNAs at the safe harbor AAVS1 locus in human K562 cells (Dalvai et al., 2015). Equivalent accumulation of the C-terminally tagged proteins (3xFlag-2xStrep) was measured and clones were selected (Figure 2.S2). Production in these clones is near physiological levels compared to endogenous KAT5. Native NuA4/TIP60 complexes were then obtained via tandem affinity purification (Doyon & Côté, 2016). Analysis of the purified fractions through the use of protein gel, immunoblotting, and mass spectrometry showed that WT and variant KAT5 normally assemble into full stoichiometric NuA4/TIP60 complexes (Figure 2.2B). Then, the histone acetyltransferase (HAT) activity of the different complexes was measured in vitro with 3H-Acetyl-CoA using free CH or native chromatin (SON) as substrates. All variants displayed impaired HAT activity to varying degrees compared to WT KAT5 (Figures 2.2C–D). The p.Cys369Ser variant showed the most dramatic effect, being unable to acetylate both free histones and chromatin, as expected based on its localization in the catalytic site. On the other hand, the complexes containing KAT5

p.Arg53His and p.Ser413Ala variants are mostly defective in their HAT activity toward chromatin, not free histones (Figure 2.2C). Strikingly, as shown through the use of gel fluorography (Figure 2.2D), this defect is clearly more specific toward nucleosomal histone H4 tail acetylation, whereas H2A acetylation is still detected. Altogether, these data clearly demonstrate that the de novo variants detected in the individuals described above cripple the lysine acetyltransferase activity of KAT5, leading to partial loss of function in vivo and impairing the ability of the NuA4/TIP60 complex to properly acetylate its targets in a chromatin context. Based on KAT5's critical role in genome expression and maintenance, control of cell proliferation, and development, these variants are likely implicated in the neurodevelopmental defects seen in these individuals.

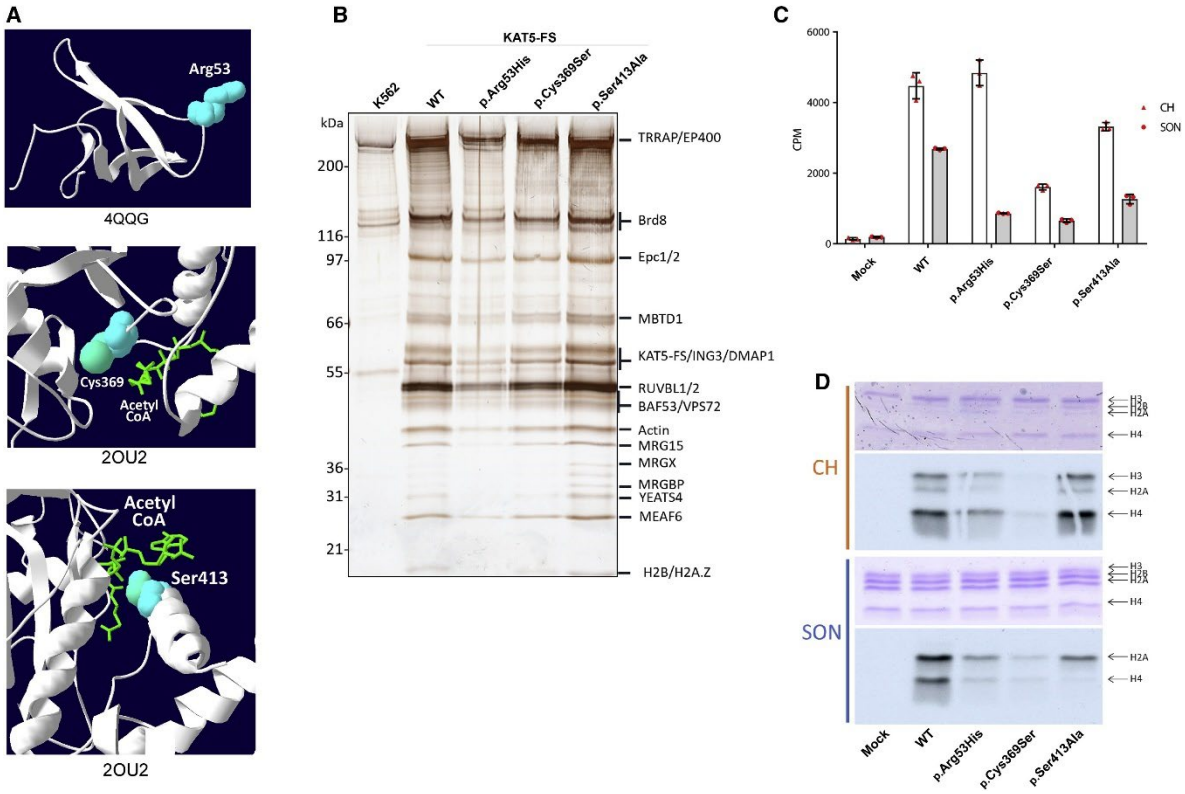

Figure 2.2. Functional Impact of KAT5 Variants on the Native NuA4/TIP60 Acetyltransferase Complex (A) Predicted variant location in 3D reconstruction of KAT5 protein. Annotations below the images refer to the RCSB PBD (Research Collaboratory for Structural Bioinformatics Protein Data Bank) structure IDs. (B) Variant KAT5 proteins assemble in normal NuA4/TIP60 complexes. WT and variant KAT5 proteins were fractionated from nuclear extracts via tandem affinity purification. Purified fractions were loaded on gel and stained with silver. Bone fide NuA4/TIP60 subunits are identified on the

right. Note: the isoform used for experiments was the commonly used 461-amino-acid isoform (RefSeq NM_182709.2), and thus variants are at positions 53, 317, and 361 in that protein, but for all figures, they were identified with the canonical isoform RefSeq NM_006388.3 numbering for consistency with the rest of the manuscript. (C) In vitro histone acetylation assay performed with purified native WT and variant complexes. The graph shows the scintillation counts of the liquid assays with 3H-Acetyl-CoA with free core histones (CH) or native short oligonucleosomes (SON). Error bars represent standard deviations of technical replicates. (D) Fluorograph of in vitro histone acetylation assays with native WT and variant complexes. Protein gels were treated with En3Hance, dried, and exposed on film to assess 3H-labeled protein bands and/or acetylation in order to visualize the effect on specific histones (lower panels). Coomassie stained gels are shown to control relative substrate amounts in the reactions (upper panels).

Transcriptomic Analyses

We performed an analysis of dysregulated genes in primary human fibroblasts from individuals 2 and 3, as described previously (Cogné et al., 2019). From the RNaseq data (cutoff Log2FC of >0.5 or <-0.5, p » 0.05 [Figure 2.3A]), we selected genes which were involved in development, neuronal function, and circadian rhythm control, and we performed qPCR analysis with additional controls (Figure 2.3B and Figure 2.S2). Genes which showed consistent downregulations were LHX9 (MIM: 606066) and KIRREL3 (MIM: 607761). Genes which showed consistent upregulations were GFPT2 (MIM: 603865), PER1 (MIM: 602260), and HDAC4 (MIM: 605314).

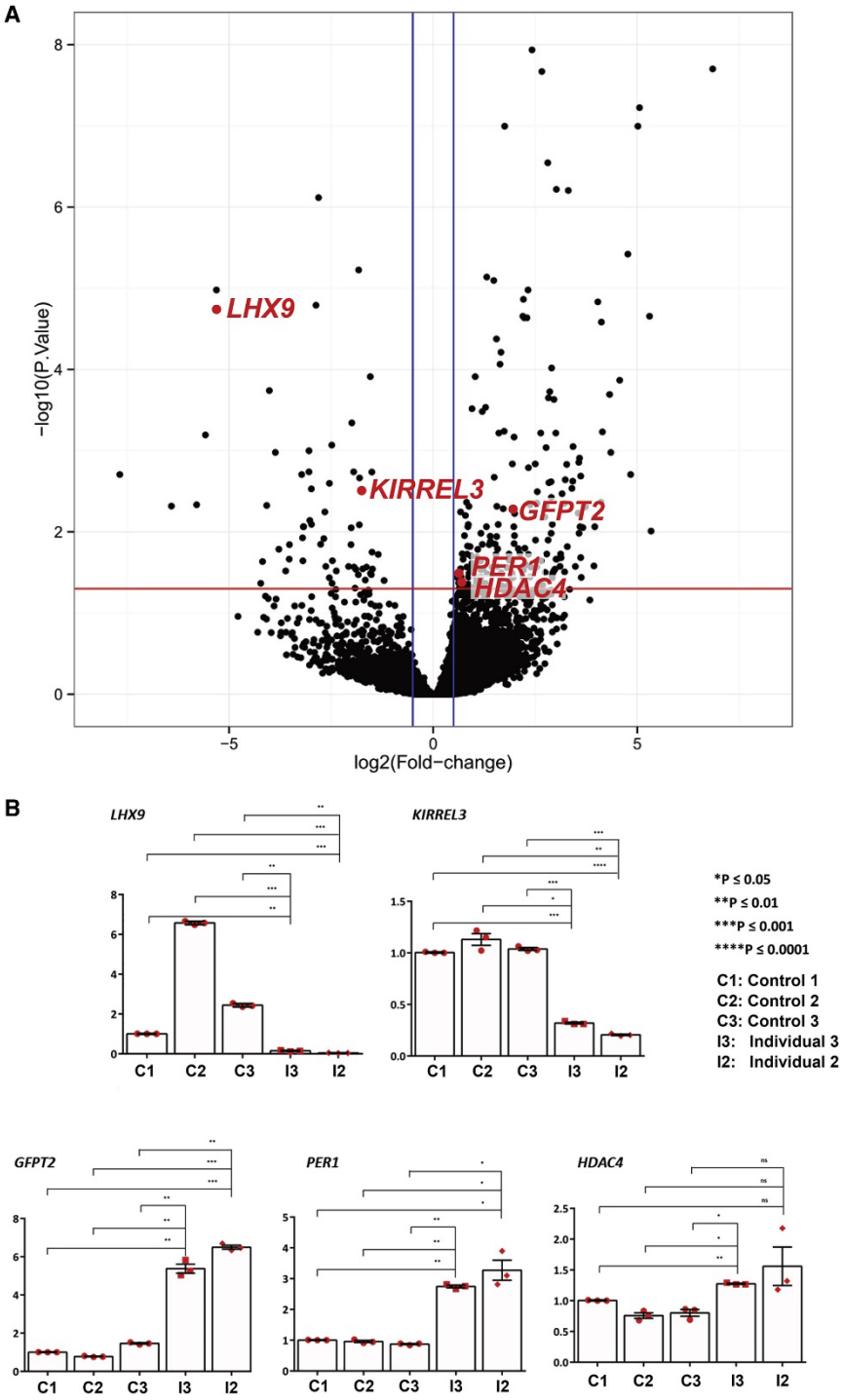

Figure 2.3. RNaseq Was Performed on Fibroblasts from Individuals 2 and 3 and Six Healthy Controls (A) Volcano plot showing common DEGs (differentially expressed genes) of individuals 2 and 3. Significant DEGs. The red line indicates a -log10 (adjusted p value) of 1.3 (padj of 0.05); and the blue line a Log2 Fold Change of -0.05 and 0.05. Significant DEGs shown in panel B are represented by red dots. (B) Reverse transcriptase-qPCR analysis of specific genes deregulated in fibroblasts from three new controls and from individuals 2 and 3. b-actin was used as the reference gene. Triplicates were used. Error

bars represent standard deviation. p values were generated through the use of two-way ANOVA with Dunnett's multiple comparisons test.

2.6 Discussion

As shown in Table 2.1, Table 2.S1, and Figure 2.1A, the three affected individuals share many features. They are moderately short (-1.95 SD, -2.1 SD, and -2.9 SD) and two have microcephaly (-2.6 SD and -2.2 SD). They have severe developmental delay or moderate-to-severe intellectual disability. All three individuals present with disruptive behavior and have severe sleep disorders. All have night waking, and individuals 2 and 3 have sleep onset delay (improved by clonidine for individual 3), and individual 1 has daytime sleepiness. Individuals 1 and 2 have anomalies of the corpus callosum, individuals 2 and 3 have cerebellar atrophy, and individual 3 has focal polymicrogyria. Facial dysmorphisms, each present in at least two individuals, include a round face with a flat facial profile, a depressed nasal bridge, downturned corners of the mouth, and prognathism. Individual 2 has a cleft lip and palate, and individual 3 has a submucous cleft palate. All three individuals have genitourinary anomalies, including cryptorchidism, hypospadias, horseshoe kidney, and vesico-ureteral reflux. Although there is some clinical overlap between the individuals we studied and individuals with SMS, such as sleep disturbances and some facial dysmorphisms, there are also several differences, such as the seizures and genitourinary anomalies seen in all individuals here, which are present in only a minority of individuals with SMS. Progressive cerebellar atrophy and CNS malformations are not observed in SMS, whereas dental anomalies and broad hands are common in SMS and are not observed here, among other differences.

KAT5 variants cause histone acetylation deficiency and gene expression deregulation, and thereby lead to a neurodevelopmental syndrome with facial dysmorphisms, various malformations, and sleep disturbances. Berger et al. (Berger et al., 2017) had already reported individual 1 in 2017 and had then proposed KAT5 as a candidate gene to explain the phenotype. The addition of two new individuals with overlapping phenotypes and having a KAT5 variant confirms the involvement of KAT5 in human diseases. The observed deficient histone acetylation by biochemical assays using native KAT5 complexes suggests an LoF mechanism during

development. Mice heterozygous for a knockout Kat5 allele have normal development, growth, and fertility in the literature (Gao et al., 2014; Gorrini et al., 2007; Hu et al., 2009). Heterozygous mice phenotyped by the International Mouse Phenotyping Consortium (IMPC) are also essentially normal (IMPC website accessed June 3, 2020) (Dickinson et al., 2016). Homozygous knockout mice are embryonic lethal both in the literature and the IMPC study. It is possible that in humans, haploinsufficiency for KAT5 does not lead to a syndrome, and it is even likely given that over 10 high-confidence LoF variants are found in gnomAD, but that missense variants abrogating KAT5 activity might cause a dominant deleterious effect. Moreover, regulatory mechanisms may lead to near-normal KAT5 protein levels in case of haploinsufficiency. This was observed in adipose tissue of Kat5 haploinsufficient mice, in which Kat5 mRNA was reduced to 50%, but protein levels were normal, and similar observations were also made in different tissues in other studies (Fisher et al., 2012; Gao et al., 2014; Gehrking et al., 2011). We hypothesize that NuA4 complexes with inactive KAT5 have widespread epigenetic consequences (as suggested by our transcriptomic studies), whereas the presence of slightly fewer NuA4 complexes does not, but future studies, ideally in vivo, will be required to assess this hypothesis. LHX9 and KIRREL3 were consistently downregulated in primary fibroblasts. LHX9 is important for thalamic neuronal differentiation (Peukert et al., 2011). Knockout mice have profound hypersomnolence, likely because Lhx9 may be important for specification or survival of a subset of hypocretin-containing neurons of the hypothalamus that are essential for the normal regulation of sleep (Dalal et al., 2013). LHX9 is downregulated in individuals with Pallister-Kallian syndrome, a neurodevelopmental disorder (Kaur et al., 2014). KIRREL3 is an IgSF-adhesion molecule implicated in synapse formation, synaptic transmission, and ultrastructure. It regulates mossy fiber synapse development in the hippocampus (Martin et al., 2015) and has been implicated in neurodevelopmental disorders (Conrad et al., 2019; Kalsner et al., 2018). GFPT2, PER1, and HDAC4 were consistently upregulated in primary fibroblasts. GFPT2 controls the flux of glucose into the hexosamine pathway involved in protein glycosylation. An individual with severe intellectual disability was reported with a de novo missense variant in this

gene (Gilissen et al., 2014). PER1 is a key component of the circadian clock and acts as a transcriptional repressor (Franken & Dijk, 2009). HDAC4 is a histone deacetylase which binds promoters through transcription factors MEF2C and MEF2D and represses transcription. Its deletion causes cognitive and behavioral issues often associated with brachydactyly (Le et al., 2019). Interestingly, genome-wide location analysis of the NuA4/TIP60 complex in human K562 cells previously reported its presence on the PER1 and HDAC4 genes (see Figure 2.S3).7 Importantly, KAT5 has been shown to be critical for learning and memory in Drosophila (Schmidt & Sheeley, 2015; Xu & Elefant, 2015; Xu et al., 2014) and has also been shown to control sleep in Drosophila by regulating axonal growth in pacemaker cells (Pirooznia et al., 2012). In addition, mammalian KAT5 has recently been reported to be an important regulator of the circadian clock cycle through direct action at gene promoters and BMAL1 acetylation (Petkau et al., 2019). Moreover, another HAT, ELP3, has also been associated with sleep anomalies in Drosophila (Singh et al., 2010), and sleep deprivation induces the expression of Hdac2 in rat hippocampi (Duan et al., 2016). The role of epigenetics in the regulation of sleep has been reviewed by Quershi and Mehler in 2014 (Qureshi & Mehler, 2014). Sleep disturbances not associated with sleep apnea are also seen in diseases caused by variants in epigenetic regulators. Dominant variants in or deletions of the histone deacetylase HDAC4 have been implicated in the pathophysiology of chromosome 2q37 deletion syndrome (MIM: 600430), in which there is a sleep disturbance, and lead to reduced expression of RAI1 (MIM: 607642), a gene for which variants cause the overlapping SMS (MIM: 182290) (Williams et al., 2010). SMS due to deletions of 17p11.2 or RAI1 variants is associated with a recognized circadian sleep disorder characterized by an advanced sleep phase and inverted melatonin secretion profile (De Leersnyder et al., 2001; Smith et al., 2019). Autosomal dominant mental retardation type 1 (MIM: 156200) is caused by variants in MBD5 (MIM: 611472) that encode Methyl-CpG-binding domain protein 5, which is part of a polycomb repressive complex that deubiquitinates a lysine of histone H2A. Interestingly, disturbed PER1 levels were noted with both MBD5 mutations and with SMS (Mullegama et al., 2015; Novakova et al., 2012). Diseases caused by mutations in

other epigenetic regulators are associated with sleep disturbances (KDM5B [MIM: 605393] (Martin et al., 2018), MECP2 [MIM: 300005] (Della Ragione et al., 2016), EHMT1 [MIM: 607001], KMT2C [MIM: 606833], and HDAC8 [MIM: 300269] (Deardorff et al., 2012)), as well as several other genetic diseases (Ansar et al., 2019; Chen et al., 2015; Gadoth & Oksenberg, 2014; Tietze et al., 2012; Trickett et al., 2018). Other HATs associated with Mendelian disorders are KAT6A and KAT6B. KAT6A variants cause autosomal dominant mental retardation 32 (MIM: 616268), and overlapping features with the syndrome described here include intellectual disability, microcephaly, epilepsy, and sleep disturbances (Kennedy et al., 2019). KAT6B variants cause Genitopatellar syndrome (MIM: 606170) and Say-Barber-Biesecker-Young-Simpson (SBBYS) syndrome (MIM: 603736). Overlapping features with both of these syndromes include intellectual disability, microcephaly, and genital anomalies. Specifically, corpus callosum anomalies (for Genitopatellar syndrome) and cleft palate (for SBBYS syndrome) are overlapping features with the syndrome described here (Philippe M Campeau et al., 2012). It will be interesting in the future to determine whether similar pathways are dysregulated in neuronal models of the various epigenetic disorders associated with sleep disturbances mentioned above, as this could perhaps lead to the development of better targeted therapies for such symptoms.

2.7 Data and Code Availability

RNaseq data are available on the National Center for Biotechnology Information (NCBI)’s Gene Expression Omnibus with accession number GSE154199.

2.8 Supplemental Data

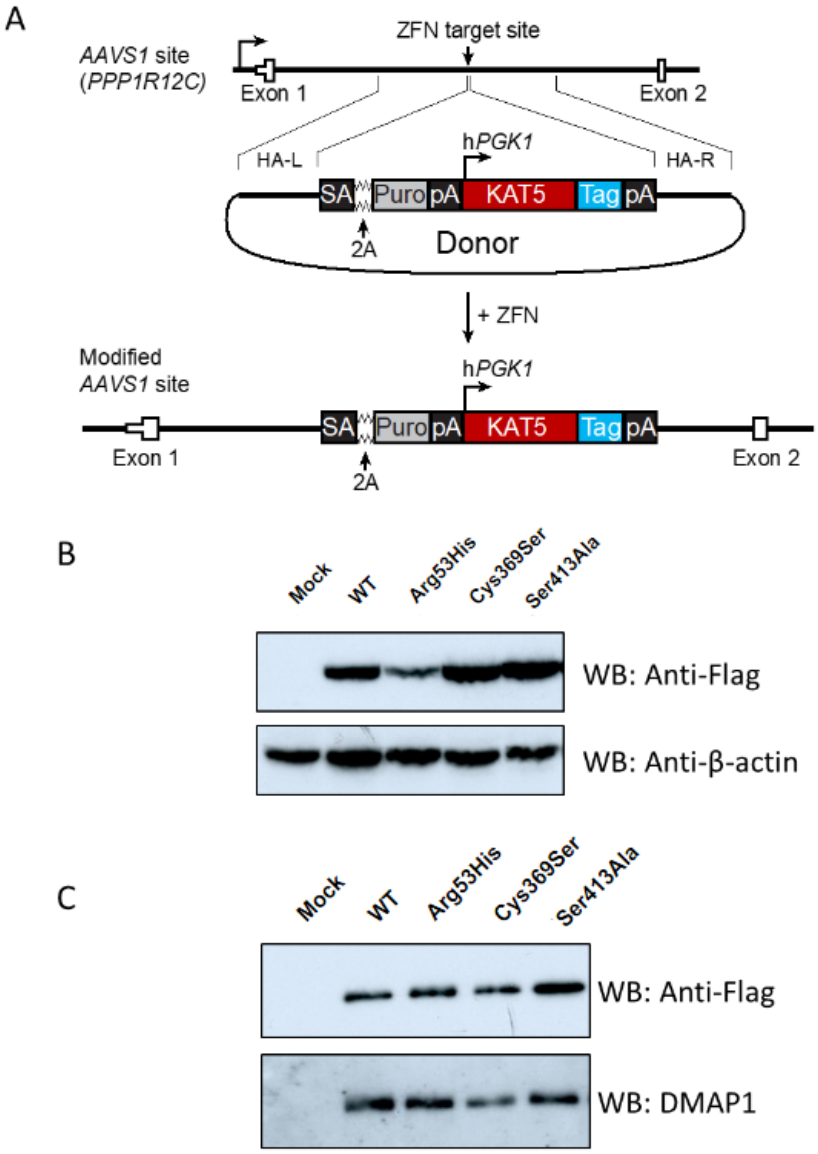

Figure 2.S1. Generation of K562 cell lines stably expressing KAT5 mutants.
A. Schematic of the strategy used to integrate KAT5 cDNA at the AAVS1 locus. The donor construct and the AAVS1 locus following KAT5 cDNA addition are represented. The first two exons of the PPP1R12C gene are shown as open boxes. Also annotated are the locations of the splice acceptor site (SA), 2A self-cleaving peptide sequence (2A), puromycin resistance gene (Puro), polyadenylation sequence(pA), human phosphoglycerate kinase 1 promoter (hPGK1), and 3xFLAG-2xSTREP tandem affinity tag (Tag); homology arms left and right (HA-L, HA-R) are respectively 800 and 840 bp.
B. Western blots showing flag-tagged KAT5 expression in whole cell extracts from selected K562 clones. Actin was used as a loading control.
C. Western blots on purified native NuA4/TIP60 complexes showing the amount of the complex subunits DMAP1 and Flag-tagged KAT5 present in HAT assays shown in Fig. 2.

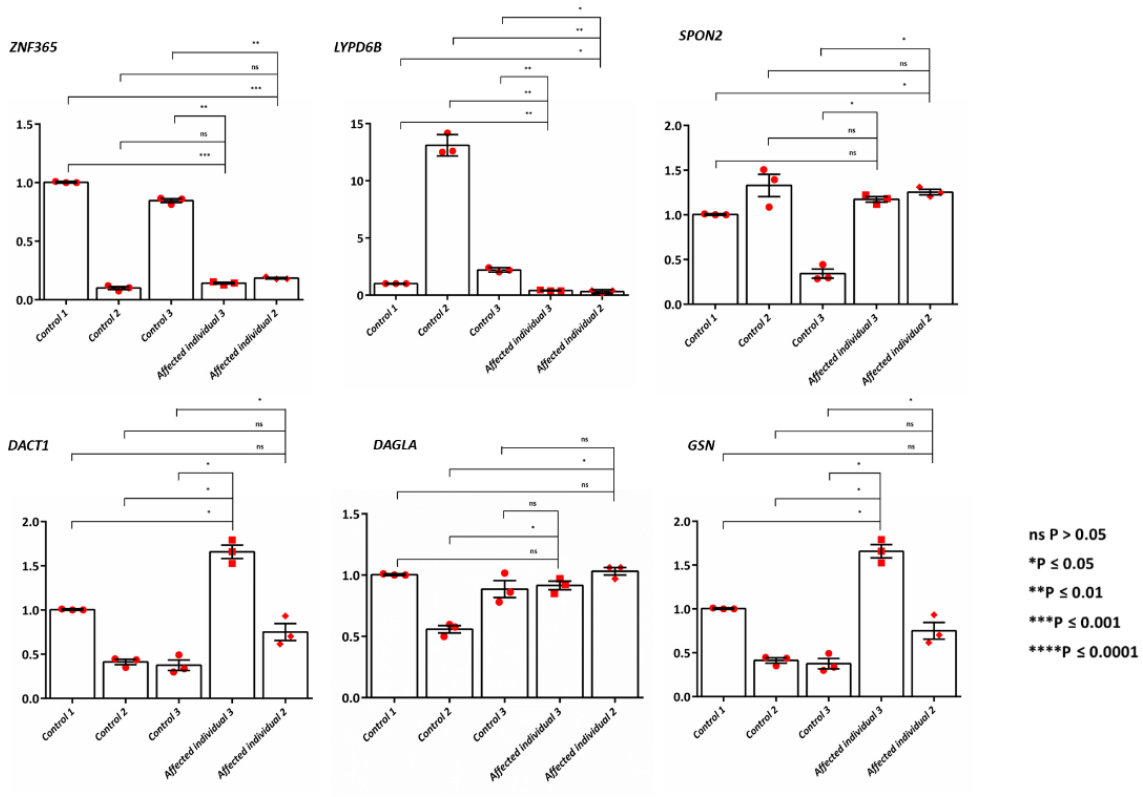

Figure 2.S2. Additional qPCR results. Fibroblast qPCR data for genes identified by RNAseq which did not show consistent and significant up or downregulation. β-actin was used as the reference gene. Triplicates were used. Error bars represent standard deviation. P-values were generated by two-way ANOVA with Dunnett's multiple comparisons test.

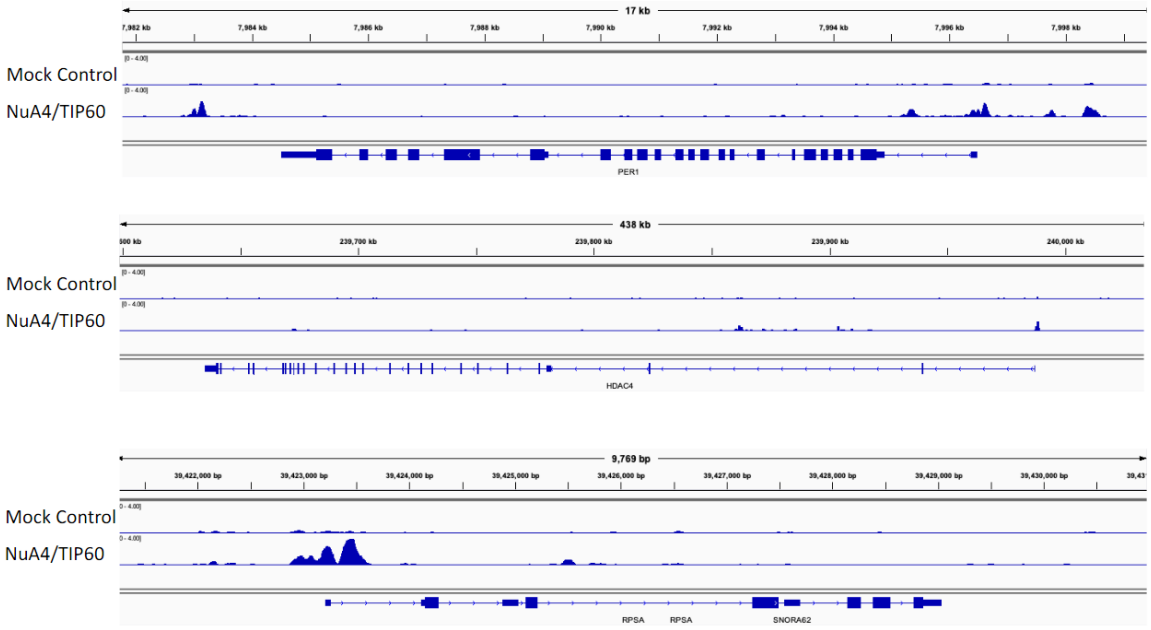

Figure 2.S3. Snapshots of ChIP-seq from Jacquet et al. 2016 for NuA4/TIP60 subunit EPC1 in K562 cells (EPC1(1-584)-3Flag-2Strep integrated at the AAVS1 locus and empty vector control. Profiles were obtained with Integrated Genomics Viewer with reads per million values and the RPSA gene is shown as positive control for NuA4/TIP60 (GEO accession GSE78027).

Table 2.S1 Additional clinical features

Individual	1	2	3
Demographic information			
Ethnicity	White	White	African-American
Gender	F	M	M
Age at last evaluation	29 years	12 years	16 months
Family history			
Affected family member	No	Cleft lip and palate in cousin once removed	No
Unaffected sibs	1	2 maternal half sibs	No
Consanguinity	No	No	No
Perinatal history			
Perinatal complications	None	None	None
At birth: duration gestation (weeks)	40	38	NA
- Weight, g	3400 (50th centile)	2740 (5th-10th centile)	2275 (2nd centile)
- Length, cm	48.3 (10th centile)	47 (3rd centile)	40.6 (-3.6 SD)
- Head circ., cm	NA	31.5 (3rd centile)	29.5 (-2.8 SD)
Weight at last visit	80.6 Kg (92nd centile)	29.7 kg (3rd centile)	11.2kg (48th centile)
Height at last visit	150.6cm (3rd centile, -1.95 SD)	133.5 cm (2nd centile, -2.1 SD)	71.1 cm (<1st centile, -2.9 SD)
Head circ at last visit	55 cm (73rd centile)	50 cm (1st centile, -2.6 SD)	44.5 cm (1st centile, -2.2 SD)
Comment	Truncal obesity		
Neurodevelopment			
Developmental delay	Yes, severe	Yes, severe	Yes, severe global developmental delay
Intellectual disabillity	Yes, FSIQ 40	Yes, IQ 20-30	NA
Speech	Expressive language at 8-year-old level and receptive language at 4-5-year-old skill level	Absent speech	NA
Feeding problems	No	No	Yes – aspiration of thin liquids on swallow study
Hypotonia	Yes	Yes- earlier	No
Medications	Valproic acid	Melatonin slow release GH injections	Risperidone and clonidine
Seizures	Yes	Yes	Yes
Age of first seizure	Adult-onset	infancy	4 months
Type of seizures	Catamenial seizures, grand mal seizures	Variable (partial tonic, late onset infantile spasms, absence) seizures	Generalized tonic clonic and myoclonic seizures
Seizure frequency	Every other month	NA	Multiple per day from 4-10 months of age, then infrequently
Seizure outcome	Seizure-free since 13 years (2004) post hysterectomy	NA	No seizures since 14 months of age
Current anti-epileptic drugs	Valproic acid 1000 mg HS	None	None currently
Anti-epileptic drugs ever tried	Phenytoin, valproic acid 500 mg QID	Valproic acid, lamotrigine, vigabatrin	Previously on Onfi and Keppra
History of Status Epilepticus	No	No	No
History of febrile/complex febrile seizures	No	No	No
Behavioral/psychiatric issues	Yes, disruptive behavior, ADHD tantrums, repetitive, aggressive, impulsive and self-injurious behaviors	Yes, multiple stereotypies, hyperactive and disruptive behavior	Yes, behavior difficulties, tantrums, head banging
Sleep disorder	Yes, severe	Yes, severe	Yes, severe
Neuroradiology	Corpus callosum variant (incomplete development)	Hypoplasia of rostrum and thickening of splenium of corpus callosum; hypoplasia of left caudate nucleus; global progressive cerebellar atrophy (vermis more than hemispheres); small anterior pituitary	Polymicrogyria of right sylvian fissure, cystic dilation of 4th ventricle with inferior cerebellar vermis atrophy, Dandy-Walker variant.

Hearing loss	No, hyperacusis	Mild bilateral asymetric neurosensory hearing deficit	Mild hearing loss with a conductive component
Vision impairment	Yes	Yes	Intermittent strabismus, no refractive error
If yes, cause	Severe myopia	Divergent strabismus; mild hypermetropia (+2 dioptries) OD; mild myopia (-1.5 dioptries) OS	NA
Malformations and dysmorphisms			
Craniofacial features	SMS-like facial dysmorphism. Round face, flat facial profile, prognathism, depressed nasal bridge, downslanting corners of mouth, low-set ears, almond-shaped eyes	Lateral thinning of eyebrows, macrostomia, prognathism, thick lower lip (N.B. repaired cleft lip/palate), bulbous and asymmetric nose	Round face, flat facial profile, downslanting corners of mouth and upturned nose with depressed nasal bridge, prominent brow ridge. Epiblepharon and epicanthal folds
Cleft palate	No	Unilateral cleft lip and cleft palate	High arched palate and submucous cleft palate
Hand and foot dysmorphisms	Brachydactyly	Bilateral unique palmar creases and bilateral 5th finger clinodactyly. Bilateral pes talus	5th finger clinodactyly bilaterally
Renal anomalies	No	Horseshoe kidney	Not assessed
Urogenital anomalies	Recurrent urinary tract infection.	Bilateral vesico-ureteral reflux (stage III R, stage II L) Unilateral cryptorchidism	Hypospadias, cryptorchidism, concealed penis
MSK anomalies	Leg length discrepancy due to "dry hip socket"	No	No
Cardiac defect	No	No	Perimembranous VSD, dysplastic pulmonary valve with supravalvular and valvular pulmonary stenosis
Gastrointestinal abnormalities	No	gastroesophageal reflux eosinophilic esophagitis severe constipation	Dysphagia, aspiration of thin liquids Umbilical hernia
Endocrine anomalies	No	Complete GH deficiency since age 2 years, treated by GH injections	No
Surgeries	Hysterectomy to reduce catamenial seizures	-	Ear tubes, hernia repair with ochiopexy
Further information	Low immunoglobulins	-	Intermittent rashes on hands and feet
Genetic tests			
Chromosomal Microarray	Yes, Normal SNP arrays	Yes, normal oligonucleotides array	Likely benign Yp11.222 duplication, 1.0 Mb
Other genetic testing	Nl karyotype Nl methylation study for PWS Nl FISH for SMS Nl sequencing of *RAI1*	Nl karyotype Nl subtelomere FISH Nl subtelomeric MLPA Nl sequencing of *ZFHX1B*	None
Biochemical genetics studies	Nl urine organic acids Nl plasma amino acids Nl urine MPS	NA	Creatine kinase 70 U/L (normal)
Hematological anomalies	NA	NA	WBC 8.4 K/ul (Nl 5-10), RBC 3.48 M/ul low (Nl 3.89-4.97), Hemoglobin 10.3 g/dl low (Nl 11-13), hematocrit 31.8% (Nl 29-41), platelets 358 K/ul (Nl 250-450)

Abbreviations: Nl, normal; GH, growth hormone; PWS, Prader-Willi syndrome; SMS, Smith-Magenis syndrome; MPS, mucopolysaccharides; OD, right eye; OS, left eye.

Table 2.S2 Sleep disorder characteristics, based on the Modified Simonds & Parraga Sleep Questionnaire (Johnson et al., 2012).

	Individual 1	Individual 2	Individual 3
Bedtime Resistance	No	No	No
Sleep Onset Delay	No	Yes	Yes (improved by clonidine)
Sleep Anxiety	No	No	No
Night-waking	Yes	Yes	Yes
Parasomnia (talks, grinds teeth, bangs head, quick movements of extremities, tongue biting, enuresis, sleepwalking)	No	No	No
Sleep Disordered Breathing	No	No	No
Daytime sleepiness	Yes	No	No
Inverted circadian rhythm:	High daytime melatonin level (of 46pg/mL), a sign of inversion.	No	No

Table 2.S3 List of primers used for RT-qPCR

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
ACTB	GTTGTCGACGACGAGCG	GCACAGAGCCTCGCCTT
LHX9	TACTTCAACGGTACGGGCAC	TCTTCTGCGAGGGTGGATAAG
KIRREL3	TGACGCTACTTTGCGCCAT	GTGGGTAACTTGAGAGGTCCC
GFPT2	ATGTGCGGAATCTTTGCCTAC	ATCGAGAGCCTTGACTTTCCC
PER1	GCCAACCAGGAATACTACCAGC	GTGTGTACTCAGACGTGATGTG
HDAC4	CCTGGGAATGTACGACGCC	CCCGTCTTTCCTGCGTAAC
ZNF365	ACGGAATCTGAGGAGGAGCT	ATCACGGACAAAGCCAGAGG
LYPD6B	CTCCTCTCGACCCTACACCA	ATGTGCTTCTTCCGTGGCTG
SPON2	AAGAACCAGTACGTCAGTAACGG	CACAAACGAGACCAGCGAGT
DACT1	TTGAACTGTTTGAGGCGAAGAG	ACTGAACACCGAGTTAGAGGAAT
DAGLA	TGTCACCCTCGGAATGGTTG	GGTTGTAGGTCCGCAGGTTAC
GSN	AACAGCAATCGGTATGAAAGACT	CTGCACCATTGGAGACCTTGT

2.9 Acknowledgments

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Chapitre 3: Integrated analysis of H2A.Z isoforms function reveals a complex interplay in gene regulation

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3.1 Résumé

Le variant d'histone H2A.Z joue un rôle majeur dans le contrôle de l'expression des gènes. Chez l'humain, H2A.Z est codé par deux gènes exprimant deux isoformes, H2A.Z.1 et H2A.Z.2, différant par trois acides aminés. Dans ces travaux, nous avons entrepris une analyse intégrée des fonctions de ces isoformes dans l'expression génique au moyen de protéines étiquetées de façon endogènes. Des analyses de RNA-seq dans des cellules non transformées montrent que le rôle des deux isoformes dans l'expression des gènes est dépendante du contexte. H2A.Z.1 et H2A.Z.2 régulent à la fois des gènes communs et distincts, et peuvent aussi jouer un rôle antagoniste dans l'expression de certains gènes. Un isoforme peut également en remplacer un autre au niveau des sites de démarrage de la transcription. Nous avons analysé l'interactome de chaque paralogue par spectrométrie de masse et révélé l'existence de partenaires spécifiques à H2A.Z.1 ou H2A.Z.2, impliqués dans leurs fonctions antagonistes dans la régulation de certains gènes. Nos travaux illustrent la façon dont la balance entre les deux isoformes de H2A.Z revêt une importance cruciale pour l'expression de nombreux gènes, ajoutant un niveau de complexité supplémentaire à notre compréhension du fonctionnement des variants d'histones.

3.2 Abstract

The H2A.Z histone variant plays major roles in the control of gene expression. In human, H2A.Z is encoded by two genes expressing two isoforms, H2A.Z.1 and H2A.Z.2 differing by three amino acids. Here, we undertook an integrated analysis of their functions in gene expression using endogenously-tagged proteins. RNA-Seq analysis in untransformed cells showed that they can regulate both distinct and overlapping sets of genes positively or negatively in a context-dependent manner. Furthermore, they have similar or antagonistic function depending on genes. H2A.Z.1 and H2A.Z.2 can replace each other at Transcription Start Sites, providing a molecular explanation for this interplay. Mass spectrometry analysis showed that H2A.Z.1 and H2A.Z.2 have specific interactors, which can mediate their functional antagonism. Our data indicate that the balance between H2A.Z.1 and H2A.Z.2 at promoters is critically important to regulate specific gene expression, providing an additional layer of complexity to the control of gene expression by histone variants.

3.3 Introduction

The H2A.Z histone variant is one of the two histone variants conserved from yeast to human. It is enriched at the −1 and +1 nucleosomes surrounding the nucleosome-depleted region of active promoters (Barski et al., 2007). It can play positive or negative roles in specific gene expression (Subramanian et al., 2015). In addition to transcriptional control, H2A.Z is important for genetic stability and DNA damage repair (Billon & Côté, 2012), although its exact function is somewhat controversial in mammals (Taty-Taty et al., 2014; Xu et al., 2012). H2A.Z is incorporated in chromatin by specialized machinery relying on the ATPase SWR1 in yeast and its orthologs in other species (Billon & Côté, 2012; Mizuguchi et al., 2004). In mammals, both SWR1 orthologs, SRCAP and p400 belong to multimolecular complexes that have been shown to mediate incorporation of the H2A.Z histone variant (Gévry et al., 2007; Ruhl et al., 2006). H2A.Z removal can be mediated by the ANP32E chaperon protein (Obri et al., 2014).

In vertebrates, H2A.Z is encoded by two different genes, *H2AFZ* and *H2AFV*, leading to the production of three proteins, one produced from the *H2AFZ* gene, called

H2A.Z.1, and two splicing variants produced from *H2AFV*, called H2A.Z.2 and H2A.Z.2.2 (Bönisch et al., 2012; Matsuda et al., 2010). Importantly, the *H2AFZ* gene is essential for mouse development (Faast et al., 2001), and conditional deletion of *H2AFZ* in the brain leads to neurogenesis defects (Shen et al., 2018), highlighting the importance of H2A.Z in mammals. Intriguingly, H2A.Z.1 and H2A.Z.2 proteins are highly similar, differing by only three amino acids (Dryhurst et al., 2009; Eirín-López et al., 2009). They are expressed at different levels as shown by the analysis of their expression in human embryonic and adult tissues (Dryhurst et al., 2009). It was recently found that they have overlapping function or can compensate each other since depleting both isoforms in the mouse intestine leads to severe homeostasis defects whereas individual mutants have no phenotype (Zhao et al., 2019). However, it was shown by ChIP-Seq analysis of exogenously expressed GFP-tagged isoforms that their genomic localisation is similar but not identical (Pünzeler et al., 2017). This could indicate that H2A.Z.1 and H2A.Z.2 functions could be divergent. Indeed, specific functions and/or expression of these isoforms in human cancer cells have been described. For example, despite the fact that the mRNA for both isoforms are overexpressed in human melanoma, only depletion of H2A.Z.2 was found to favour melanoma cell proliferation (Vardabasso et al., 2015). In contrast, H2A.Z.1 is specifically overexpressed in hepatocellular carcinoma (HCC) (Yang et al., 2016). Differences in the physiological roles of H2A.Z.1 and H2A.Z.2 have also been documented. In highly recombinogenic DT40 chicken cells, Nishibuchi et al. (Nishibuchi et al., 2014) found that H2A.Z.2, but not H2A.Z.1, is recruited to and functionally important for the repair of double strand breaks (Dunn et al., 2017). However, no study has extensively investigated the independent and interdependent roles of both isoforms in their physiological context in specific gene expression in non-transformed cells. Furthermore, the lack of specific antibodies, preventing the investigation of endogenous proteins, is a strong limitation to understand the molecular bases of these differences.

Here, we performed an integrated study of H2A.Z.1- and H2A.Z.2-dependent gene expression in untransformed cells by combining RNA-seq, RT-qPCR and ChIP assays. This unveiled the parallel and antagonistic functions of H2A.Z.1 and H2A.Z.2

in gene regulation. Furthermore, by tagging endogenous H2A.Z.1 and H2A.Z.2 isoforms, we were able to identify proteins interacting specifically with one isoform or the other. Our study thus reveals major antagonism between H2A.Z.1 and H2A.Z.2 regarding control of gene expression, mediated by specific interactors.

3.4 Results

H2A.Z.1 and H2A.Z.2 are major regulators of gene expression

In an effort to identify genes regulated by H2A.Z isoforms H2A.Z.1 and H2A.Z.2 in non-transformed cells, we transfected Telomerase-immortalized WI38 human primary fibroblasts with specific siRNAs silencing either H2A.Z.1, H2A.Z.2, or both at the same time. Analysis of each H2A.Z isoform mRNA expression by RT-qPCR indicated that both siRNAs are efficient and specific (Figure 3.1A), although transfection of the H2A.Z.2 siRNA slightly affected the expression of H2A.Z.1 mRNA. Using U2OS cells in which the two alleles coding for H2A.Z.1 or H2A.Z.2 were tagged with a 3xFlag-2xStrep tag by genome editing (see Figure 3.1—figure supplement 1A for the characterization of the cell lines) (Dalvai et al., 2015), we found that siRNA-mediated depletion efficiently decreased the expression of one isoform without affecting the expression of the other (see Figure 3.1B for a H2A.Z western blot and Figure 3.1—figure supplement 1B for a Flag western blot). Note that in these western blots, we observed a band migrating above H2A.Z and decreasing upon siRNA depletion. This band probably corresponds to a post-translational modification of H2A.Z, most likely its ubiquitination (see below). No obvious difference could be observed between H2A.Z.1 and H2A.Z.2 with respect to this band (Figure 3.1—figure supplement 1B). By performing a western blot analysis using an antibody recognising total H2A.Z, we found that the strong depletion of total H2A.Z in WI38 cells required transfection of both siRNAs together, as we observed only a moderate or weak effect upon H2A.Z.1 or H2A.Z.2 depletion, respectively (Figure 3.1C). Quantification of this experiment is consistent with the interpretation that total H2A.Z is composed of about 2/3rd of H2A.Z.1 and 1/3rd of H2A.Z.2 in WI38 cells (Figure 3.1C).

We next performed RNA-Seq experiments to identify the genes regulated by both isoforms in an unbiased fashion. Two entirely independent experiments were performed and differential analysis showed that the expression levels of 3573 mRNAs were significantly affected upon H2A.Z.1 depletion and 1500 upon H2A.Z.2 depletion (see Supplementary files 3.1–4 for the list of deregulated genes). 41.5% and 50.4% of regulated genes were activated upon H2A.Z.1 and H2A.Z.2 depletion, respectively. In addition, 691 mRNAs were significantly affected only upon depletion of the two isoforms together (371 activated and 320 repressed) (Supplementary file 3.5), suggesting that at these promoters H2A.Z isoforms can compensate each other. These results indicate that H2A.Z.1 and H2A.Z.2 are major regulators of gene expression in non-transformed cells, both acting as gene activators or repressors. Note that the expression of H2A.Z isoforms is strongly reduced but not abolished upon transfection of the siRNAs. We thus may have missed some H2A.Z-regulated genes in this analysis, when a residual amount of H2A.Z is sufficient to bring about the correct regulation.

Gene ontology analyses indicate that genes activated upon H2A.Z1 knock-down are enriched in negative regulators of cell proliferation whereas genes repressed are mostly enriched in mitosis-linked genes (Figure 3.1D), in agreement with the known cell proliferation arrest observed upon H2A.Z.1 depletion. Genes induced upon H2A.Z.2 knock-down are also enriched in cell cycle-linked genes (Figure 3.1D), although no obvious effect on cell proliferation could be observed upon H2A.Z.2 depletion (data not shown).

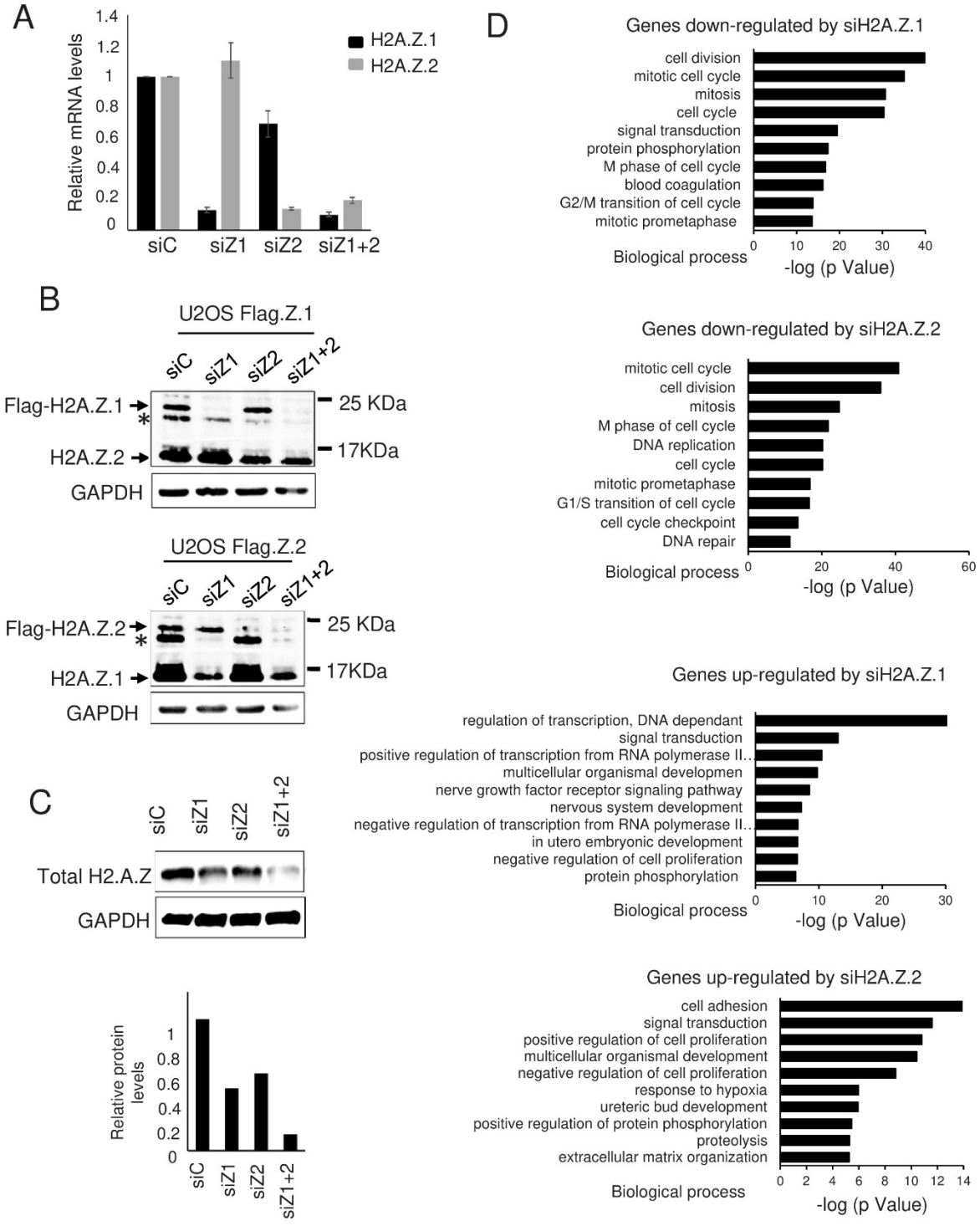
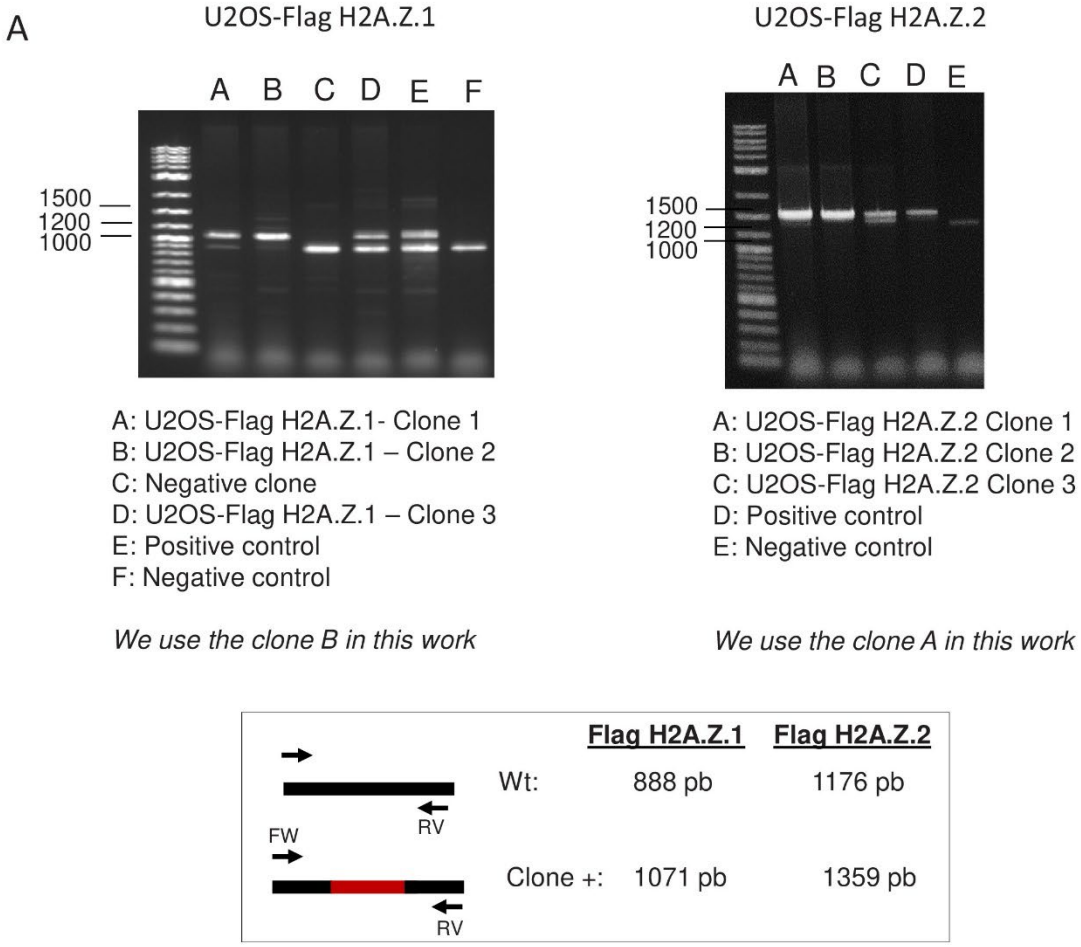


Figure 3.1. Effect of H2A.Z.1 and H2A.Z.2 depletion on gene expression.
(A) WI38 cells were transfected with the indicated siRNAs. 72 hr later, total RNA was prepared. The amount of H2A.Z.1 and H2A.Z.2 mRNA was quantified by RT-qPCR, standardised using GAPDH mRNA levels and calculated relative to one for cells transfected with the control siRNA. The mean and SDOM from five independent experiments are shown. (B) Genome edited U2OS cells expressing either tagged H2A.Z.1 (top) or tagged H2A.Z.2

(bottom) were transfected with the indicated siRNAs. 72 hr later, total cell extracts were prepared and subjected to western blot analysis using an anti H2A.Z antibody. The star * indicates a band probably corresponding to a post-translationally modified untagged H2A.Z isoform. (C) Same as in A, except that total cell extracts were prepared and subjected to western blot analysis using the indicated antibody, then protein signals were standardised using GAPDH protein levels and calculated relative to1 for cells transfected with the control siRNA. A representative experiment out of two is shown. (D) Gene ontology analyses (Genecodis) of genes downregulated upon H2A.Z.1or H2A.Z.2 depletion or upregulated upon H2A.Z.1 or H2A.Z.2 depletion (from top to bottom). The top 10 most significant enrichments are shown.



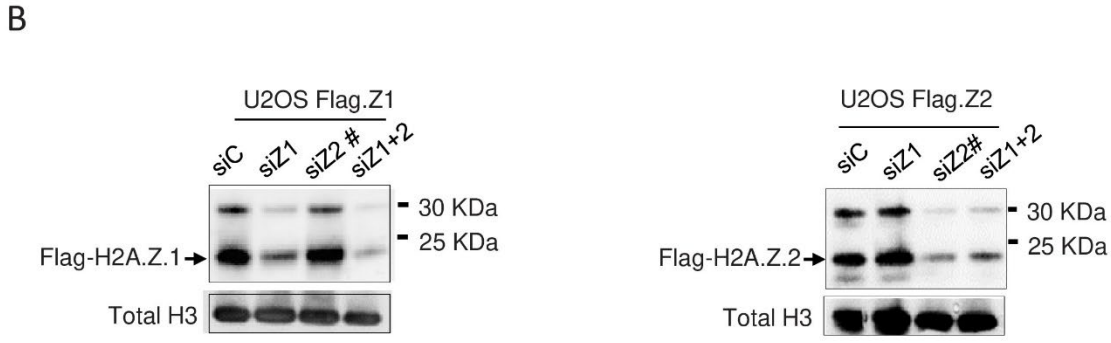


Figure 3.1-figure supplement 1. Characterisation of U2OS cells genome-edited to express 3xFlag-2xStrep H2A.Z.1 or H2A.Z.2.
(A) Genomic DNA samples were subjected to a PCR reaction using primers located outside of the inserted sequence as shown in the scheme (Bottom). The top panels show an analysis on agarose gel of the PCR products obtained on negative clones and on the genome-edited clones used in this study. Note the shift of the band in the genome-edited clones expressing Flag-H2A.Z.1 (Left) or Flag-H2A.Z.2 (Right), indicating the recombination. We chose the B clone from U2OS-Flag H2A.Z.1 and the A clone from U2OS-Flag H2A.Z.2 for further analyses. The bands were excised and sequenced to check the accuracy of the recombination. (B) Nuclear extracts from U2OS cells expressing Flag-H2A.Z.1 (Left) or Flag-H2A.Z.2 (Right) transfected with the indicated siRNA were subjected to a Flag western blot. Note the band migrating close to 30 kDa which likely corresponds to a post-translationally modified tagged histone. Note that the H2A.Z.2 siRNA is the one used in Figure 3.2—figure supplement 2 (si Z2#), *ie* a different siRNA than in Figure 1.

H2A.Z.1 and H2A.Z.2 isoforms regulate both distinct and overlapping sets of genes

We next analysed whether H2A.Z.1 and H2A.Z.2 regulate the same set of genes. We found that among the 3573 genes regulated by H2A.Z.1, 759 are also regulated by H2A.Z.2 (Figure 3.2A), whereas the expected overlap for random lists of gene of the same size would be 255. Actually, more than half of the genes regulated by H2A.Z.2 are also regulated by H2A.Z.1. This intersection is highly significant (p value = $9{,}38E^{-283}$), indicating that H2A.Z isoforms regulate overlapping sets of genes. However, they also have independent functions, since 2814 and 741 genes are regulated specifically by H2A.Z.1 and H2A.Z.2 respectively. The lists of genes up-regulated or down-regulated upon H2A.Z.1 or H2A.Z.2 depletion in WI38 cells are shown in Supplementary files 3.1–4.

We next crossed the lists of genes regulated by each H2A.Z isoform considering whether the genes were activated or repressed. We found 325 genes activated and 192 repressed by both H2AZ.1 and H2A.Z.2, both overlaps being highly significant (Figure 3.2B). Taking into account the 691 genes which were found deregulated only when we depleted the two isoforms, H2A.Z isoforms regulate 1208 genes in a similar way. This result indicates that both H2A.Z isoforms can play similar roles in the regulation of specific genes. However, we also found 72 genes repressed by

H2A.Z.1 and activated by H2A.Z.2 and 170 activated by H2A.Z.1 and repressed by H2A.Z.2 (Figure 3.2B). This latter overlap is much more than expected by chance and is also highly significant, indicating that H2A.Z.1 and H2A.Z.2 can also regulate gene expression in an opposite fashion for a significant proportion of genes.

Using RT-qPCR, we analysed the expression of 6 selected mRNAs (ZDHHC20, RRM2, PLAT, COLEC12, AKAP12 and ADAMTS1) deregulated upon either H2A.Z isoform depletion. This showed a striking similarity with RNA-Seq results, validating the analysis (Figure 3.2—figure supplement 1). Moreover, results concerning the effects of H2A.Z.2 were confirmed with a second independent siRNA (Figure 3.2—figure supplement 2), ruling out the possibility of off-target effects at least for H2A.Z.2. Note however that we have not been able to find another efficient and specific siRNA against H2A.Z.1 (they were either inefficient or also decreased H2A.Z.2 levels). Some individual genes we identified here as regulated by H2A.Z.1 could thus be due to off-target effects. However, the highly significant intersection between genes differentially-expressed upon H2A.Z.1 and H2A.Z.2 depletion that we observed in WI38 as well as following experiments (see below) strongly suggests that off-target effects are modest. In particular, data on the ZDHHC20, RRM2 and PLAT gene were reproduced using a siRNA targetting a H2A.Z.1 interactor (see below), ruling out the possibility of off-target effects at least for these genes.

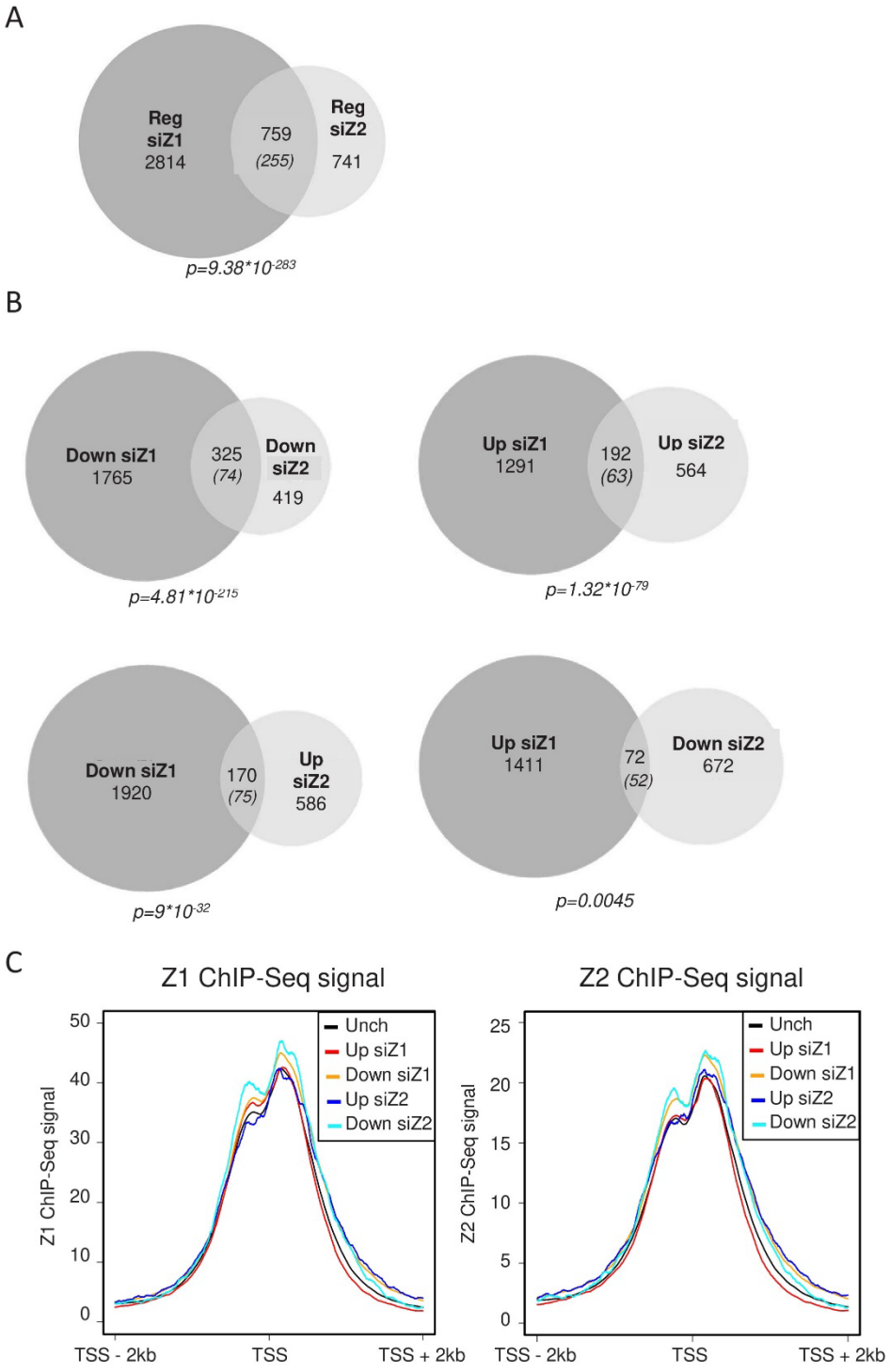


Figure 3.2 H2A.Z.1 and H2A.Z.2 regulate both distinct and overlapping sets of genes. RNA-Seq data was analysed for differential gene expression in samples transfected with either H2A.Z.1 siRNA or H2A.Z.2 siRNA versus the control siRNA sample. (A) Venn diagram showing the intersection between genes differentially expressed upon H2A.Z.1 and H2A.Z.2 inhibition. The p value indicated below the diagram indicates the significance of the intersection calculated using the Chi square test considering all expressed genes. The

numbers in brackets indicate the expected number of genes considering the total number of expressed genes if intersection was random. (B) Same as in A, except that the intersections indicate genes that are up-regulated or down-regulated in each sample and those that are regulated in an opposite way. Note that the intersection between genes up-regulated upon H2A.Z.1 depletion and down regulated upon H2A.Z.2 depletion is not highly significant. (C) U2OS cells expressing endogenously Flag-tagged H2A.Z.1 or H2A.Z.2 were subjected to ChIP-Seq experiments using anti-Flag antibodies. Metadata showing ChIP-Seq signals around TSS were computed for the five classes of genes (Unch: unchanged upon H2A.Z.1 or H2A.Z.2 depletion) defined from RNA-Seq data obtained in U2OS upon H2A.Z.1 or H2A.Z.2 depletion (see Figure 3.2—figure supplement 3). A representative experiment is shown. A replicate is shown in Figure 3.2—figure supplement 5A.

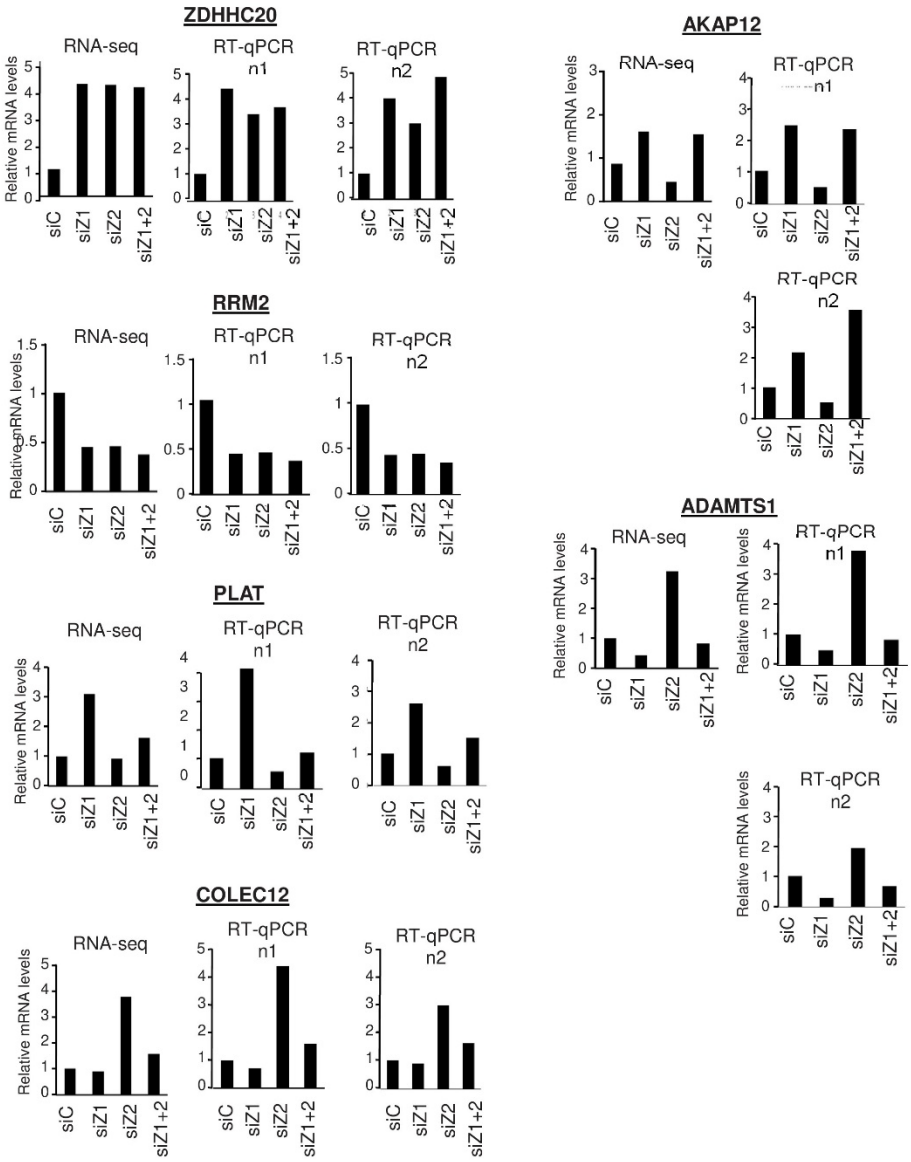


Figure 3.2-figure supplement 1. Validation of RNA Seq results.
WI38 cells were transfected with the indicated siRNAs. 72 hr later, total RNA was prepared and further purified to be subjected to RNA-Seq or analysed by RT-qPCR. RNA-Seq signals

were averaged for the indicated genes and calculated relative to one for the control siRNA sample. RT-qPCR data for the indicated genes was standardised using GAPDH mRNA levels and calculated relative to one for cells transfected with the control siRNA. The data for the two samples sent to RNA-Seq are shown.

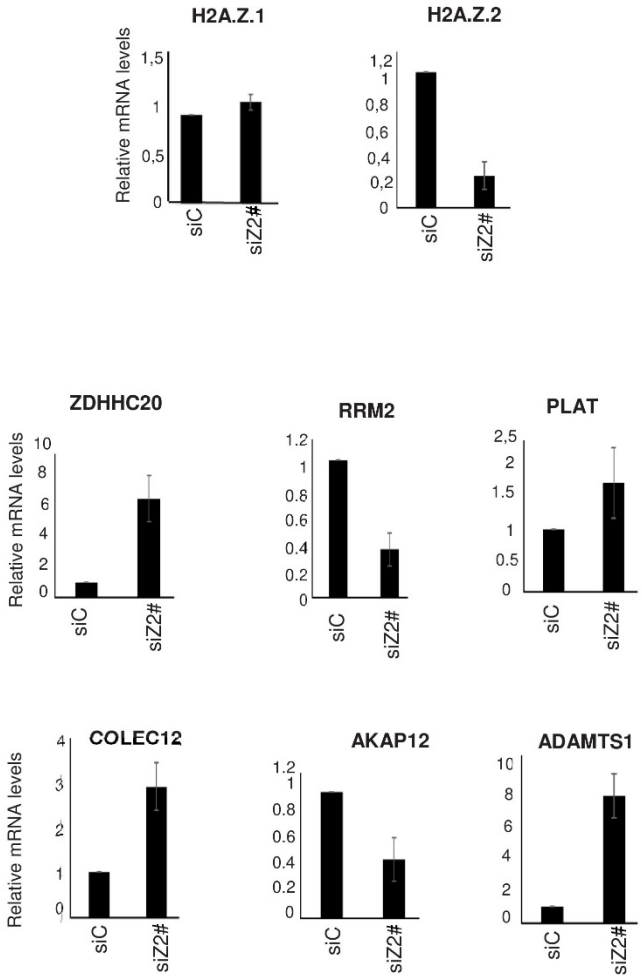


Figure 3.2-figure supplement 2. Effect of a second siRNA H2A.Z.2 (siZ2#).
WI38 cells were transfected using a control siRNA or a second independent siRNA against H2A.Z.2 (siZ2#). 72 hr later, total RNAs was prepared. The amount of the indicated mRNA was quantified by RT-qPCR, and was standardised using GAPDH mRNA levels and calculated relative to one in cells transfected using the control siRNA. The mean and SDOM from three independent experiments are shown.

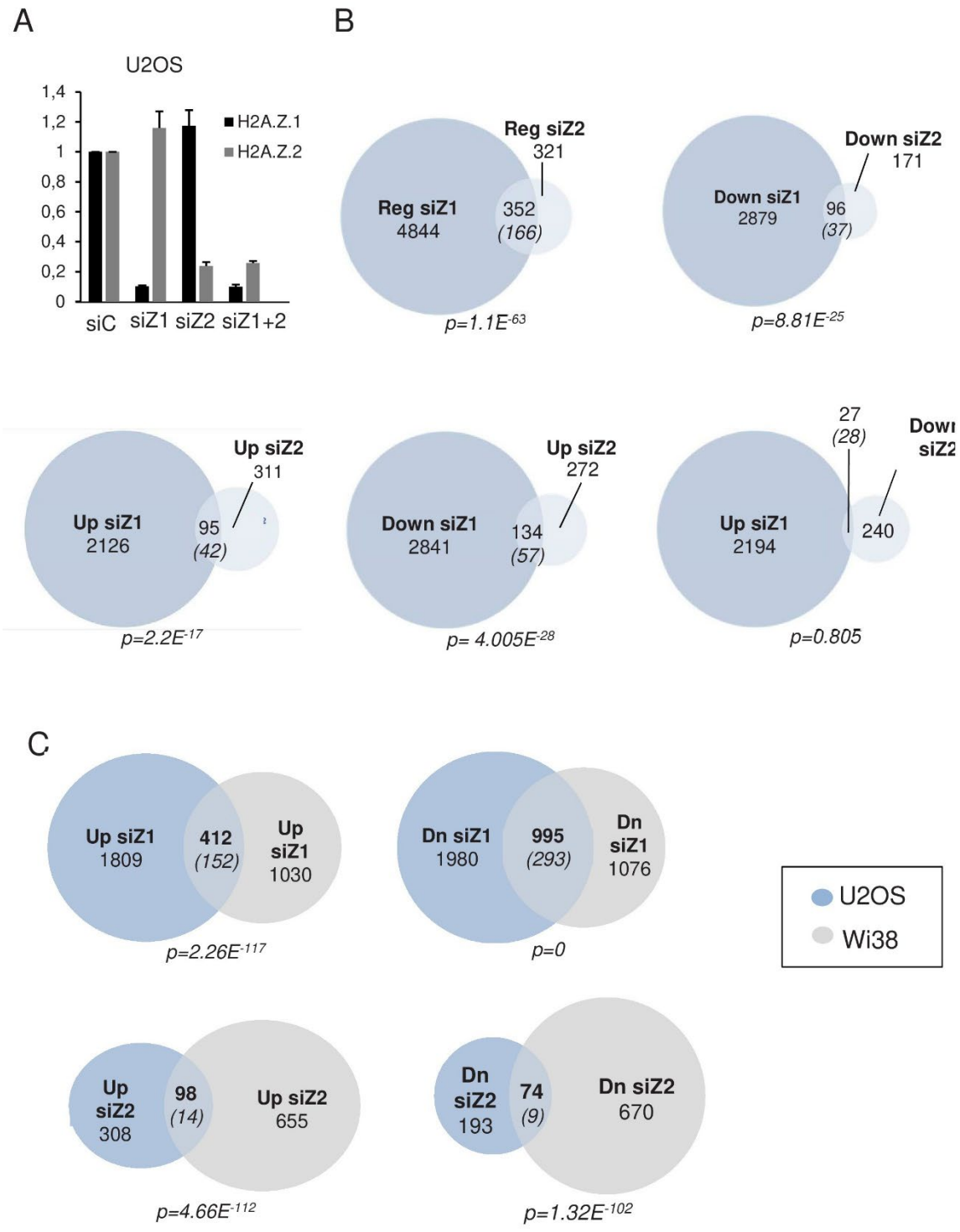


Figure 3.2-figure supplement 3. RNA-seq analysis after H2A.Z.1 and H2A.Z.2 depletion in U2OS cells.

(A) U2OS cells were transfected with the indicated siRNAs. 72 hr later, total RNA was prepared. The amount of H2A.Z.1 and H2A.Z.2 mRNA was quantified by RT-qPCR, standardised using GAPDH mRNA levels and calculated relative to one for cells transfected with the control siRNA. The mean and SDOM from three independent experiments are

shown. (B) H2A.Z.1 and H2A.Z.2 regulate specific and common genes in U2OS cells. RNA Seq data from U2OS cells were analysed for differential gene expression in samples transfected by either the H2A.Z.1 siRNA or the H2A.Z.2 siRNA versus the control siRNA sample. Venn diagram showing the intersection between the genes differentially expressed upon H2A.Z.1 and H2A.Z.2 inhibition, between genes up-regulated in the two samples, down-regulated in the two samples or regulated in an opposite way in the two samples. Note that, as for WI38, the intersection between genes upregulated upon H2A.Z.1 depletion and down-regulated upon H2A.Z.2 depletion is not highly significant. The numbers in brackets indicate the expected number of genes considering the total number of expressed genes if intersection was random. The p-value was calculated using the Chi-square test considering all expressed genes. (C) H2A.Z.1 and H2A.Z.2 regulate different genes in WI38 and U2OS cells. Venn diagram showing the intersection between lists of differentially expressed genes from RNA Seq data from WI38 and U2OS cells. Note that despite significant overlap, gene lists were mostly different. The numbers in brackets indicate the expected number of genes considering the total number of expressed genes if intersection was random. The p-value was calculated using the Chi-square test considering all expressed genes.

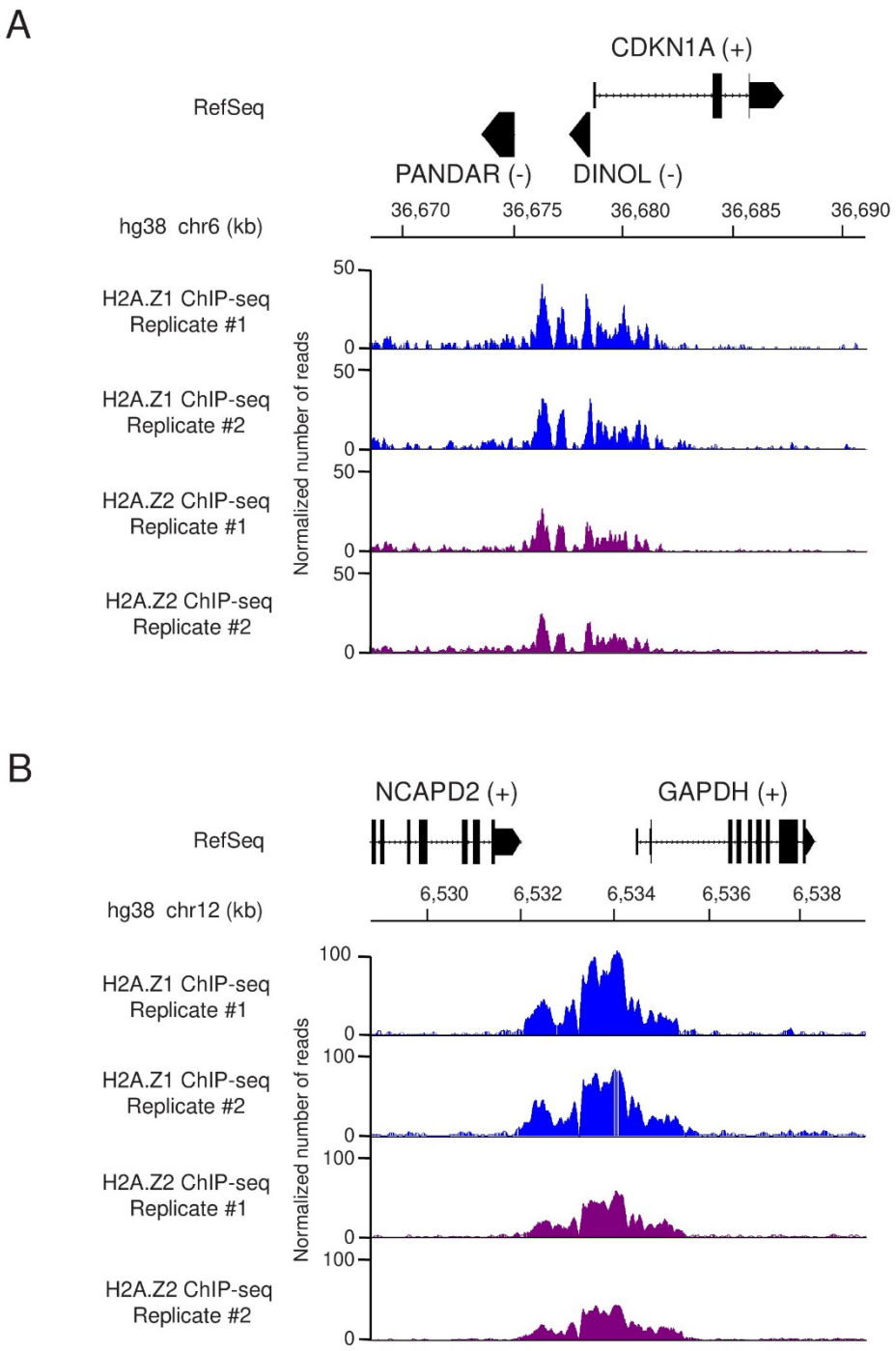


Figure 3.2-figure supplement 4. Profiles of tagged H2A.
Z.1 and H2A.Z.2 ChIP-Seq data at the *CDKN1A/p21* and *GAPDH* loci. The two replicates of ChIP-Seq experiments using Flag antibodies from U2OS cells expressing either genome edited H2A.Z.1 or H2A.Z.2 were visualized on IGB (Integrated Genome Browser). The tracks show the normalized number of aligned reads of ChIP-seq datasets at the *CDKN1A/p21* (A) and *GAPDH* (B) genes. RefSeq genes (hg38) are also shown with their DNA strands in brackets (if several transcript variants exist, only the 1st transcript variant is shown for simplicity).

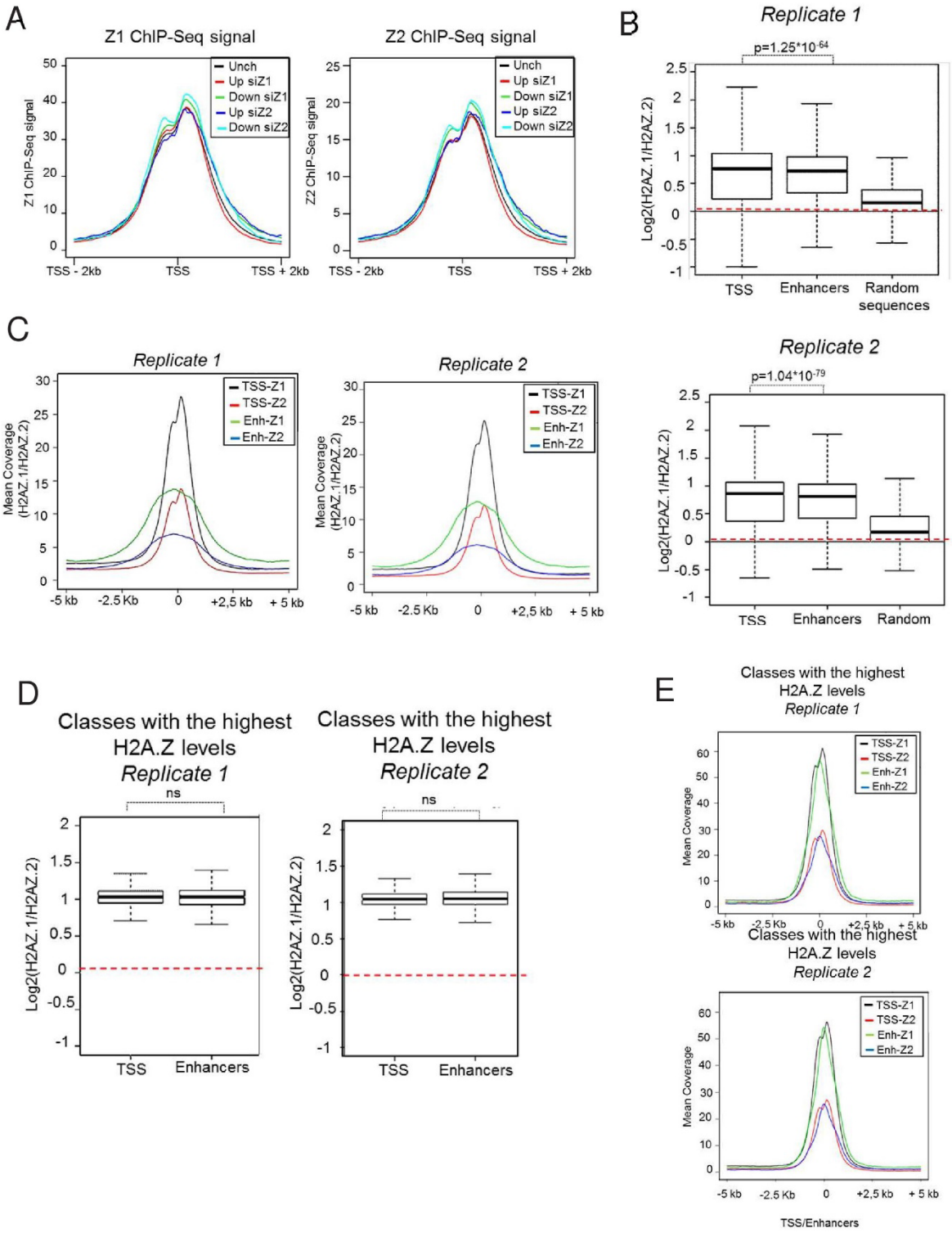


Figure 3.2-figure supplement 5. Analysis of H2A.Z.1 or H2A.Z.2 presence around TSS and enhancers.

(A) Replicates of ChIP-Seq experiments using Flag antibodies from U2OS cells expressing either genome edited H2A.Z.1 (left) or H2A.Z.2 (right) analysed as in Figure 3.2C. (B) Box

plots representing the ratio of the mean amount of H2A.Z isoforms ChIP-Seq signals around all TSS (59,553 TSSs), at all U2OS enhancers (obtained through enhanceratlas: http://www.enhanceratlas.org/, 14,764 enhancers) and at 1000 random genomic sequences of 1000 bases. (C) Metadata showing the mean of H2A.Z.1 and H2A.Z.2 ChIP Seq signals on the 10 kB region encompassing all TSSs and all enhancers. For enhancers, the '0' position corresponds to the centre of enhancers defined in enhanceratlas. (D) TSSs and enhancers were sorted in five classes according to the total levels of H2A.Z. The box plots show the ratio of the mean amount of H2A.Z isoforms ChIP-Seq signals around TSS (24,695/22,263 TSSs), and U2OS enhancers (1,707/1,459 enhancers) falling into the highest class (4 < ln (H2A.Z.1 + H2A.Z.2 ChIP-Seq signals)<5). (E) Metadata showing the mean of H2A.Z.1 and H2A.Z.2 ChIP Seq signals on the 10 kB region encompassing the TSS and enhancer populations defined in D). For enhancers, the '0' position corresponds to the centre of enhancers defined in enhanceratlas.

Importantly, very similar results were observed in RNA-Seq data obtained following depletion of H2A.Z.1 or H2A.Z.2 in tumoral U2OS cells. We found more genes regulated by H2A.Z.1 (5196), less by H2A.Z.2 (673) than in WI38 cells, with roughly an equivalent number of activated and repressed genes (Figure 3.2—figure supplement 3B). The lists of genes up-regulated or down-regulated upon either H2A.Z.1, H2A.Z.2 or both depletion in U2OS cells are shown in Supplementary files 3.6–10. Again, crossing the results obtained for H2A.Z.1 and H2A.Z.2 indicated that, besides genes regulated by only one isoform, significantly enriched gene populations are activated or repressed by both H2A.Z.1 and H2A.Z.2, or activated by H2A.Z.1 and repressed by H2A.Z.2 (Figure 3.2—figure supplement 3B), confirming that H2A.Z.1 and H2A.Z.2 can regulate specific gene expression similarly or in an opposite way.

Finally, we crossed the results obtained in U2OS cells with those obtained in WI38 cells (Figure 3.2—figure supplement 3C). Despite significant overlap, these lists were mostly different, indicating that H2A.Z.1 and H2A.Z.2 regulate different sets of genes in different cells, consistent with the fact that transcription regulation by H2A.Z isoforms H2A.Z.1 and H2A.Z.2 is highly specific of the promoter context.

The presence of H2A.Z isoforms at TSS does not determine their transcriptional effect

We next tested whether genes specifically regulated by a given isoform were characterized by a specific feature with respect to the presence of this isoform at

their promoters. Thanks to the U2OS cells lines with tagged endogenous isoforms, we performed H2A.Z.1 and H2A.Z.2 ChIP-Seq experiments. ChIP-Seq profiles on the CDKN1A/p21, GAPDH genes were very similar and showed accumulation of H2A.Z.1 and H2A.Z.2 signal mostly at the TSS (Figure 3.2—figure supplement 4). We then integrated these data with the RNA-Seq following depletion of H2A.Z.1 or H2A.Z.2 in the same cells. Metadata analyses showed that both isoforms accumulate around the Transcription Start Sites (TSS) of expressed genes at the −1 and +one nucleosome surrounding the Nucleosome Depleted region (Figure 3.2C), as already shown for total H2A.Z (Barski et al., 2007). Importantly, binding of H2A.Z.1 appeared to be similar on genes that were unchanged upon depletion of H2A.Z.1 or H2A.Z.2 than on genes which responded to H2A.Z.1 depletion, either positively or negatively (Figure 3.2C and Figure 3.2—figure supplement 5A). Although genes which are repressed by H2A.Z.1 depletion showed a slightly higher level of H2A.Z.1, they also showed a higher level of H2A.Z.2 (Figure 3,2C and Figure 3.2—figure supplement 5A). Similarly, genes responding to H2A.Z.2 depletion do not show major differences in the amount of H2A.Z.2 bound to their promoters. These data thus indicate that genes affected by the depletion of a given isoform are not characterized by the amount of this isoform around the TSS. A recent report described a higher H2A.Z.2/H2A.Z.1 ratio at active enhancers (Greenberg et al., 2019). To test whether this is also true in U2OS cells, we recovered U2OS enhancers through enhancer atlas (http://www.enhanceratlas.org/). We next computed the ratio of H2A.Z.1 to H2A.Z.2 ChIP-Seq signals at these enhancers as well as at all TSS or on control genomic regions. As previously found by (Greenberg et al., 2019), we found that the H2A.Z.1/H2A.Z.2 ratio was significantly lower at enhancers than at TSS (See Figure 3.2—figure supplement 5B and C for box plots and metadata). However, it was even lower at regions chosen arbitrary along the genomes (Figure 3.2—figure supplement 5B and C). To rule out any effect due to differences in the total amount of H2A.Z, we calculated this total amount by adding the H2A.Z.1 and H2A.Z.2 signal (which is feasible following internal spike-in normalisation) and sorted enhancers and promoters according to this amount. Strikingly, we did not observe a lower H2A.Z.1/H2A.Z.2 ratio at enhancers compared to promoters, whichever the

class we considered (see Figure 3.2—figure supplement 5D and E for box plots and metadata) for the class with the highest level of total H2A.Z). Thus, in U2OS cells, the ratio H2A.Z.2/H2A.Z.1 is dependent on the total amount of H2A.Z present but not on any functional differences between enhancers and promoters.

H2A.Z.1 and H2A.Z.2 isoforms can have antagonistic roles

We next asked whether the depletion of both isoforms together in WI38 non-transformed cells could lead to cumulative effects, as could be guessed if the total amount of H2A.Z was functionally important. As mentioned, only upon transfection of both siRNAs could we achieve efficient inhibition of total H2A.Z expression (see Figure 3.1C). We thus tested whether depletion of H2A.Z.2 could amplify the effects observed upon depletion of H2A.Z.1 alone. For the genes up-regulated upon H2A.Z.1 depletion, we plotted the effect of H2A.Z.1 depletion alone for each gene (calculating Log2 (siH2A.Z.1/siCtrl) for each gene) and the effect of both H2A.Z.1 and H2A.Z.2 depletion (Log2 (siH2A.Z.1+.2)/siCtrl)). Strikingly, we observed no cumulative effect of H2A.Z.1 and H2A.Z.2 depletion (Figure 3.3A). On the contrary, the effect of the double depletion was significantly lower than the effect of depleting H2A.Z.1 alone (Figure 3.3A). Importantly, similar findings were observed when analysing genes down-regulated upon H2A.Z.1 depletion (Figure 3.3A) and genes up- or down-regulated upon H2A.Z.2 depletion (Figure 3.3B). Thus, on these genes, the transcriptional effect of depleting one isoform on gene expression is attenuated upon depletion of the other isoform. This is not due to a lower siRNA effect upon co-transfection as shown in Figure 3.1. These results suggest that the effect of the loss of one H2AZ isoform on gene expression depends on the presence of the other H2AZ isoform. Thus, altogether these data uncover an antagonistic function of both H2A.Z isoforms on specific gene expression. Importantly, these findings were confirmed by RT-qPCR for the PLAT, AKAP12, ADAMTS1 and COLEC12 mRNAs (see Figure 3.2—figure supplement 1).

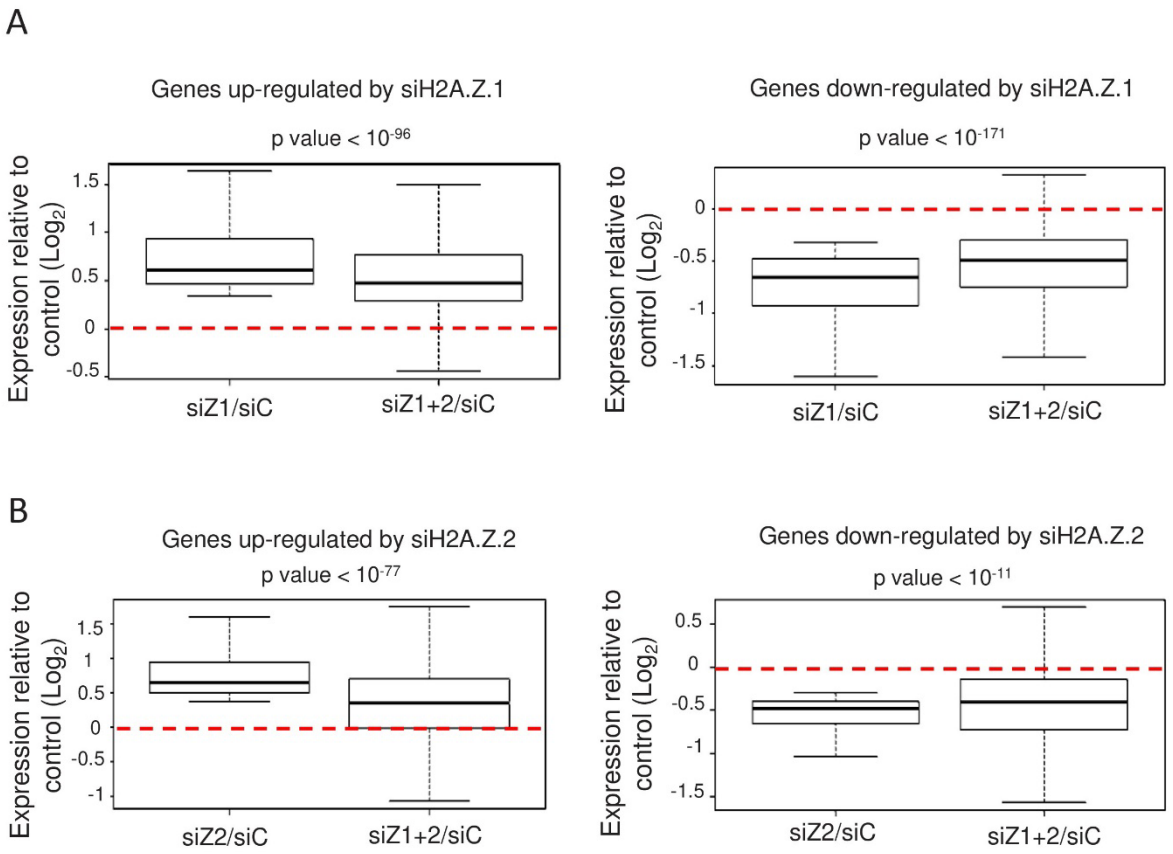


Figure 3.3. H2A.Z isoforms exert an antagonistic regulation on gene expression.
(A) For each gene up-regulated (left) or down-regulated (right) upon H2A.Z.1 depletion, we calculated the ratio between its expression in either H2A.Z.1 depleted cells (siZ1/siC) or cells depleted for H2A.Z.1 and H2A.Z.2 versus control cells (siZ1+2/SIC). The boxplots show the median, the 25% percentiles and the extrema of the Log2 of this ratio within the gene population (without outliers). The p value shows the significance of the difference between the two populations (paired welch test). (B) Same as in A, except that the calculation was done for genes up-regulated (left) or down-regulated (right) upon H2A.Z.2 depletion.

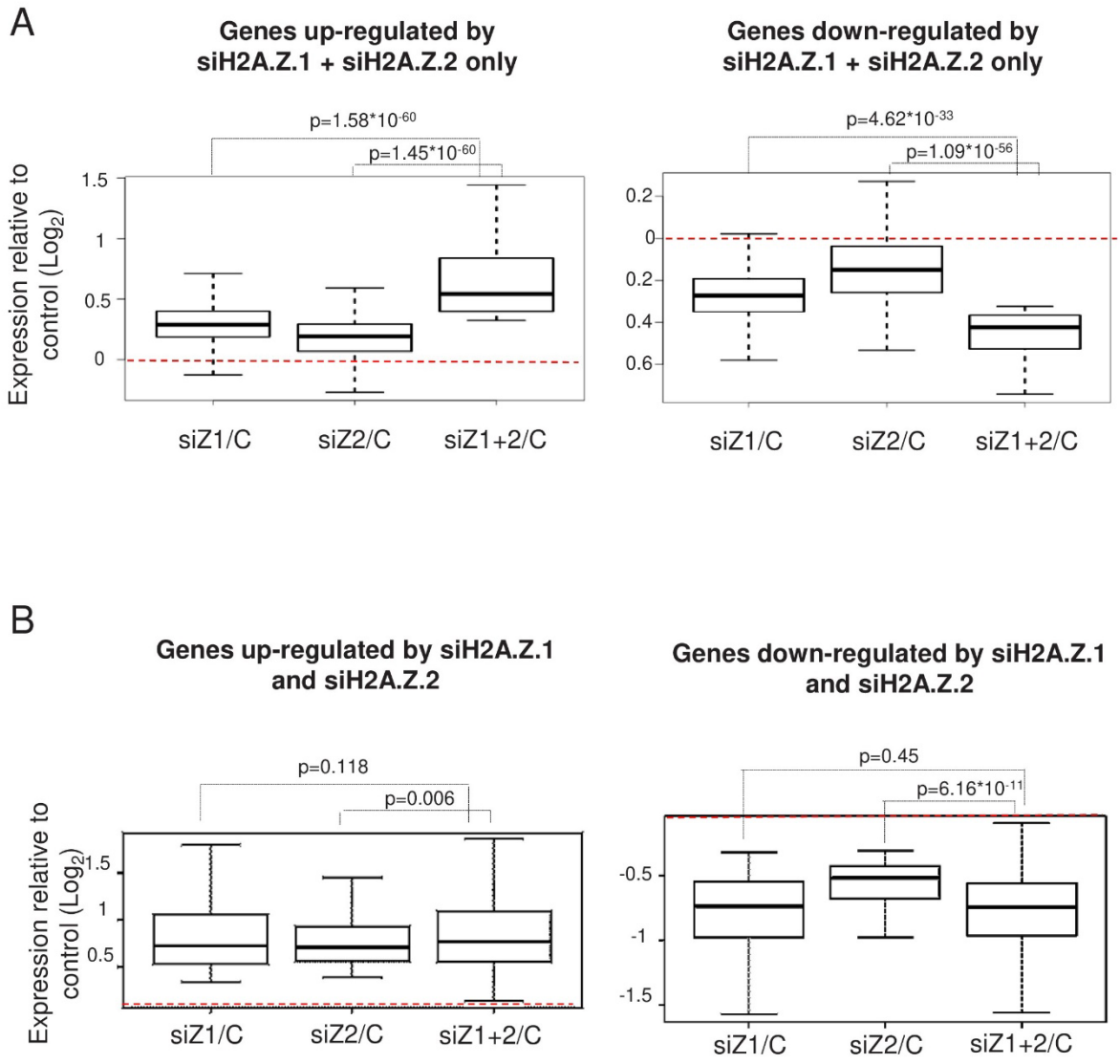


Figure 3.3-figure supplement 1. Effect of the double depletion of H2A.Z.1 and H2A.Z.2 on genes similarly regulated by H2A.Z.1 and H2A.Z.2.
(A) For genes up-regulated (left) or down-regulated only after the combined depletion of H2A.Z.1 and H2A.Z.2, we calculated the ratio between its expression in either H2A.Z.1-depleted cell, H2A.Z.2-depleted cells, or cells depleted for H2A.Z.1 and H2A.Z.2 together versus control cells. Boxplots show the median, the 25% percentiles and the extrema within the gene population of the Log2 of this ratio (without outliers). The p value shows the significance of the difference between the two populations (paired welch test). (B) Same as in A for genes up-regulated (left) or down-regulated (right) after the depletion of H2A.Z.1 and H2A.Z.2 alone.

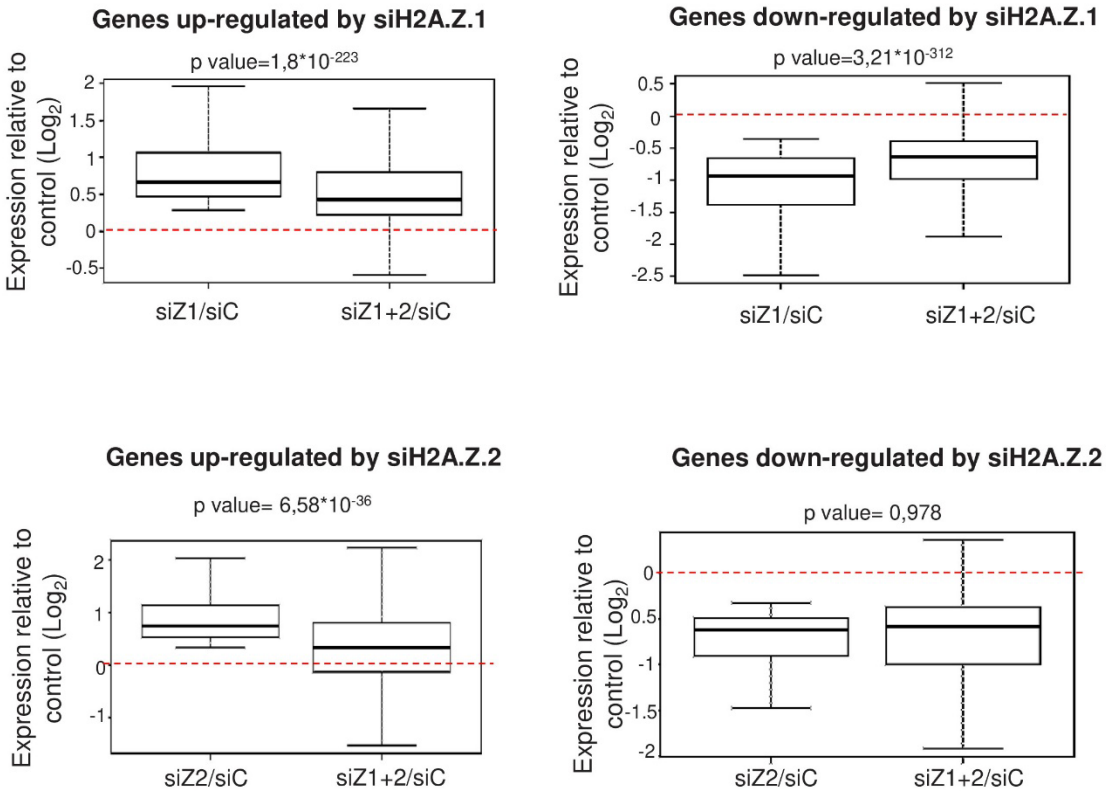


Figure 3.3-figure supplement 2. Antagonistic regulation by H2A.Z isoforms in U2OS cells.
For each gene up-regulated (Top left) or down-regulated (Top, right) upon H2A.Z.1 depletion, we calculated the ratio between its expression in either H2A.Z.1 depleted cells or cells depleted for H2A.Z.1 and H2A.Z.2 versus control cells. Boxplots shows the median, the 25% percentiles and the extrema within the gene population of the Log2 of this ratio (without outliers). The p value shows the significance of the difference between the two populations (paired welch test). Bottom: genes up-regulated (left) or down-regulated (right) upon H2A.Z.2 depletion were analysed.

Note however that cumulative effects can be observed on genes which are deregulated only upon the combined depletion of the isoforms, as expected, as well as on some genes which are similarly regulated by H2A.Z.1 and H2A.Z.2 (Figure 3.3—figure supplement 1).

Again, very similar results were observed in U2OS cells, in which the effects of depleting one isoform were attenuated upon depletion of the other (Figure 3.3—figure supplement 2), with the notable exception of genes down-regulated upon H2A.Z.2 depletion.

Altogether, these data indicate a conserved complex interplay between H2A.Z.1 and H2A.Z.2 in specific gene regulation, with two types of H2A.Z-regulated genes: genes that they regulate similarly or on which they can compensate each other and genes that they differentially regulate and on which there is a rather general antagonism between the two isoforms.

H2A.Z isoforms can replace each other at promoters

We next investigated the molecular mechanism underlying this complex interplay. H2A.Z is proposed to regulate specific gene expression by binding around gene Transcription Start Sites (TSSs) (Subramanian et al., 2015). One possibility could be that H2A.Z.1 and H2A.Z.2 can compete with each other for binding to the same promoters, with one isoform replacing the depleted one. Indeed, this would result in compensatory mechanisms where they play similar roles or in an antagonism where they bring about a different consequence regarding gene expression.

In agreement with this hypothesis, analysis of ChIP-Seq profiles (Figure 3.2—figure supplement 5) and ChIP-qPCR experiments (Figure 3.4A) using Flag antibodies indicate that both isoforms are recruited to the *GAPDH* and *CDKN1A/p21* promoters in U2OS cells expressing endogenous H2A.Z with a 3xFlag-2xStrep tag. Moreover, at the genome-wide level, we observed a strong correlation between the levels of H2A.Z.1 and H2A.Z.2 binding to gene promoters (Figure 3.4B). Thus, these data indicate that H2A.Z.1 and H2A.Z.2 bind to the same genomic regions. We next tested whether depletion of one isoform could result in its replacement by the other. We transfected U2OS cells expressing 3xFlag-2xStrep-tagged H2A.Z.2 with siRNA against H2A.Z.1 and analysed chromatin recruitment of tagged H2A.Z.2 by performing a ChIP assay with Flag antibodies. Spike-in DNA was added to increase accuracy of the ChIP results. We found that H2A.Z.2 levels strongly increased upon H2A.Z.1 depletion at two loci at which H2A.Z.1 was bound, that is the *CDKN1A/p21* and *GAPDH* promoters, underlining the replacement of H2A.Z.1 by H2A.Z.2 upon H2A.Z.1 depletion (Figure 3.4C). In reciprocal experiments, we did not observe a significant increase in Flag-tagged H2A.Z.1 occupancy following depletion of H2A.Z.2 (Figure 3.4D), most likely because H2A.Z.2 is less expressed than H2A.Z.1

so that replacement of a significant amount of H2A.Z.2 by H2A.Z.1 does not increase much the total amount of H2A.Z.1. Nevertheless, these data suggest that H2A.Z isoforms can replace each other at TSS, therefore explaining the complex interplay we observed between H2A.Z isoforms for specific gene regulation.

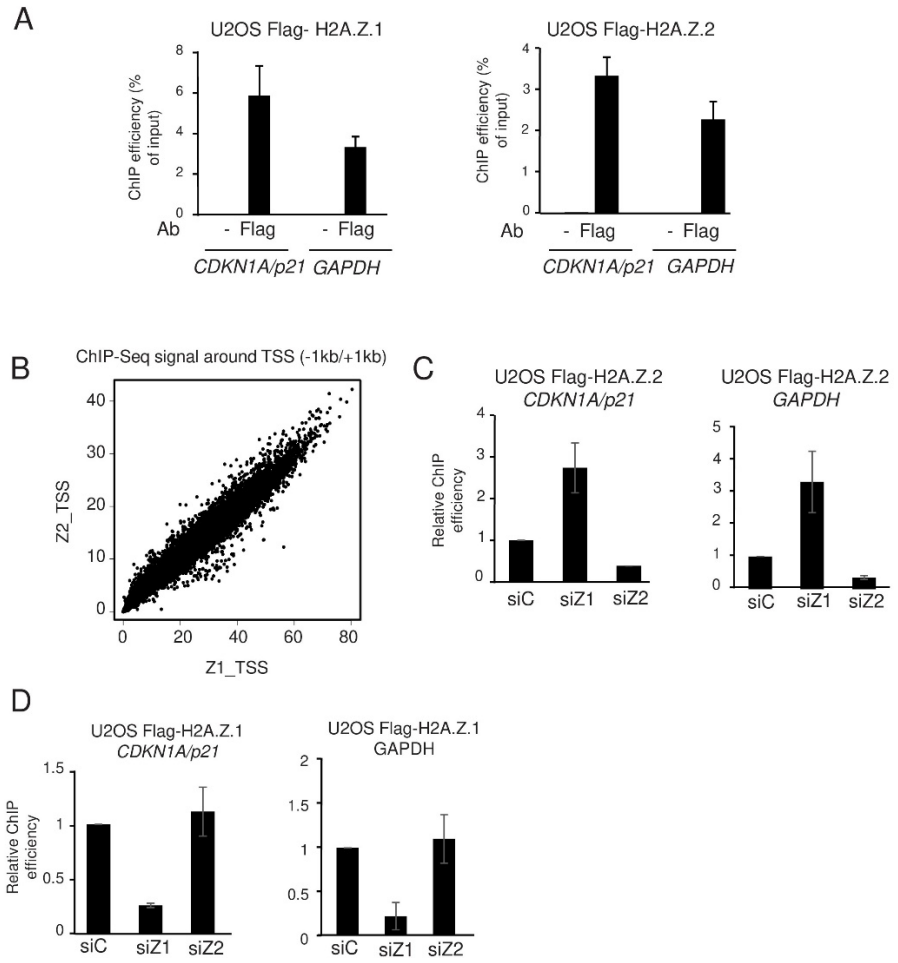


Figure 3.4. H2A.Z.1 and H2A.Z.2 replace each other at genes promoters.
(A) U2OS cells expressing endogenously tagged H2A.Z.1 or H2A.Z.2 as indicated were subjected to a ChIP assay using the Flag antibody or no antibody as a control. The amount of the indicated sequences was measured by qPCR and calculated relative to the input DNA. The mean and SDOM from three independent experiments are shown. (B) A ChIP Seq assay was performed on the same samples. The amount of H2A.Z.1 or H2A.Z.2 from −1000 to +1000 of each protein-coding gene TSS was calculated and plotted against one another. Note the striking correlation between the binding of H2A.Z.1 and H2A.Z.2. (C) U2OS cells expressing endogenously tagged H2A.Z.2 were transfected with the indicated siRNA. 72 hr later cells were harvested and subjected to a ChIP experiment in the presence of spike-in chromatin. The amount of the indicated promoter was measured by qPCR, calculated relative to spike-in signals and relative to one for cells transfected with the control siRNA. The mean and SDOM from three independent experiments are shown. (D) Same as in C, except that U2OS cells expressing endogenously tagged H2A.Z.1 were used.

At promoters at which they play a similar role, this could result in compensation for specific gene regulation, and deregulation would be seen only when depleted both isoforms. We observe this situation for 691 genes in WI38 and 800 genes in U2OS cells. At promoters at which H2A.Z.1 and H2A.Z.2 play a differential role, they would compete with each other, and that would result in the general antagonism we observe on the genes differentially regulated upon depletion of one isoform.

H2A.Z.1 and H2A.Z.2 are differently associated with specific proteins

We then investigated the mechanism by which the presence of H2A.Z.1 or H2A.Z.2 could have differential functional consequences for gene transcription, despite their strong similarities. We thus asked whether they could be associated with different proteins and whether this could lead to different outcomes regarding gene expression. We tagged either isoform with a 3xFlag-2xStrep tag through genome editing in K562 cells (see the characterisation of both cell lines in Figure 3.5—figure supplement 1; (Dalvai et al., 2015)). Indeed, since these cells are non-adherent cells, they are much more convenient to grow in amounts large enough to purify and identify by mass spectrometry interactors of endogenous proteins. Flag western blots on these clones indicate that, like in U2OS cells, a band of higher molecular weight can be observed with both isoforms Figure 3.5—figure supplement 1, most likely representing ubiquitinated H2A.Z considering its size and the fact that it represents 20% to 25% of non ubiquitinated H2A.Z when extraction is performed in the presence of DUB inhibitors (data not shown). ChIP-Seq analysis indicate that in this cell line as in U2OS cells, the two isoforms localise at the TSS of active genes and we observed a strong correlation between levels of H2A.Z.1 and H2A.Z.2 around TSS (Figure 3.5—figure supplement 1). We next performed mass spectrometry analysis of proteins interacting with each isoform expressed at endogenous levels. Since endogenous H2A.Z.2 is less expressed than H2A.Z.1, we used clones heterozygously-tagged H2A.Z.1 vs homozygously-tagged H2A.Z.2 to adequately compare interactomes with similar expression levels of the bait (Figure 3.5A and Figure 3.5—figure supplement 1, Supplementary file 3.8). We found that

the tandem affinity purification of each isoform from soluble nuclear extracts led to the co-fractionation of the previously characterized H2A.Z-incorporating complexes, that is p400- and SRCAP-containing complexes, and H2A.Z/H2B histone chaperones (see protein gel, mass spectrometry and western blot analysis in Figure 3.5B–D and Figure 3.5—figure supplement 2), in agreement with our findings that both isoforms can be incorporated at the same locations. Strikingly though, some proteins are found at higher levels in H2A.Z.1 purifications, such as chromatin proteins PHF14, HMG20A/iBRAF, TCF20 and RAI1, which were already found co-purifying together (Eberl et al., 2013), whereas others, such as SIRT1 are found at higher levels in H2A.Z.2 purifications (Figure 3.5C–D and Figure 3.5—figure supplement 2A). Importantly, similar preferential binding of PHF14 and SIRT1 to H2A.Z.1 and H2A.Z.2 respectively was also observed in U2OS cells (Figure 3.5—figure supplement 2B).

PHF14 and SIRT1 are major H2A.Z1 and H2A.Z.2 effectors

We next tested whether the proteins interacting specifically with H2A.Z.1 and H2A.Z.2 could be recruited to chromatin through their interaction with H2A.Z.1 and H2A.Z.2. We could not obtain specific ChIP signals with commercially-available antibodies against PHF14. We thus raised a stable U2OS cell line in which one PHF14 allele was edited in order to express a flag tagged PHF14 protein (see Figure 3.6—figure supplement 1 for the characterisation of this cell line). Despite epitope tagging, PHF14 ChIP experiments did not give any specific signal on the genes we tested (data not shown). To understand the molecular interplay of SIRT1 and PHF14 with H2A.Z isoforms, we performed fractionation experiments using the U2OS flag-tagged PHF14 cell line. We first began by a cell fractionation procedure relying on EDTA-mediated bivalent ions chelation. In these conditions, SIRT1 is mostly in the nuclear and cytoplasmic fractions, with detectable amount in the chromatin fraction (Figure 3.6A). Interestingly, depletion of H2A.Z.2 resulted in a decrease in SIRT1 presence in the chromatin fraction (Figure 3.6A, see Figure 3.6—figure supplement 2 for the quantification and a replicate), indicating that H2A.Z.2 favours the chromatin localisation of SIRT1.

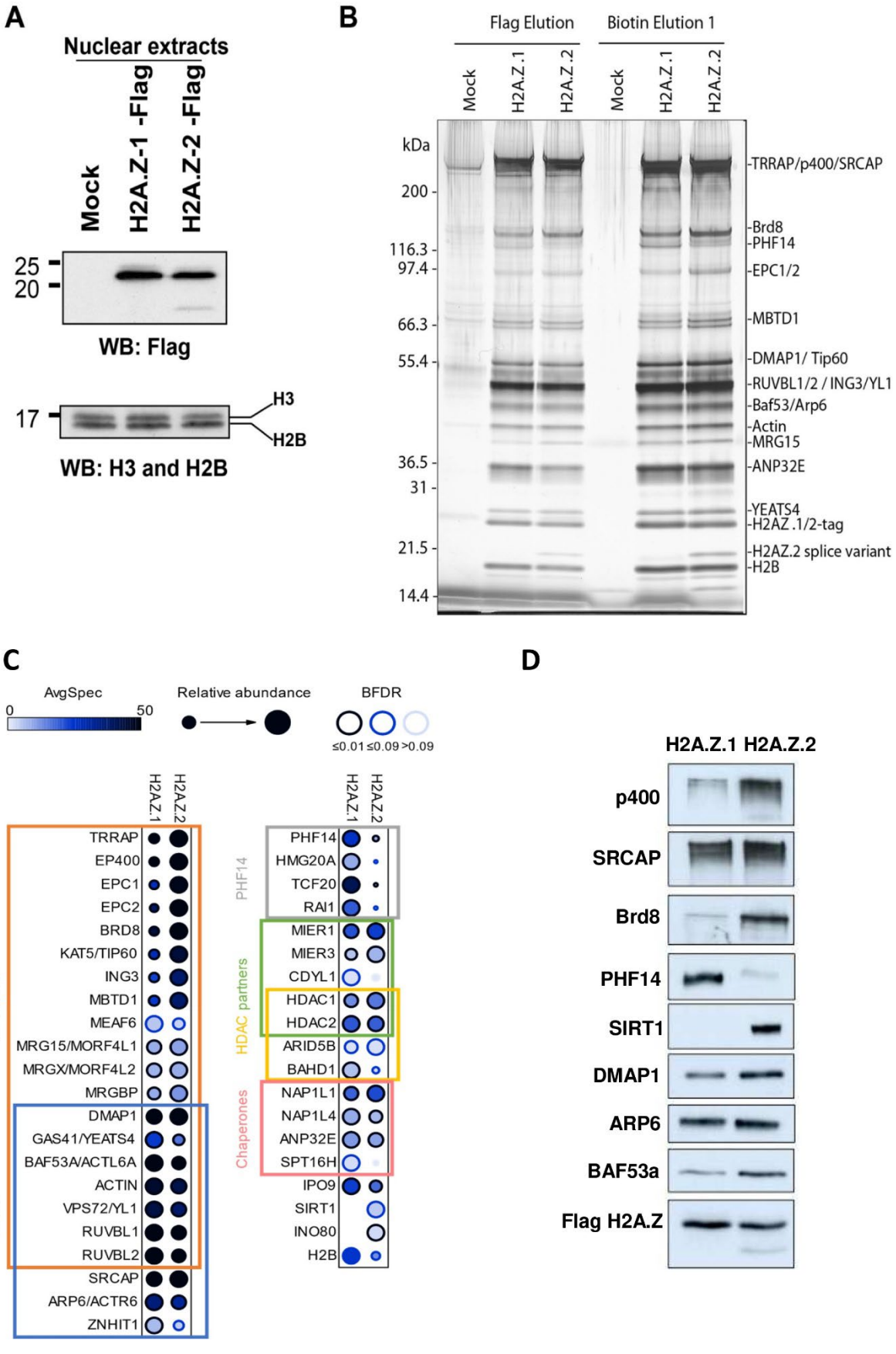

A
Nuclear extracts
Mock
H2A.Z-1 -Flag
H2A.Z-2 -Flag
25
20
WB: Flag
17
H3
H2B
WB: H3 and H2B
B
Flag Elution
Biotin Elution 1
Mock
H2A.Z.1
H2A.Z.2
Mock
H2A.Z.1
H2A.Z.2
kDa
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
-TRRAP/p400/SRCAP
-Brd8
-PHF14
-EPC1/2
-MBTD1
-DMAP1/ Tip60
-RUVBL1/2 / ING3/YL1
-Baf53/Arp6
-Actin
-MRG15
-ANP32E
-YEATS4
-H2AZ.1/2-tag
-H2AZ.2 splice variant
-H2B
C
AvgSpec
0
50
Relative abundance
BFDR
≤0.01 ≤0.09 >0.09
H2A.Z.1
H2A.Z.2
TIP60/p400
TRRAP
EP400
EPC1
EPC2
BRD8
KAT5/TIP60
ING3
MBTD1
MEAF6
MRG15/MORF4L1
MRGX/MORF4L2
MRGBP
SRCAP
DMAP1
GAS41/YEATS4
BAF53A/ACTL6A
ACTIN
VPS72/YL1
RUVBL1
RUVBL2
SRCAP
ARP6/ACTR6
ZNHIT1
PHF14
PHF14
HMG20A
TCF20
RAI1
HDAC partners
MIER1
MIER3
CDYL1
HDAC1
HDAC2
ARID5B
BAHD1
Chaperones
NAP1L1
NAP1L4
ANP32E
SPT16H
IPO9
SIRT1
INO80
H2B
D
H2A.Z.1 H2A.Z.2
p400
SRCAP
Brd8
PHF14
SIRT1
DMAP1
ARP6
BAF53a
Flag H2A.Z

Figure 3.5. Identification of differential H2AZ.1 and H2AZ.2 interactors.
(A) Comparison of expression levels of tagged endogenous H2A.Z.1 (heterozygous) and H2A.Z.2 (homozygous) clones used in tandem affinity purification from nuclear extracts (see Figure 3.5—figure supplement 1). (B) Silver-stained gel of fractions obtained for the purification of H2A.Z.1 and H2A.Z.2 from nuclear extracts of K562 cells shown in (A). A mock non-tagged cell line is used as control. Flag peptide elution is obtained from the first purification step (M2-Flag resin) and biotin elution from the second final step (Strep-Tactin resin). Known components of protein complexes interacting with histone H2A.Z are identified on the right (PHF14 is also indicated). (C) Dot-blot representation of AP-MS experiments using tagged H2A.Z.1 and H2A.Z.2 as baits. Circle filling represents average spectral counts, while circle diameter represents relative enrichment in one bait versus the other and circle border represents BFDR confidence level. Known/expected partners based on the literature and large-scale public data (BioGrid) include TIP60/p400, SRCAP and HDAC complexes. Data represent two replicates for each bait and were normalized on H2AZ-H2B chaperone levels (ANP32E, NAP1L1 and NAP1L4). (D) Western-blot validation of interactions shown in (B–C). TAP-purified fractions were normalized based on Flag-H2A.Z signals, loaded on SDS-PAGE gels, and blotted with the indicated antibodies.

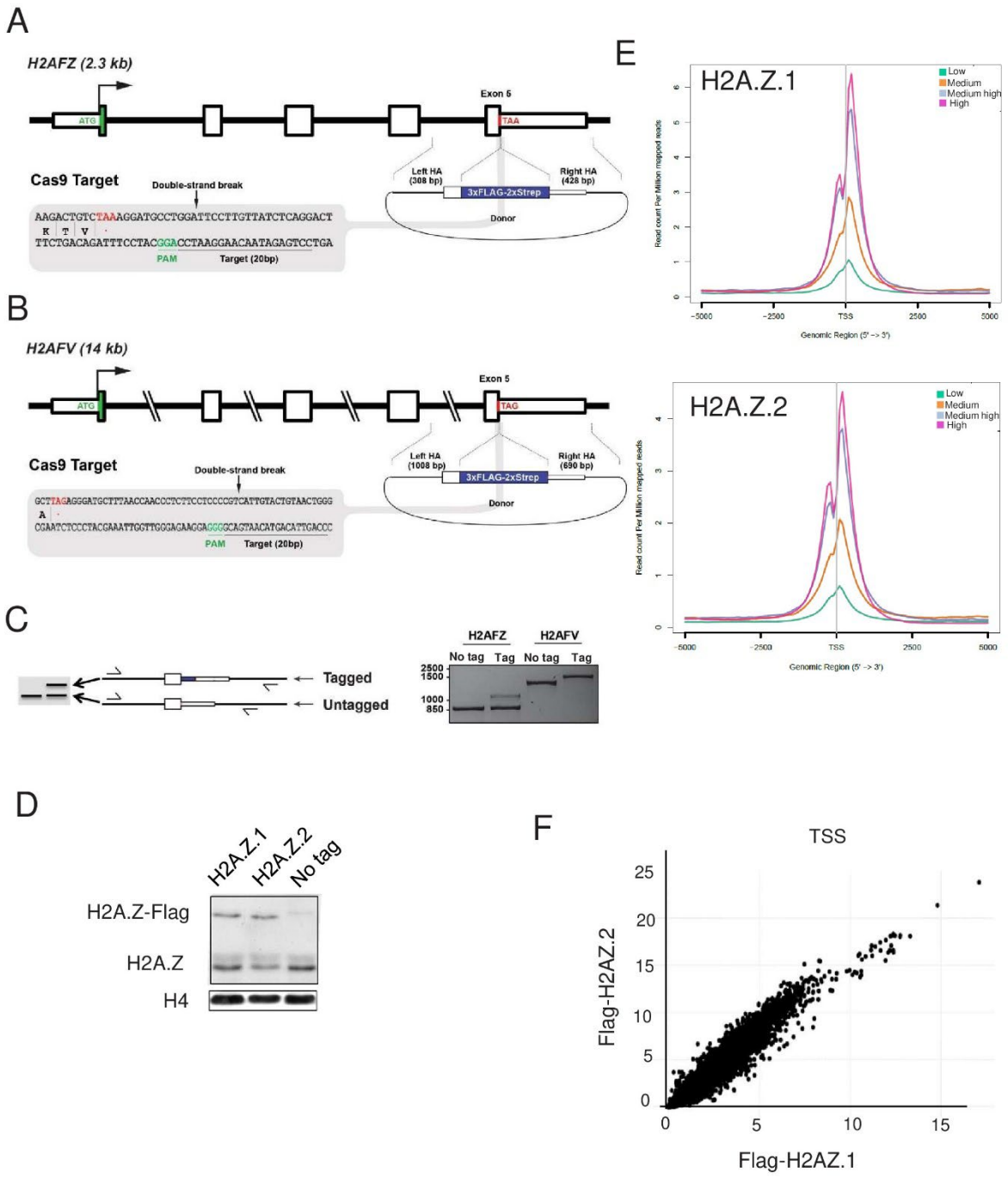


Figure 3.5-figure supplement 1. Tagging of H2A.Z isoforms by CRISPR/Cas9 in K562 cells used for characterisation of interactomes.

(A) Schematic of the H2AFZ locus (4q23), Cas9 target site, and donor construct used to insert the 3xFlag-2xStrep tag to the C-terminus of the H2A.Z.1 protein. Annotated are the positions of the stop codon (red), the PAM (green) that specifies the cleavage site, the gRNA target sequence, and the left and right homology arms (HA) used for HR-directed insertion. (B) Schematic of the H2AFV locus (7p13), depicted as in (A). (C) Schematic and results of a PCR-based assay (out-out PCR) on genomic DNA to detect targeted integration (TI) of

the tag sequence in single-cell-derived K562 clones obtained by limiting dilution. Primers are located outside of the homology arms and are designed to yield a longer PCR product if the tag is inserted, as described in Figure 3.1—figure supplement 1. Note that the H2A.Z.1-tagged clone is heterozygous, whereas the H2A.Z.2-tagged clone is homozygous. These two clones were used for subsequent analyses since tag expression levels were similar (D) Chromatin-enriched nuclear extracts from the indicated cell line were subjected to a total H2A.Z western blot. (E) K562 cells expressing endogenously tagged H2A.Z.1 or Z2 were subjected to a ChIP-Seq experiments. Protein-coding genes were ranked in 4 classes of equal number based on their expression levels. Meta data showing binding of H2A.Z.1 or H2A.Z.2 ChIP Seq signal around the TSS for the 4 classes of genes are shown. (F) The amount of H2A.Z.1 or Z2 in the −1000 to +1000 from each transcription start site of each protein-coding gene were calculated and plotted against each other. Note the striking correlation between the binding of Z1 and Z2.

A

		H2A.Z.1	H2A.Z.2
TIP60	TRRAP	321	492
	EP400	193	316
	EPC1	33	55
	EPC2	43	85
	BRD8	47	70
	KAT5/Tip60	33	52
	ING3	28	40
	MBTD1	31	42
	MRG15/MORF4L1	5	5
	MRGX/MORF4L2	2	2
	MRGBP	4	5
SRCAP	DMAP1	64	57
	GAS41/YEATS4	12	8
	BAF53a/ACTL6A	98	77
	Actin	43	33
	VPS72/YL1	48	37
	RUVBL1	180	182
	RUVBL2	172	172
	SRCAP	255	275
	ARP6/ACTR6	37	29
	ZNHIT1	3	0

		H2A.Z.1	H2A.Z.2
PHF14	PHF14	27	15
	HMG20A	12	2
	TCF20	44	15
	RAI1	18	7
HDAC partners	MIER1	24	25
	MIER3	6	8
	CDYL1	3	1
	HDAC1	12	13
	HDAC2	25	19
	ARID5B	3	3
	BAHD1	4	1
Chaperones	NAP1L1	20	22
	NAP1L4	8	8
	ANP32E	11	9
	SPT16H	4	1
	IPO9	31	20
	SIRT1	0	8
	INO80	0	2
	H2A.Z	8	7
	H2B	6	5

B

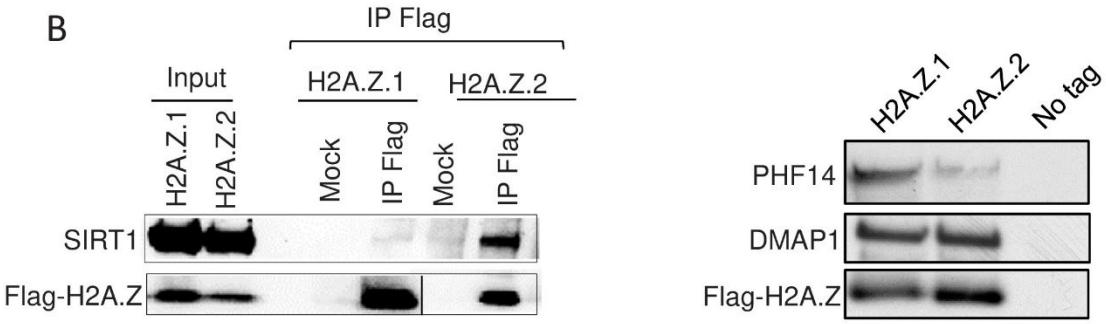


Figure 3.5-figure supplement 2. Mass spectrometry analysis of the H2A.Z.1/2 purifications shown in Figure 3.5B and validation in U2OS cells.
(A) Total spectral counts obtained in one experiment with each biotin elution fraction are shown and grouped by known complexes based on the literature and BIOGRID. (B) Nuclear extracts from U2OS expressing tagged H2A.Z.1 or tagged H2A.Z.2 were subjected to an immunoprecipitation using the indicated antibody or no antibody as a control. Immunoprecipitates were analysed by western blot using anti-SIRT1 and anti-Flag antibodies. Note that despite lower expression of H2A.Z.2, more SIRT1 was found associated with it. Note that PHF14 was undetectable in these experiments (data not shown), probably because of lack of good antibodies. We thus used higher amount of cells to purify H2A.Z-isoforms interacting proteins as performed in Figure 3.5 for mass spectrometry analysis (see below in (C)). (C) Soluble nuclear extracts from U2OS cells expressing either tagged H2A.Z.1, tagged H2A.Z.2 or no Tag as indicated was subjected to tandem affinity purification (anti-Flag resin followed by Flag peptide elution). The amount of cells was adapted to have approximately the same amount of immunoprecipitated H2A.Z isoforms. Flag-eluted proteins were analysed by western blot using the indicated antibody. Note the higher amount of PHF14 found in the H2A.Z.1 fraction although amounts of purified H2A.Z1 and DMAP levels were higher in the H2A.Z.2 fraction.

In these conditions, PHF14 was found exclusively at the chromatin (Figure 3.6A), and this irrespective of the siRNA used (data not shown). We thus tested fractionation conditions in which we extract proteins from chromatin using high salt conditions (420 mM NaCl). In these conditions, we observed that PHF14 is present both in the chromatin fraction and in the soluble nuclear fraction (Figure 3.6B). Depletion of H2A.Z.1 (see the H2A.Z western blot shown in Figure 3.6—figure supplement 1) does not lead to a reproducible decrease of PHF14 in the chromatin fraction (Figure 3.6B and Figure 3.6—figure supplement 2 for the quantification and a replicate). However, we consistently observed an increase of PHF14 amounts in the soluble nuclear fraction. Since there is no PHF14 in the cytoplasmic fraction in any of the conditions tested, this fractionation procedure resulted in higher PHF14 extraction from chromatin upon H2A.Z.1 knockdown, suggesting that H2A.Z.1 favours the interaction of PHF14 with chromatin.

Taken together, these results are consistent with the hypothesis that H2A.Z.1 and H2A.Z.2-containing nucleosomes provide docking sites in chromatin for PHF14 and SIRT1 respectively.

To investigate whether PHF14 and SIRT1 could be responsible for the effect of H2A.Z.1 and H2A.Z.2 on gene expression, we analysed whether PHF14 and SIRT1

depletion could phenocopy H2A.Z isoform depletion. To do so, we transfected immortalized WI38 human primary fibroblasts with siRNAs against SIRT1 or PHF14. These siRNAs were efficient as verified at the mRNA level by RT-qPCR and at the protein level by western blot analysis (Figure 3.6—figure supplement 3). Furthermore they had no effect on total H2A.Z or H2A.Z.1 or H2A.Z.2 mRNA expression levels (Figure 3.6—figure supplement 3). Analysis of specific gene expression by qPCR indicated that on four out of the five genes we analysed, PHF14 depletion induces changes in a similar way compared to H2A.Z.1 depletion (Figure 3.6C, compared to Figure 3.2—figure supplement 1). Similar results were obtained when comparing the effects of SIRT1 depletion with H2A.Z.2 depletion, with three out of the five analysed genes regulated in a similar manner (Figure 3.6C). Note however that fold changes on these genes upon depletion of H2A.Z isoforms or their effectors can be very different, indicating that other mechanisms may take place.

To confirm this finding at the genome wide level, we performed RNA-Seq analysis following depletion of SIRT1 and PHF14. We found 4189 and 2405 genes significantly regulated by PHF14 and SIRT1 respectively (see Supplementary files 3.11 and 3.12 for the complete list of de-regulated genes upon PHF14 and SIRT1 depletion, respectively). Strikingly, we observed that 30.6% of up-regulated genes and 38.5% of down-regulated genes in the siH2A.Z.1 condition were similarly regulated when PHF14 was silenced, which is a highly significant intersection ($p=8,4*10^{-197}$and 0 respectively) (Figure 3.6D). Likewise, 24.7% of up-regulated genes and 27.2% of down regulated genes in the siH2A.Z.2 condition were de-regulated in a similar way in the siSIRT1 condition (Figure 3.6E).

Thus, taken together, these data indicate that PHF14 and SIRT1 are major mediators of H2A.Z.1- and H2A.Z.2-specific gene regulation, since i) depletion of H2A.Z isoforms specifically affects their localisation ii) they affect transcription of a significant proportion of H2A.Z-regulated genes in a manner similar to the H2A.Z isoforms. Given that these effects can be positive or negative, our data further underline the function of PHF14 and SIRT1 in mediating the context-dependent regulation of specific gene transcription by H2A.Z isoforms.

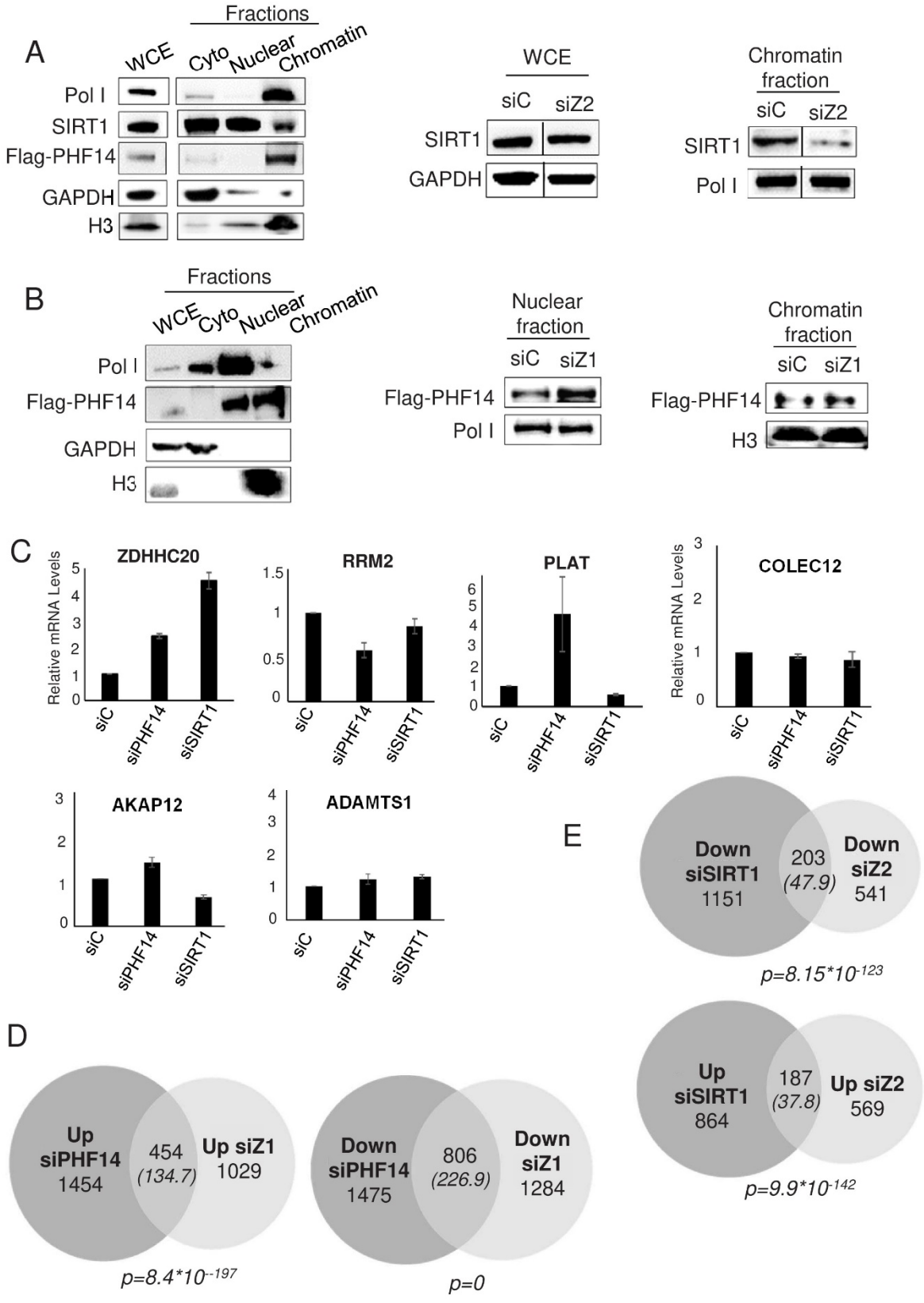


Figure 3.6. PHF14 and SIRT1 mediate H2A.Z.1 and H2A.Z.2 functions respectively.

(A) Left panel: U2OS cells expressing endogenously tagged PHF14 were subjected to cell fractionation experiments in mild conditions. Cell fractions were then subjected to western blot analysis using the indicated antibody. Right panel: U2OS cells expressing endogenously tagged PHF14 were transfected with the indicated siRNA and analysed 72 hr later as in the left panel. A representative experiment from three independent experiments are shown (see Figure 3.6—figure supplement 2 for a replicate) B) Left panel: U2OS cells expressing endogenously tagged PHF14 were subjected to cell fractionation experiments using NP40. Cell fractions were then subjected to western blot analysis using the indicated antibody. Right panel: U2OS cells expressing endogenously tagged PHF14 were transfected with the indicated siRNA and analysed 72 hr later as in the left panel. A representative experiment from three independent experiments are shown (see Figure 3.6—figure supplement 2 for a replicate) C) WI38 cells were transfected with the indicated siRNA. 72 hr later, total RNA was prepared and analysed by RT-qPCR to assess the expression of the indicated genes. Data was standardised using GAPDH mRNA levels and calculated relative to one for cells transfected with the control siRNA. The mean and SDOM from three independent experiments are shown. (D and E) WI38 cells were transfected with the indicated siRNA. 72 hr later, total RNA was prepared and further purified to be subjected to RNA-Seq. Genes differentially expressed upon PHF14 or SIRT1 depletion were characterised. The Venn diagrams indicating the intersection of genes regulated by PHF14 and H2A.Z.1 (D) or by SIRT1 and H2A.Z.2 (E) are shown. The p value indicates the significance of the intersection (Chi square test) considering the total number of expressed genes. The numbers in brackets indicate the expected number of genes considering the total number of expressed genes if intersection was random.

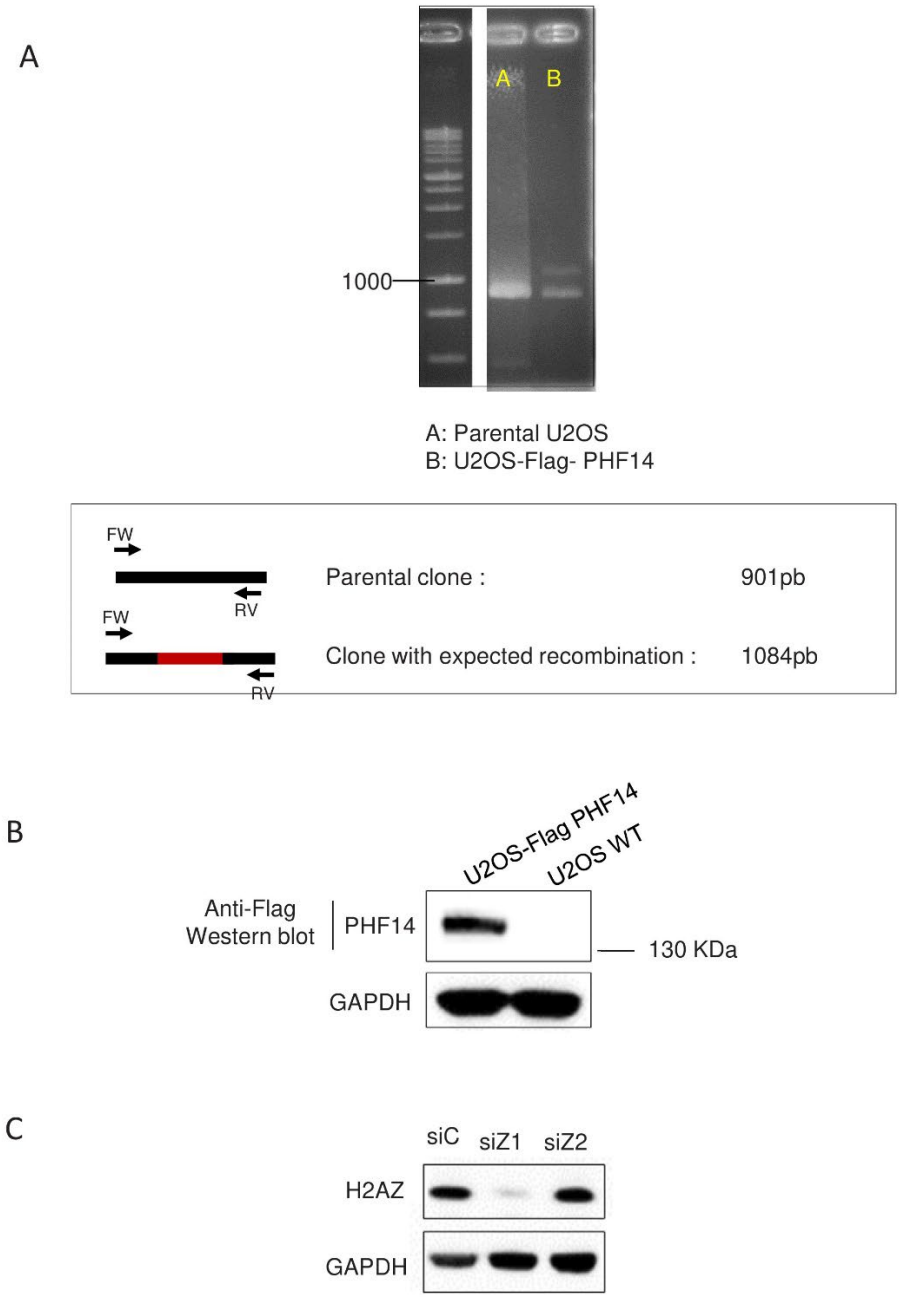


Figure 3.6-figure supplement 1. Characterisation of U2OS cells expressing 3xFlag-2xStrep tagged PHF14.
(A) Genomic DNA samples were subjected to a PCR reaction using primers located outside of the inserted sequence as shown in the scheme (Bottom). The top panels show an analysis on agarose gel of the PCR product obtained on a negative clone and on the genome-edited clone used in this study. Note the upper band which appeared in the genome-edited clone expressing Flag-PHF14, indicating recombination at one allele. The bands were excised and sequenced to check the accuracy of the recombination. (B) Total cell extracts from U2OS-Flag PHF14 cells or from parental U2OS cells were prepared and analysed by western blot using anti-Flag antibody. (C) H2A.Z western blot performed on total cell extracts in the experiment shown in Figure 3.6A.

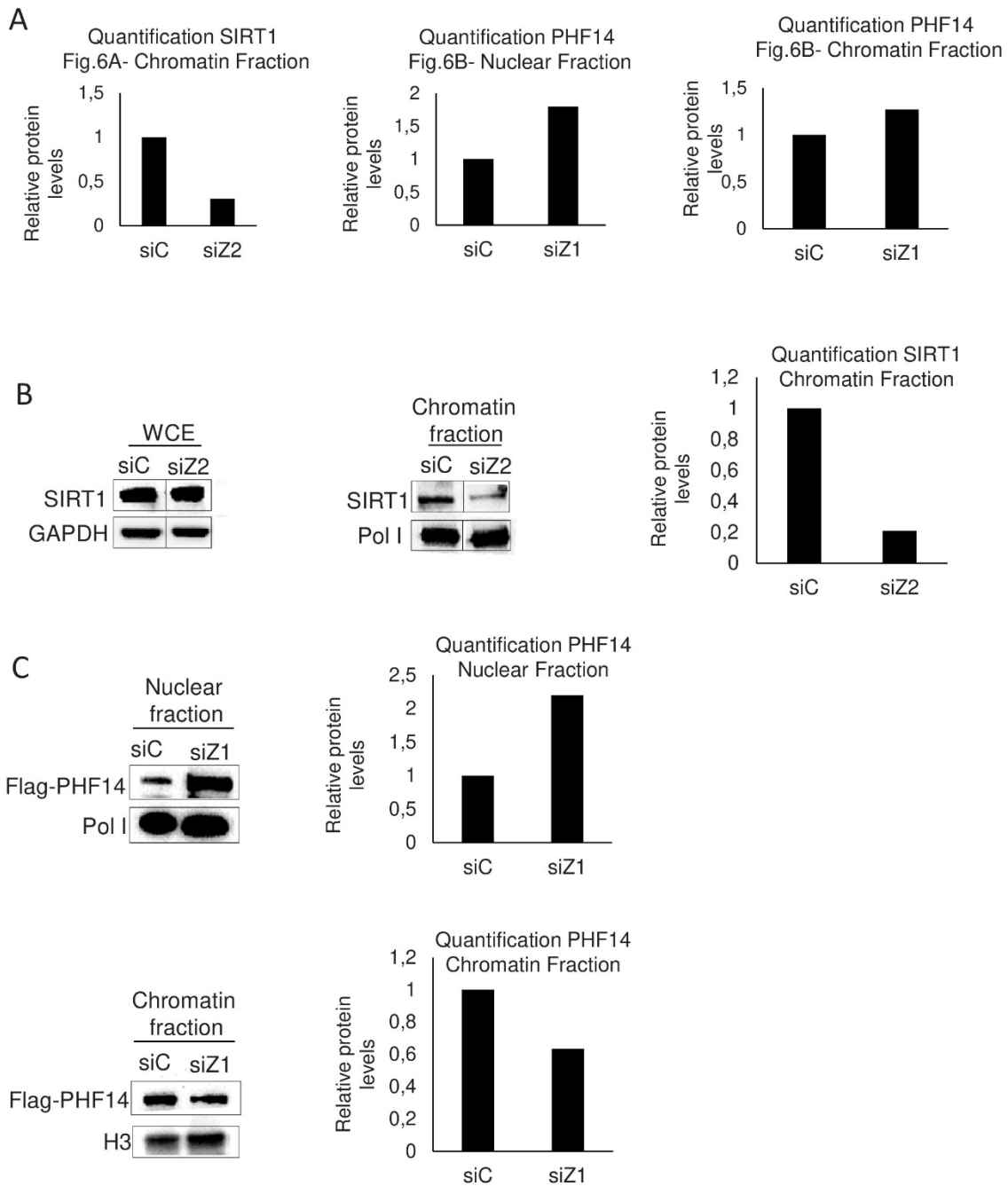


Figure 3.6-figure supplement 2. Quantification and replicate of Figure 3.6A and B experiments.

(A) The indicated bands from the experiment shown in Figure 3.6A and B were quantified using image J. (B) Replicate of Figure 3.6A experiments and its quantification. (C) Replicate of Figure 3.6B experiments and its quantification. Note the reproducible increase of PHF14 expression in the soluble nuclear fraction upon Z1 depletion, although no decrease of PHF14 expression in the chromatin fraction can be observed, which suggests that PHF14 expression is slightly induced upon H2A.Z.1 depletion. Since PHF14 mRNA expression levels do not change (data not shown), it perhaps reflects a slightly increased stability of PHF14 when H2A.Z.1 expression is depleted.

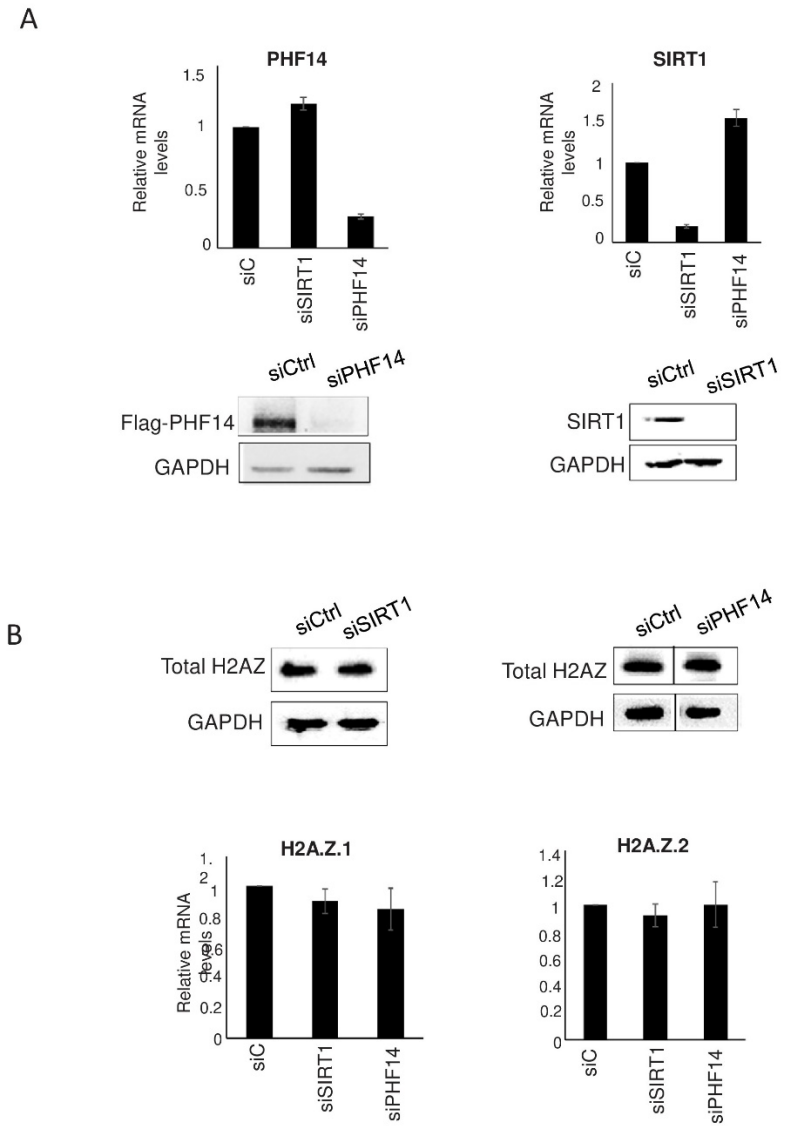


Figure 3.6-figure supplement 3. Validation of siRNAs targetting SIRT1 and PHF14. (A) *Validation of PHF14 and SIRT1 siRNA.* Top: WI38 cells were transfected using siRNA against SIRT1 or PHF14. 72 hr later, total RNAs was prepared. The amount of SIRT1 and PHF14 mRNA was quantified by RT-qPCR, and was standardised using GAPDH mRNA levels and calculated relative to one in cells transfected using the control siRNA. The mean and SDOM from seven independent experiments are shown. Note that SIRT1 depletion seems to slightly favours PHF14 expression and vice-versa. Bottom: Total cell extracts from WI38 cells were also prepared and the depletion of SIRT1 protein was verified by western blot using specific antibody. Since commercial PHF14 antibody has poor quality in western blot, total cell extracts from U2OS Flag-PHF14 cells were used to perform western blot with anti-Flag antibody after siPHF14 transfection. (B) *Effect on H2A.Z.1 and H2A.Z.2 expression.* WI38 cells were transfected as in A. Levels of total H2A.Z protein were analysed by western blot. Total RNA was also prepared and the amount of the two isoforms levels was verified by RT-qPCR, standardised using GAPDH mRNA levels and calculated relative to one in cells transfected using the control siRNA. The mean and SDOM from three independent experiments are shown.

PHF14 and SIRT1 can mediate the antagonistic relationship between H2A.Z.1 and H2A.Z.2

Strikingly, when we analysed the PLAT mRNA, we found that co-depletion of SIRT1 along with H2A.Z.1 or PHF14 also abolishes the effects of H2A.Z.1 and PHF14 depletion (Figure 3.7A). This shows that SIRT1 mediates the antagonistic effect of H2A.Z.1 and H2A.Z.2 on PLAT expression. Very similar results were observed for the AKAP12 mRNA concerning it specific repression by H2A.Z.2 (which is mimicked by depletion of SIRT1). Indeed, not only is this repression attenuated upon co-depletion of H2A.Z.1 along with H2A.Z.2 or SIRT1, as shown in Figure 3.3, but also upon co-depletion of PHF14 along with H2A.Z.2 or SIRT1 (Figure 3.7A). Consistently, H2A.Z.2-dependent activation of the ADAMTS1 and COLEC12 mRNAs was attenuated upon depletion of H2A.Z.1 or PHF14 (Figure 3.7—figure supplement 1A). Thus, these results indicate that PHF14 and SIRT1 can antagonise each other, at least for the four genes we tested.

We next investigated the mechanism involved in this antagonism. Given that SIRT1 is a protein deacetylase, we tested whether PHF14 could favour protein acetylation. Indeed, PHF14 and associated proteins were already proposed to function by counteracting the function of the repressive LSD1-CoREST complex, which contains histone deacetylases along the H3K4 demethylase (Garay et al., 2016; Wynder et al., 2005). Therefore, we depleted PHF14 using a specific siRNA and assessed whether it affected global site-specific histone acetylation by western blot analysis. Importantly, depletion of PHF14 strongly decreased global histone H3K9 acetylation, a known SIRT1-target sites (Vaquero et al., 2004), while it had only a mild effect, if any, on histone H3K14 acetylation levels (Figure 3.7B, see Figure 3.7—figure supplement 1B for a replicate) To test whether this global change in H3K9 acetylation could also be observed at promoters regulated by H2A.Z.1 and H2A.Z.2, we performed a ChIP assay against this acetylation mark. Spike-in chromatin was added to the samples as an internal control to increase accuracy of the ChIP measurements. We found that PHF14 depletion led to a decrease in H3K9 acetylation at two promoters regulated by H2A.Z.1 and H2A.Z.2, the *RRM2* and *AKAP12* promoters (Figure 3.7C). Note that no major effect could be observed on

two not targeted promoters (*GAPDH* and *β-actin*) and on the H2A.Z.1-regulated *PLAT* promoter Thus, altogether these data indicate that PHF14 favours histone acetylation, at least at the *RRM2* and *AKAP12* promoters.

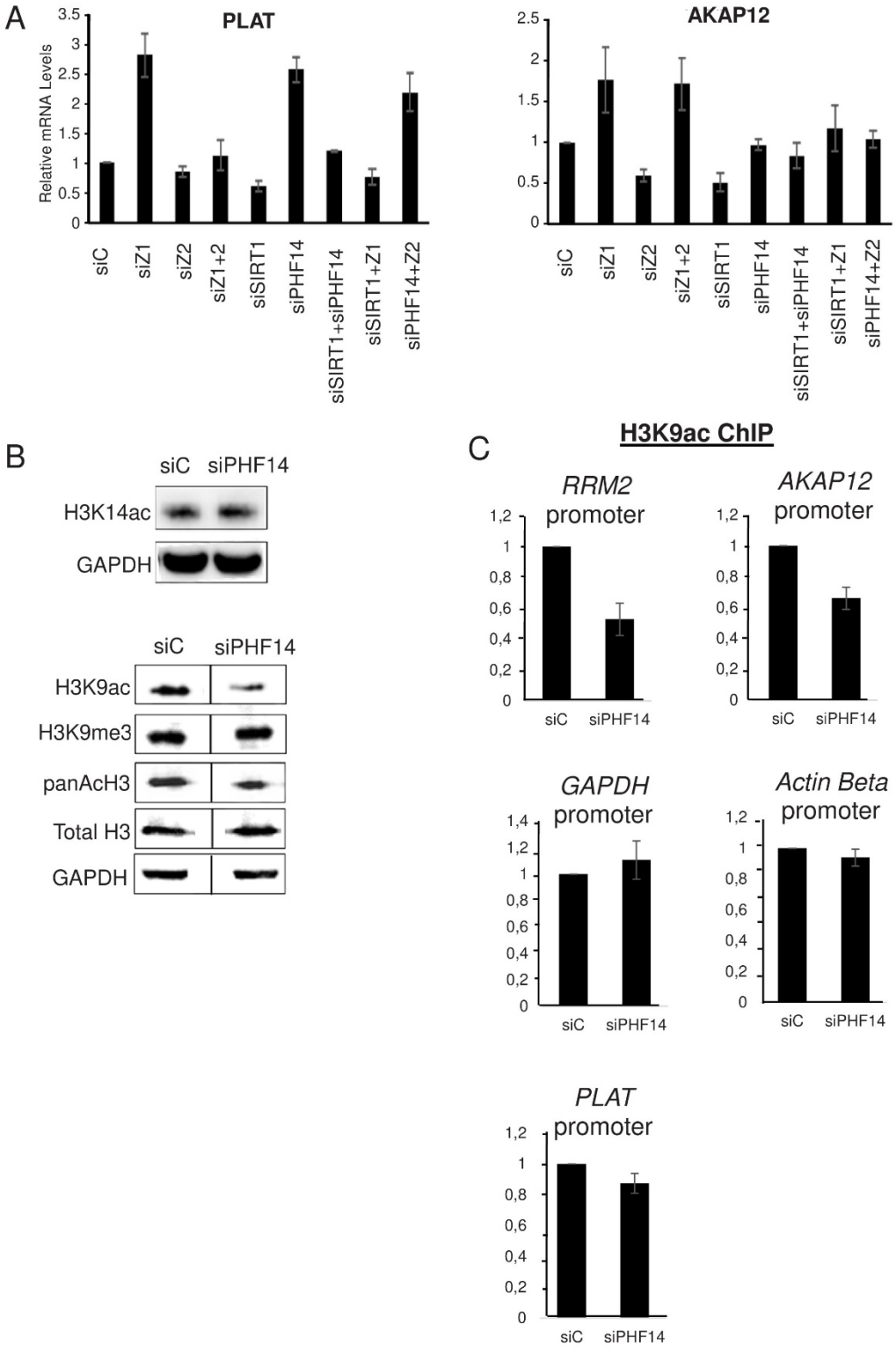


Figure 3.7. PHF14 and SIRT1 can mediate the antagonism between H2A.Z.1 and H2A.Z.2.
(A) WI38 cells were transfected with the indicated siRNAs alone or in combination. 72 hr later, total RNA was prepared and analysed by RT-qPCR to assess the expression of the indicated genes. Data was standardised using GAPDH mRNA levels and calculated relative to one for cells transfected with the control siRNA. The mean and SDOM from three independent experiments are shown. (B) WI38 cells were transfected with the indicated siRNA. 72 hr later, whole cell extracts were prepared and subjected to western blot analysis using the indicated antibody. A replicate is shown in Figure 3.7—figure supplement 1. (C) WI38 cells were transfected with the indicated siRNA. 72 hr later, transfected cells were subjected to a ChIP assay using antibodies directed against H3K9ac (left) or anti-H3 (right).*Drosophila*or mouse spike-in chromatin was added after sonication as an internal control. The amount of the indicated human promoters was quantified by qPCR, standardised using the spike-in signal and calculated relative to one for control cells. The mean and SDOM from three independent experiments are shown.

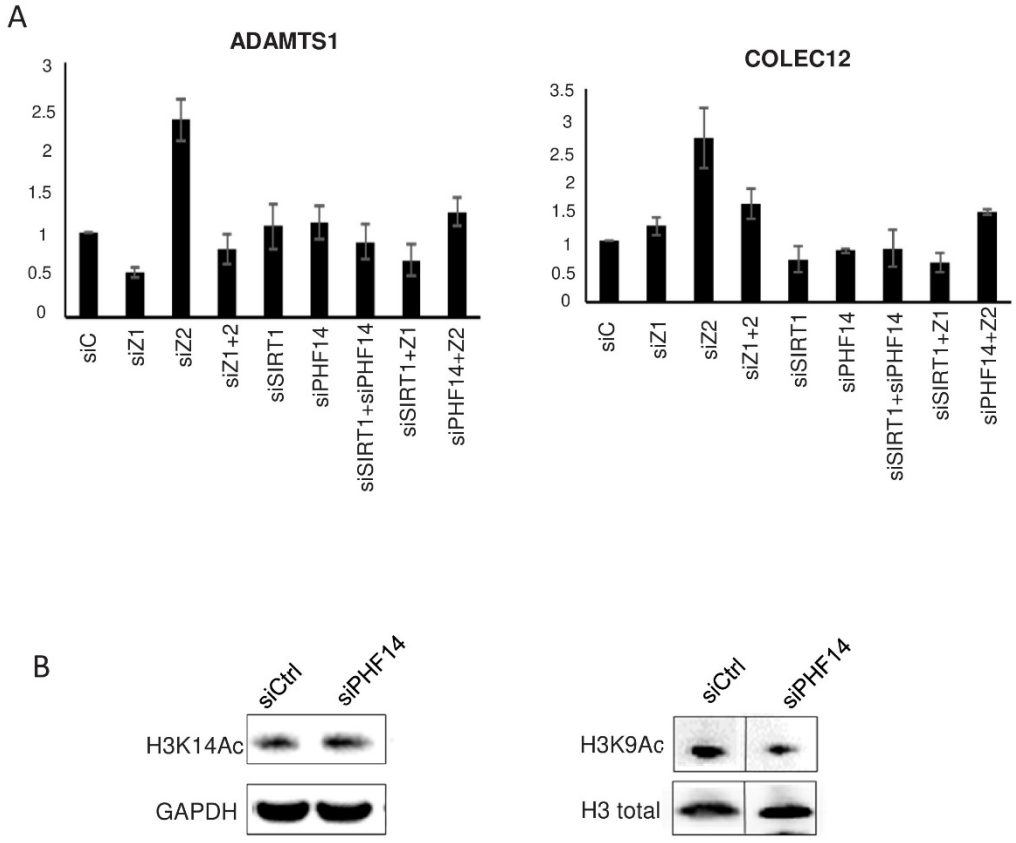


Figure 3.7-figure supplement 1. PHF14 mediates antagonistic effect of H2A.Z.1 and H2A.Z.2 on ADAMTS1 and COLEC12 mRNA expression.
(A) WI38 cells were transfected using the indicated siRNA alone or in combination. 72 hr later, total RNA was prepared and analysed by RT-qPCR to assess the expression of the indicated genes. Data were standardised using GAPDH mRNA levels and calculated relative to one in cells transfected using the control siRNA. The mean and SDOM from three independent experiments are shown. (B) Replicate of Figure 3.7B experiment.

3.5 Discussion

Here, we performed an integrated study of the role of H2A.Z isoforms H2A.Z.1 and H2A.Z.2 on gene expression in non-transformed cells.

We first confirmed that H2A.Z is a major regulator of specific gene expression. If we take into accounts all genes differentially regulated upon depletion of H2A.Z.1, H2A.Z.2 or both together, we found that 5005 and 6317 genes are deregulated in WI38 and U2OS cells, which represent roughly 1/4th of all expressed genes. Interestingly, the number of genes activated by either H2A.Z isoform is roughly equivalent to the number of genes they repress, in agreement with previous studies underlining a positive and negative role for H2A.Z in gene expression (Dunn et al., 2017; Subramanian et al., 2015).

We also found that there are many genes at which H2A.Z isoforms play similar roles including some on which they can compensate each other. This compensation probably explains why a strong phenotype is observed upon depletion of both H2A.Z isoforms in mouse intestinal stem cells whereas depletion of individual isoforms had no or weak effects (Zhao et al., 2019). At these promoters, the presence of H2A.Z could regulate gene expression by affecting nucleosome structure or stability, or by recruiting proteins leading to similar outcomes on gene expression.

We further found that both isoforms regulate different sets of genes, as previously shown (Dunn et al., 2017; Greenberg et al., 2019), again confirming that despite their high similarity, they can play different roles. Finally, we found that on a significant number of genes, H2A.Z.1 and H2A.Z.2 can play opposite roles, with H2A.Z.1 acting as an activator and H2A.Z.2 as a repressor. Interestingly, at these two gene populations, analysis of the double depleted samples uncovers a general antagonism between H2A.Z.1 and H2A.Z.2.

Altogether, these data underline that H2A.Z isoforms H2A.Z.1 and H2A.Z.2 can regulate gene expression similarly and antagonistically depending on the gene, uncovering a complex interplay.

We also provide major insights into the mechanism of this interplay. By ChIP-Seq analysis, we found H2A.Z isoforms are present on most promoters of expressed genes or genes prone to be expressed, as already shown for total H2A.Z (Barski et al., 2007). Clearly, the presence of a H2A.Z isoform around a TSS is not sufficient to predict the effect of this isoform on gene transcription. Rather, our ChIP-Seq data indicate that H2A.Z.1 and H2A.Z.1 are incorporated at the same promoters and we found that they can replace each other. This replacement could explain the compensation between the two isoforms we observe for the regulation of many promoters.

These data also suggest that the two isoforms compete each other for the binding to gene promoters. As a consequence, at a promoter specifically regulated by a given isoform, the presence of the other isoform would counteract the presence and the function of the regulatory isoform. Such a model would explain the rather general antagonism we observe on genes specifically regulated by an isoform. It underlines the functional importance of the relative levels between the two isoforms.

Thus, the same mechanism, that is the ability to be incorporated at the same promoters, could explain both the compensation between H2A.Z isoforms at some promoters and their antagonism at others. It also fits with the fact that they can both interact with incorporation machineries (p400- and SRCAP complexes).

We next investigated how H2A.Z isoforms could differentially regulate gene expression. We identified proteins interacting preferentially with one isoform or the other, that is PHF14 with H2A.Z.1 and SIRT1 with H2A.Z.2. To our knowledge, this is the first demonstration that endogenous H2A.Z.1 and H2A.Z.2 can differentially bind to proteins. Note however that it was previously found using overexpressed proteins that H2A.Z.1 nucleosomes were slightly more efficient than H2A.Z.2 nucleosomes in interacting with BRD2 as well as with PHF14, HMG20A and TCF20 (Draker et al., 2012; Pünzeler et al., 2017).

A functional interaction between H2A.Z and SIRT1 in the nucleus was also already described (Baptista et al., 2013; Chen et al., 2006). The authors propose that

deacetylation of H2A.Z by SIRT1 leads to its destabilization. However, we did not find any effect of SIRT1 depletion on H2A.Z expression in our hands. Moreover, such a mechanism would not explain the similarities in gene expression control by H2A.Z.2 and SIRT1. We rather found that depletion of H2A.Z.2 decreased the nuclear retention of SIRT1, in agreement with the interpretation that the H2A.Z.2/Sirt1 interaction is required for recruitment of SIRT1 in the nucleus.

Functional analysis of PHF14 and SIRT1 indicates that they can mediate, at least in part, the specific effects of H2A.Z.1 and H2A.Z.2 on gene expression. In particular, we provide evidence that PHF14 and SIRT1 can mediate the functional antagonism between both H2A.Z isoforms. These two proteins would function as readers of the presence of a specific H2A.Z isoform at gene promoters. Upon depletion of one isoform, the other isoform takes over, resulting in changes in the amount of promoter-bound PHF14 and/or SIRT1. These changes may in turn affect gene expression in a context-dependent manner. Importantly, we found that PHF14 favours acetylation of histones, at least on H3K9 residues at its target promoters. Thus, this may provide a mechanism by which PHF14 and SIRT1 mediate opposite effects on gene expression: when H2A.Z.1 is depleted, there is less recruitment of PHF14 and more recruitment of SIRT1, shifting the balance towards local deacetylation, whereas when H2A.Z.2 is depleted, the balance is shifted towards local acetylation. Such changes in acetylation of histones or non-histone proteins could mediate the context-dependent regulation of gene expression. However, the fact that there are many genes regulated by H2A.Z isoforms H2A.Z.1 and H2A.Z.2 but not by PHF14 and SIRT1 suggests that other mechanisms mediate H2A.Z gene expression regulation, such as changes in nucleosome stability, as previously proposed, or interaction with other specific effectors.

Our data thus indicate that the relative levels of H2A.Z isoforms is probably a major determinant of gene regulation and that changes in these levels lead to important changes in gene expression. Interestingly, the two H2A.Z isoforms are produced by two genes with two independent promoters (Matsuda et al., 2010), providing a basis for the regulation of the relative isoforms levels by signaling pathways. In this

respect, it will be of great interest to decipher the transcription factors and the signalling pathways regulating H2A.Z promoters. For example, we recently showed that the *H2AFZ* promoter is a direct target of the Wnt signalling pathway (Rispal et al., 2019). Furthermore, it is known that the expression of both isoforms is regulated in a tissue- and developmental stage- dependent manner (Dryhurst et al., 2009). Our data suggest that these changes are essential for establishing the correct gene expression pattern associated with specific developmental pathways.

In addition to regulation of the H2A.Z.1/H2A.Z.2 relative levels, the potential regulation of the association between PHF14, SIRT1 or other readers with H2A.Z isoforms is clearly worth investigating. Our work points to the importance of the three residues differing between the two isoforms in the binding of specific partners. Strikingly, one of these differences (T14 in H2A.Z.1, A14 in H2A.Z.2) is located in the N-terminal tail basic patch important for H2A.Z function and target of multiple post-translational modifications such as acetylation, methylation or ubiquitylation. Furthermore, T14 of H2A.Z.1 could itself be phosphorylated which would likely affect adjacent lysine modifications (K13/15). Acetylation of H2A.Z has indeed been linked to transcriptional activation by many studies (Bruce et al., 2005; Millar et al., 2006; Valdés-Mora et al., 2012). It is tempting to speculate that post-translational modifications of this T in H2A.Z.1 or surrounding amino acids in H2A.Z.1 or H2A.Z.2 could regulate the recruitment of H2A.Z.1 and H2A.Z.2 effectors, among which PHF14 and SIRT1, and therefore participate in the regulation of specific gene expression. In agreement with this hypothesis, the PHF14-complex contains many different PhD fingers, suggesting that it could function as a reader of H2A.Z.1 post-translational modifications.

Interestingly, a recent study showed that a S38T substitution in H2A.Z.1, mimicking T38 found in H2A.Z.2, rescues the SRCAP-dependent Floating-Harbor Syndrome (Greenberg et al., 2019). Understanding the molecular determinants of PHF14 complex and SIRT1 binding to H2A.Z isoforms will be a first step towards the identification of the mechanisms underlying the context-dependent regulation of transcription by H2A.Z isoforms.

3.7 Materials and methods

Cell cultures and transfections

Lung Fibroblastic cells WI38 hTERT RAF1-ER, which are immortalized by hTERT expression and contain an inducible RAF1 oncogene fused to estrogen receptor (ER) (Jeanblanc et al., 2012), were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), sodium pyruvate, L-glutamine, non-essential amino acids and penicillin-streptomycin, in normoxic culture conditions (5%O2).

Osteosarcoma U2OS cells were obtained from the ATCC and were grown in DMEM media with Glutamax (1 g/L glucose), supplemented with 10% of FBS, sodium pyruvate and penicillin-streptomycin.

K562 cells were obtained from the ATCC and maintained at 37°C under 5% CO_2 in RPMI medium supplemented with 10% newborn calf serum (Wisent) and GlutaMAX. When cultivated in spinner flasks, 25 mM HEPES-NaOH (pH 7.4) was added. Cells were transfected using the Amaxa 4D-Nucleofector (Lonza) per the manufacturer's recommendations.

All cell lines were regularly checked for the absence of mycoplasma contamination.

siRNA transfection was performed using the Dharmafect four reagent (Dharmacon) according to the manufacturer's instructions. 24 hr before transfection, 1.4 million of Wi-38 cells, and 850,000 of U2OS cells were plated in 10 cm dish. 100 nM of siRNA were used, and an equal volume of the culture medium was added 24 hr after transfection. 48 hr later, cells were harvested. The list of siRNAs used is indicated in Supplementary file 3.13. DNA plasmids were transfected with jet-PEI reagent (polyplus) according to manufacturer's instructions.

Tagging of endogenous proteins in K562 cells

Cytomegalovirus (CMV)-driven human-codon optimized Cas9 nuclease (Addgene #41815) was used. gRNA sequences targeting *H2AFZ* and *H2AFV* were selected using a web-based tool (https://www.benchling.com/, using algorithms from (Hsu et

al., 2013) and (Doench et al., 2016) and validated by Surveyor assay (Integrated DNA Technologies). gRNA expression vectors were built in the MLM3636 (Addgene #43860) backbone. The donor plasmid for *H2AFZ* was synthesized as a gBlock gene fragment (Integrated DNA Technologies) and assembled using the Zero Blunt TOPO cloning kit (Invitrogen). The donor plasmid for *H2AFV* was obtained by cloning. Briefly, a PCR fragment of 1.7 kb genomic DNA (1008 bp before stop codon and 690 bp after stop codon) was integrated in Zero Blunt TOPO plasmid following manufacturer's instructions. Subsequently, the PAM motif corresponding to the gRNA was mutated and the 3xFlag-2xStrep sequence (Dalvai et al., 2015) was integrated before the stop codon using Gibson assembly kit (NEB, E5510).

The i53 53BP1 inhibitor (Addgene #74939, (Canny et al., 2018), gift from Amélie Fradet-Turcotte) was used to increase tagging efficiency of *H2AFZ* by homology directed repair. One million K562 cells were transfected using 2 µg gRNA plasmid, 2 µg Cas9 vector, and 4 µg donor (plus 600 ng i53 plasmid for *H2AFZ*). Limiting dilution cloning was performed 3 days post-transfection, and targeted clones were identified via out-out PCR as before (Dalvai et al., 2015).

Generation of genome-edited cell clones in U2OS cells

We used the ouabaine based co-selection strategy described by (Agudelo et al., 2017). This consists of the co-transfection of an RNA guide + DNA donor that are able to give the cell a resistance to a drug, ouabaine, by inducing a mutation in the ATP1A1 gene (Na+/K+ pump). For this, we cloned our RNA guides of interest in a modified pX330 plasmid (Addgene #86616, ATP1A1 G3 dual sgRNA) containing in addition to the CRISPR cas9 enzyme, the RNA guide needed to mutate ATP1A1 gene.

RNA guides for targetting *H2AFZ* and *H2AFV* are described above. RNA guides for the Flag-PHF14 CRISPR were selected using the CRISPOR website (http://crispor.tefor.net/). Primers were then chosen according to the website proposition. To clone guides, a phosphorylation step consisting on incubation of 1 µl of each oligo (100 µM) at 37°C for 30 min in the presence of the PNK enzyme

(Promega) was performed. Then, samples were heated for 5 min at 95°C, and cooled over night at room temperature. The annealed phosphorylated product was then digested-ligated in a one step reaction into the ATP1A1 G3 dual sgRNA plasmid in the presence of bbs1 restriction enzyme (NEB) and T7 ligase (NEB), and the reaction was subjected to the following PCR cycle (37°C 5 min, 25°C 5 min, 6 times). The product of the ligation was exonuclease-digested using exonuclease V RecBCD for 30 min at 37°C. Competent cells were then transformed with the final product, and positive clones were selected by bbs1 digestion. 200,000 U2OS cells plated in 6-well plates were transfected with this plasmid, a double stranded 3xFlag-2xStrept tagged PHF14 DNA donor (300 bp, ordered from GeneScript) or donor plasmids for H2AFZ and H2AFV described above and Ouabaine-resistance donor (ordered from Addgene #66551, ATP1A1 plasmid donor) and guide to a final DNA concentration of 500 ng, using jetPEI polyplus reagent according to manufacturer's instructions. 48 hr later, cells were trypsinised and transferred into 10 cm dish, and ouabaine (sigma) was added to a final concentration of 0.5 µM, for one week. Clones were then recovered and screened by PCR for the presence of Flag-tag. Positive clones were verified by sequencing. Primers for each guide are detailed in Supplementary file 3.13.

Antibodies and immunoblotting

Total Cell extracts were prepared and analysed by standard Western blotting protocol, using antibodies against total H2AZ (ab4174, Abcam), GAPDH (clone 6C5, MAB374, Millipore), PHF14 (SAB3500960, Sigma and proteintech 24787–1-AP), SIRT1 (Clone E104, ab32441, Abcam), Flag (Clone M2, F1804, Sigma), Pol I (Clone RPA194, sc48385, Santa Cruz), H3 (ab1791, Abcam), pan Acetyl-H3 (6599, Upstate), Acetyl-H3K9 (06942, Upstate), Flag-HRP (Sigma, A8592, lot #GR08726011-2013), Brd8 (Bethyl A300-219A), DMAP1 (Aff. BioReag., PA1-886), ACTR6(ARP6) (Abcam, ab208830), p400 (Abcam, ab5201), SRCAP (gift from J. Chrivia), BAF53a (Abcam, ab3882, lot #9118237), EPC1 (Abcam, ab5514, lot#98723).

Tandem-affinity purification of endogenous H2AZ.1 and H2AZ.2

Purification of endogenously tagged H2AZ.1 and H2AZ.2 was performed basically as described (Doyon & Côté, 2016). Typically, soluble nuclear extracts (Abmayr et al., 2006) were prepared from 3E9 cells (3 L cultures at 0.6–1.0 million cells per ml), adjusted to 0.1% Tween-20, and centrifuged at 100,000 g for 45 min. Extracts were precleared with 300 µl Sepharose CL-6B (Sigma), then 250 µl anti-FLAG M2 affinity resin (Sigma) was added for 2 hr at 4°C. The beads were then washed in Poly-Prep columns (Bio-Rad) with 40 column volumes (CV) of buffer #1 (20 mM HEPES-KOH).

[pH 7.9], 10% glycerol, 300 mM KCl, 0.1% Tween 20, 1 mM DTT, 1 mM PMSF, 2 µg/mL Leupeptin, 5 µg Aprotinin, 2 µg/mL Pepstatin, 10 mM Na-butyrate, 10 mM β-glycerophosphate, 100 µM Na-orthovanadate, 5 mM N-Ethylmaleimide, 2 mM Ortho-Phenanthroline) followed by 40 CV of buffer #2 (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 150 mM KCl, 0.1% Tween 20, 1mMDTT, 1 mM PMSF, 2 µg/mL Leupeptin, 5 µg Aprotinin, 2 µg/mL Pepstatin, 10 mM Na-butyrate, 10 mM β-glycerophosphate, 100 µM Na-orthovanadate, 5 mM N-Ethylmaleimide, 2 mM Ortho-Phenanthroline). Complexes were eluted in two fractions with 2.5 CV of buffer #2 supplemented with 200 ug/ml 3xFLAG peptide (Sigma) for 1 hr at 4°C. Next, fractions were mixed with 125 µl Strep-Tactin Sepharose (IBA) affinity matrix for 2 hr at 4°C, and the beads were washed with 20 CV of buffer #2. Complexes were eluted in two fractions with 2 CV of buffer #2 supplemented with 4 mM D-biotin, flash frozen in liquid nitrogen, and stored at −80°C. Typically, 15 ul of the first elution (3% of total) was loaded on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and analyzed by silver staining.

Mass-spectrometry analysis

The analyses were performed at the proteomic platform of the Quebec Genomics Center.

The peptides were directly loaded at 300 nL/min onto a New Objective PicoFrit column (15 cm ×0.075 mm I.D; Scientific Instrument Services, Ringoes, NJ) packed with Jupiter 5 µm C_{18} (Phenomenex, Torrance, CA) stationary phase. The peptides were eluted from the column by a gradient generated by an Agilent 1200 HPLC

system (Agilent, Santa Clara, CA) equipped with a nano electrospray ion source coupled to a 5600+ Triple TOF mass spectrometer (Sciex, Concord, ON). A 65 min linear gradient of a 5–35% mixture of acetonitrile, 0.1% formic acid injected at 300 nL/min was used to elute peptides. Data dependent acquisition mode was used in Analyst version 1.7 (Sciex) to acquire mass spectra. Full scan mass spectrum (400 to 1250 m/z) were acquired and followed by collision-induced dissociation of the twenty most intense ions. A period of 20 s and a tolerance of 100 ppm were set for dynamic exclusion.

Protein Pilot version 5.0 (Sciex) was used to generate MS/MS peak lists. Mascot (Matrix Science, London, UK; version 2.4.0) was used to analyze MGF sample files. Mascot was set up to search the UniprotKB *Homo sapiens* database (release 11/2014, 162831 sequences) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance and a parent ion tolerance of 0.1 Da. Oxidation of methionine and deamidation of asparagine and glutamine were specified as variable modifications and carbamidomethylation as fixed modification. Two missed cleavages were allowed. Scaffold (version 4.0.1), Proteome Software Inc, Portland, OR) was used to validate MS/MS based peptide and protein identifications. Proteins/peptides FDR rate was set to 1% or less based on decoy database searching. The Protein Prophet algorithm assigned protein probabilities. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Data were further analyzed using the CRAPome online tool (www.crapome.org; (Mellacheruvu et al., 2013)) with SAINTexpress default parameters, and visualized using ProHits-viz (https://prohits-viz.lunenfeld.ca/; (Knight et al., 2017)). Two replicates were used for each bait and normalized on Histone H2A.Z-H2B chaperones (histones themselves have too low spectral counts).

Cell fractionation

Cell fractionation using NP40

Cell pellets (5 million cells) were resuspended in 250–300 µl of lysis buffer (10 mM Tris pH 8.0, 10 mM NaCl, 2 mM MgCl2) and incubated at 4°C for 5 min. 10 µl were kept to prepare whole cell extracts. 50 µl/ml of NP40 were then added and samples were incubated for additional 10 min at 4°C and then centrifuged at 3000 g for 5 min. The supernatant was collected and represented the cytoplasmic fraction. The remaining pellet was resuspended in 35 µl of nuclear buffer (20 mM Hepes pH 7.9, 150 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 10% Glycerol) and incubated 30 min at 4°C. Samples were centrifuged at full speed for 5 min. The supernatant was removed and a second extraction was performed on the remaining pellet with a nuclear buffer high in salt (20 mM Hepes pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 10% Glycerol). After 30 min of incubation at 4°C, samples were centrifuged at full speed. The supernatant was collected and represented the soluble nuclear fraction. The pellet corresponding to the chromatin was resuspended in boiling buffer (1% SDS, 1% Triton, 10 mM Tris PH 7.4, 0.5 M NaCl) and sonicated 5 times at 25% amplitude for 10 s. Whole cell extracts were also resuspended in boiling buffer and sonicated. All the buffers were supplemented with EDTA-free protease inhibitor cocktail (Roche).

Cell fractionation under mild conditions

Cells were trypsinated, counted, and resuspended at a concentration of 4×10^7 cells/ml in buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 0.34 M Sucrose, 10% glycerol, 0.1% Triton X-100 and 1 mM DTT) and incubated at 4°C for 8 min. Small volume was kept to prepare whole cell extracts. The samples were then centrifugated at 1300 g for 5 min. The supernatant was collected and represented the cytoplasmic fraction. The nuclei pellet was washed once in buffer A and lysed for 30 min on ice in buffer B (3 mM EDTA, 0.2 mM EGTA and1 mM DTT). Samples were centrifuged at full speed for 5 min. The supernatant was collected and represented the soluble nuclear fraction. The pellet corresponding to the insoluble chromatin was washed once in buffer B. The chromatin was then extracted with buffer A and sonicated 3 times at 25% amplitude for 10 s. Whole cell extracts were sonicated with the same protocol. All fractions were clarified by full speed

centrifugation. The buffers were supplemented with EDTA-free protease inhibitor cocktail (Roche).

RNA extraction and reverse transcription

Total RNA was prepared using the MasterPure RNA Purification Kit (Epicentre) supplemented with Baseline-ZERO DNase, according to the manufacturer's instructions. For random-primed RT-qPCR, 200 ng of RNA were used for each reverse transcription reaction. The reverse-transcription was performed using random primers and superscript III reverse transcriptase (Invitrogen) at 50°C according to manufacturer's protocol. qPCR analysis was performed on CFX96 devices (BioRad) using the SYBR Premix Ex Taq II (Takara), according to the manufacturer's instructions. All samples was analysed in duplicates. All data was normalized relative to GAPDH mRNA levels. The list of primers can be found in Supplementary file 3.13.

ChIP experiments

15 million cells transfected with siRNA were crosslinked for 15 min using 1% formaldehyde directly in the culture medium. 0.125 M of Glycine were then added for 5 min. After two washes with PBS, cells were scraped and frozen at −80°C. Cells were lysed with 3 ml of a lysis buffer (5 mM Pipes PH 8, 85 mM KCl, 0.5% NP40) and homogenized 40 times with a dounce (20 times, pause 2 min, 20 times). After centrifugation, nuclear pellets were resuspended in 1.5 ml of nuclear lysis buffer (50 mM Tris PH 8.1, 10 mM EDTA, 0.2% SDS), and sonicated 10 times for 10 s (power setting 5% and 50% duty cycle, Branson Sonifier 250), to obtain DNA fragments of about 500 bp. DNA concentration was determined using a Nanodrop and samples were adjusted to the same concentration of chromatin. Samples were diluted at least one time in dilution buffer (0.01% SDS, 1.1% Triton, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl) and precleared for 2 hr with 250 µl of previously blocked protein-A and protein-G beads (Sigma P-7786 and P-3296 respectively). Blocking was achieved by incubating the beads with 0.5 mg/ml of Ultrapure BSA for 3 hr at 4°C. ChIP reaction was performed in 1 ml final volume. 100 µl of chromatin were kept for inputs. 50 µg of pre-cleared samples per ChIP supplemented with 10 ng of

Drosophila melanogaster chromatin (spike in chromatin, Active motif), and 1 ug of an antibody recognizing H2Av, a *Drosophila* specific histone variant, (spike in antibody, active motif), were incubated overnight with 4 µg of antibody at 4°C. A mock sample without antibody was processed similarly. Then, 20 µl of blocked A/G beads were added for 2 hr at 4°C to recover immune complexes. Beads were washed once in dialysis buffer (2 mM EDTA, 50 mM Tris pH 8, 0.2% Sarkosyl), four times in wash buffer (100 mM Tris pH 8.8, 500 mM LiCl, 1% NP40, 1% NaDoc) and twice in TE buffer (10 mM Tris pH 8, 1 mM EDTA). The bead/chromatin complexes were resuspended in 200 µl of TE buffer and incubated 30 min at 37°C with 10 µg of RNase A (Abcam), as well as input DNA. Formaldehyde crosslink was reversed in the presence of 0.2% SDS at 70°C overnight with shaking. After 2 hr of proteinase K (0.2 mg/ml) treatment at 45°C, immunoprecipitated and input DNA were purified on columns using Illustra GFX kit (GE Healthcare). All buffers for ChIP experiment were supplemented with EDTA-free protease inhibitor cocktail (Roche) and filtered 0.2 µM. Results were analysed by qPCR. The list of primers used can be found in Supplementary file 3.13.

Immunoprecipitation

8 million cells were lysed with 600 µl the lysis buffer (10 mM Tris PH 8, 1% NP40, 420 mM NaCl, 2 mM EDTA). Samples were incubated 15 min on ice and vortexed each 5 min. After 15 min of centrifugation at full speed, supernatant was recovered and diluted 3 times with dilution buffer (20 mM Tris PH 8, 2 mM EDTA, 20 µl DNase I epicenter, 25 mM CaCl2). NP40 final concentration was adjusted to 0.5%, and lysates were quantified. 1 mg was used per immunoprecipitation reaction. Preclearing was done by incubating lysates 2 hr at 4°C with 15 µl of A/G beads (Sigma). 4 µg of Flag antibody were then added ON at 4°C. 15 µl of A/G beads were added for 2 hr at 4°C, and immuno-complexes were washed 4 times with wash buffer (20 mM Tris PH 8, 0.5% NP40, 140 mM NaCl, 2 mM EDTA). Results were analysed by Western blot.

RNA-Seq

In WI38 cells, to identify the interplay between H2A.Z. isoforms, two samples of siCtrl, siH2A.Z.2, and siH2A.Z.1 + siH2A.Z.2 and one sample of siH2A.Z.1 were transfected in parallel. The other siH2A.Z.1 sample came from (Muniz et al., 2017). To identify genes regulated by PHF14 and SIRT1, two samples of siCtrl, siPHF14 and siSIRT1 were transfected in parallel.

In U2OS cells, two samples of siCtrl, two sample of si Ctrl#, siH2A.Z.1, siH2A.Z.2 and siH2A.Z.1+siH2A.Z.2 were transfected in parallel.

We used strand-specific RNA-Seq method, relying on UTP incorporation in the second cDNA strand. For each sample, 5–10 µg of total RNA, extracted as described above in (RNA extraction and Reverse transcription), was submitted to EMBL-GeneCore, Heidelberg, Germany. Paired-end sequencing was performed by Illumina's NextSeq 500 technology. Two replicates of each sample were sequenced.

ChIP-seq

For the ChIP-seq in U2OS cells expressing Flag-H2AZ.1 and Flag-H2AZ.2, 100 µg of chromatin supplemented with 10 ng of spike in chromatin (active motif) were used per ChIP experiment. For each reaction, 4 ug of Flag M2 antibody (sigma) and 1 ug of spike in antibody (active motif), were used. About 10 ng of immunoprecipated DNA (quantified with quantiFluor dsDNA system, Promega) were obtained at each time, and samples were submitted to EMBL-GeneCore Heidelberg for sequencing, that was performed by Illumina's NextSeq 500 technology.

ChIP-seq in K562 cells expressing Flag-H2A.Z.1 and Flag-H2A.Z.2 were performed and analysed as previously described (Jacquet et al., 2016; Lalonde et al., 2013).

RNA-Seq processing

RNA-Seq samples were sequenced using Illumina NextSeq 500 sequencer, paired-end, 80 bp reads, at EMBL Genomics core facilities (Heidelberg, Germany). The quality of each raw sequencing file (fastq) was verified with FastQC (Andrews, 2010). Files were aligned to the reference human genome (hg38) in paired-end mode with

STAR Version 2.5.2b (Agudelo et al., 2017) and processed (sorting and indexing) with samtools (Li et al., 2009). Raw reads were counted, per gene_id, using HT-seq Version 0.6.1 (Anders et al., 2015) on the NCBI refseq annotation gtf file from UCSC in a strand specific mode with default parameters.

RNA-Seq analysis and figures

Several differential analyses (siH2A.Z.1 vs siCtrl, siH2A.Z.2 vs siCtrl, siH2A.Z1 and siH2A.Z.2 vs siCtrl, siSIRT1 vs siCtrl, siPHF14 vs siCtrl) were done with DESeq2 Bioconductor R package, Version 1.22.1 (Love et al., 2014) with default parameters. In U2OS cells, four control samples obtained using two different siRNAs were used. Genes of interest were selected when |log2FoldChange| higher $\log_2(1.25)$ and adjusted p-value lower than 0.05. RPKM shown in Supplementary files 3.1–12 were calculated for each gene in the different datasets by taking the raw counts from HT-seq multiplied by 1E+09/(total number of aligned reads * sum of exons' sizes of the gene).

Lists of gene of interest were crossed to identify common genes, and results were represented using Venn Diagrams with R and VennDiagram package. For each crossing, the Chi-square test (chisq.test() function in R) was applied to the associated contingency table. The test evaluates if the 2 lists of genes are independent that is whether there is a significant association between the categories of the two variables. Chi-square p-values were then corrected for multiple testing (one test per crossing) with the Benjamini–Hochberg method.

Boxplots representing the RPKM value were generated with R-base based on the mean of replicates from Tables S1-8. The center line represents the median, box ends represent respectively the first and third quartiles, whiskers represent the minimum and maximum values without outliers. Outliers were defined as below 1stQuartile − 1.5 × InterquartileRange and above 3rdQuartile + 1,5 × InterquartileRange. Nonparametric Mann–Whitney–Wilcoxon test (wilcoxon.test() function in R) was applied to test distribution differences between two populations.

ChIP-Seq processing and analysis

ChIP-Seq samples were sequenced using Illumina NextSeq 500 sequencer, single-end, 80 bp reads, at EMBL Genomics core facilities (Heidelberg, Germany).

The quality of each raw sequencing file was verified with FastQC. Files were aligned to the reference human genome (hg38) in single-end mode with (Li & Durbin, 2009) and processed (sorting, PCR duplicates removing and indexing) with samtools. The coverage was computed with the GenomicAlignments Bioconductor R package (Lawrence et al., 2013).

ChiP-Seq mean coverage per base was computed for each annotated gene, in a window of +/- 2 kb around Transcription Start Site (TSS). For each gene, the log2 mean value in these windows was computed and plotted using R-base, in a dot-plot representing H2AZ1 versus H2AZ2. Lastly, the log2 ratio of the mean value in these windows for H2AZ1 divided by the mean value in these windows for H2AZ2 was computed for 5 list of genes selected through the RNA-Seq differential analysis: genes up-regulated from siH2AZ1 versus siCtrl, genes down-regulated from siH2AZ1 versus siCtrl, genes up-regulated from siH2AZ2 versus siCtrl, and un-regulated genes in both analyses. Results of these calculations were shown in a boxplot.

3.8 Data availability

Deep Sequencing Data are available at GEO (accession number: # GSE131579). MS and scaffold files generated in this study were deposited at MassIVE (http://massive.ucsd.edu) and assigned the MassIVE accession numbers MSV000084836. Source data files have been added for all histograms.

3.9 Supplementary files

Supplementary file 3.1

Genes upregulated upon H2A.Z.1 depletion in WI38 cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp1-v1.xlsx

Supplementary file 3.2

Genes upregulated upon H2A.Z.2 depletion in WI38 Cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp2-v1.xlsx

Supplementary file 3.3

Genes down-regulated upon H2A.Z.1 depletion in WI38 cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp3-v1.xlsx

Supplementary file 3.4

Genes down-regulated upon H2A.Z.2 depletion in WI38 cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp4-v1.xlsx

Supplementary file 3.5

Genes regulated upon the combined depletion of H2A.Z.1 and H2A.Z.2 in WI38 cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp5-v1.xlsx

Supplementary file 3.6

Genes upregulated upon H2A.Z.1 depletion in U2OS cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp6-v1.xlsx

Supplementary file 3.7

Genes upregulated upon H2A.Z.2 depletion in U2OS Cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp7-v1.xlsx

Supplementary file 3.8

Genes down-regulated upon H2A.Z.1 depletion in U2OS cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp8-v1.xlsx

Supplementary file 3.9

Genes down-regulated upon H2A.Z.2 depletion in U2OS cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp9-v1.xlsx

Supplementary file 3.10

Genes regulated upon the combined depletion of H2A.Z.1 and H2A.Z.2 in U2OS cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp10-v1.xlsx

Supplementary file 3.11

Genes regulated upon PHF14 depletion in WI38 cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp11-v1.xlsx

Supplementary file 3.12

Genes regulated upon SIRT1 depletion in WI38 cells.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp12-v1.xlsx

Supplementary file 3.13

List of siRNA and primers.

https://cdn.elifesciences.org/articles/53375/elife-53375-supp13-v1.xlsx

Conclusion

L'organisation de la chromatine joue un rôle crucial dans l'ensemble des processus impliquant le génome des cellules, et l'étude des phénomènes régulant cette organisation constitue un champ de recherche aussi étendu qu'important pour notre compréhension du fonctionnement du vivant et le traitement des nombreuses pathologies.

Le complexe NuA4/TIP60 intègre plusieurs activités affectant cette organisation et joue un rôle central dans de nombreux processus tels que l'expression des gènes et le maintien de l'intégrité génomique. Même si le complexe et ses fonctions ont à ce titre fait l'objet de nombre de travaux et sont aujourd'hui relativement bien caractérisés, plusieurs questions demeurent à son sujet. En particulier, NuA4/TIP60 est composé de 17 sous-unités (18 en comptant la nouvelle sous-unité JAZF1 (Procida et al., 2021; Sudarshan et al., 2022)), dont certaines n'ont été identifiées comme telles que récemment, et dont le rôle précis au sein du complexe est encore peu connu pour plusieurs d'entre elles. Il en va de même pour certains domaines lecteurs de la chromatine présents au sein du complexe comme le domaine SANT de DMAP1 ou le chromodomaine de KAT5/Tip60. Un autre aspect encore peu connu concerne l'incorporation du variant H2A.Z, accomplie par deux complexes différents (NuA4/TIP60 et SRCAP) chez l'humain, et l'importance de cette redondance apparente reste à élucider.

Par les travaux présentés dans cette thèse, nous avons cherché à répondre à certaines de ces questions, et certains éléments de réflexion seront présentés ici.

C.1 Le chromodomaine de KAT5/Tip60 : un 'lecteur' analphabète?

Comme évoqué en introduction et au chapitre 1, le chromodomaine de KAT5/Tip60 a fait l'objet de rapports contradictoires quant à sa fonction et à sa capacité à reconnaître des marques d'histones particulières. Nos résultats (chapitre 1) indiquent que ce domaine ne fonctionnerait en fait pas comme un chromodomaine canonique puisqu'il semble incapable de reconnaître par lui-même des marques d'histones dans un contexte de chromatine, tout en étant essentiel pour la viabilité

cellulaire et l'activité acétyltransférase de NuA4/TIP60 selon un mécanisme structural qui reste à déterminer. Comment expliquer des conclusions aussi différentes selon les études ? Il s'agit selon nous d'un bon exemple de l'influence du choix de conditions expérimentales, de réactifs et de substrats, sur les résultats observés, en particulier en ce qui concerne des essais enzymatiques *in vitro*. De nombreuses KATs ont ainsi une spécificité différente selon qu'on leur présente des histones libres ou des nucléosomes. C'est le cas pour KAT5 qui est capable d'acétyler H3, H2A et H4 en présence d'histones libres, mais seulement H2A et H4 au sein de nucléosomes, un substrat dont l'acétylation nécessite la présence d'autres sous-unités du complexe en plus de KAT5 (Allard et al., 1999; Boudreault et al., 2003; Chittuluru et al., 2011; Doyon et al., 2004; Smith et al., 1998). Pour trancher la question de la spécificité de liaison du chromodomaine de KAT5/Tip60, nous avons choisi d'utiliser le chromodomaine seul en présence du substrat 'physiologique' du complexe, à savoir des nucléosomes, portant différentes PTMs sur leurs histones, là où les études précédentes utilisaient des peptides. Cependant, nos essais et d'autres (Zhang et al., 2018) sur des peptides n'ont pas non plus montré d'interaction spécifique.

Une objection possible est que le chromodomaine a besoin, pour se lier à son substrat spécifique, du reste de la protéine KAT/Tip60, et il s'agit d'un test qui pourrait s'avérer intéressant à l'avenir. Néanmoins, nos résultats avec des complexes natifs purifiés montrent que les mutations dans le chromodomaine affectent l'activité acétyltransférase du complexe indépendamment des marques d'histones. De plus, nos conclusions sont appuyées par des observations d'ordre structural, la cage aromatique du chromodomaine KAT5/Tip60 ne semblant pas compatible avec la reconnaissance d'un résidu lysine méthylé (Zhang et al., 2018). Chez la levure, il a été montré que l'absence du chromodomaine de KAT5/Esa1 n'affecte pas la reconnaissance du nucléosome par NuA4, mais seulement la stabilité de l'interaction (P. Xu et al., 2016), en cohérence avec nos résultats qui semblent indiquer que des mutations dans le chromodomaine n'affectent pas le recrutement de NuA4/TIP60 à la chromatine.

Malgré tout, le chromodomaine de NuA4/TIP60 est essentiel pour l'activité acétyltransférase du complexe, en particulier sur H4, expliquant sa forte conservation de la levure à l'humain. Nous en sommes réduits à des hypothèses quant au mécanisme précis par lequel ce domaine fonctionne à cet égard, mais le plus probable est qu'il aide le complexe à adopter une conformation correcte une fois lié au nucléosome, permettant le contact entre la queue de H4 et le site catalytique. Cette hypothèse est renforcée par l'observation déjà mentionnée selon laquelle l'absence du chromodomaine de KAT5/Esa1 provoque des changements relativement subtils dans la structure de NuA4 lié à un nucléosome (Figure C.1) (P. Xu et al., 2016).

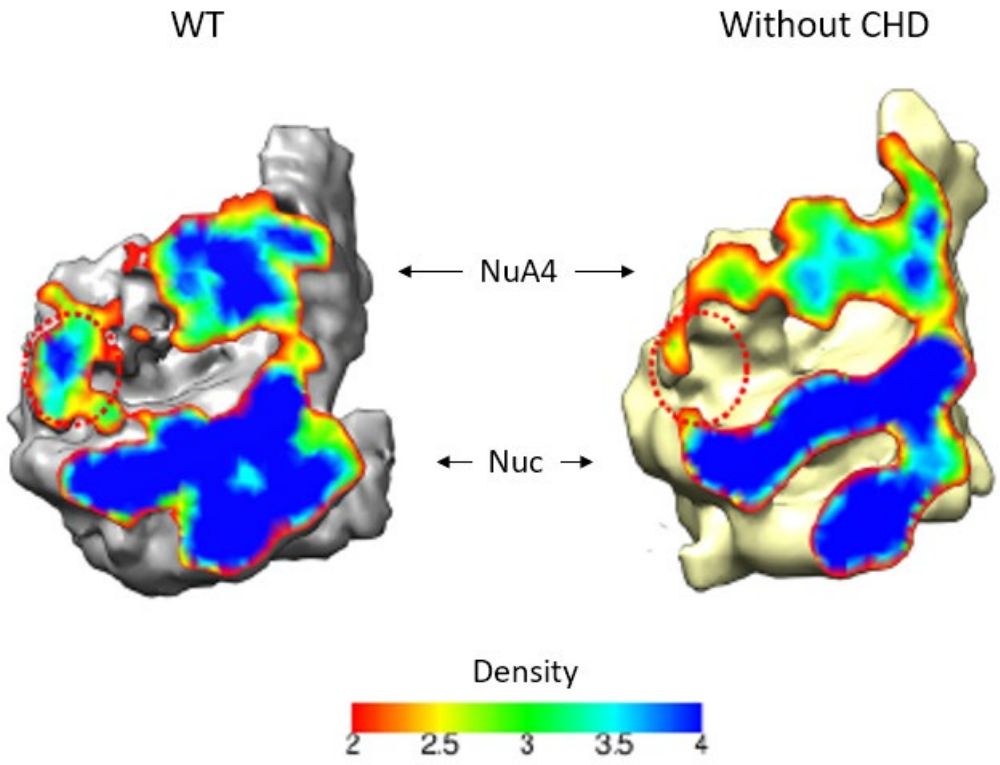


Figure C.1. Structure du 'cœur' de NuA4 de S. cerevisiae lié à un nucléosome, en présence (à gauche) ou non (à droite) du chromodomaine de KAT5/Esa1. (Adapté de P. Xu et al., 2016)

Enfin, il n'est pas à exclure que le chromodomaine de KAT5/Tip60 puisse lui-même adopter une conformation différente en fonction du contexte, amenant potentiellement à libérer la cage aromatique pour la reconnaissance d'un résidu spécifique. Étudier la structure de NuA4/TIP60 lié au nucléosome en présence de mutations au sein du chromodomaine pourrait permettre d'élucider la façon dont ce dernier influence l'activité catalytique du complexe, un objectif qui semble de plus en plus réalisable au vu des dernières avancées concernant la détermination de la structure du complexe (Ji et al., 2022; Qu et al., 2022; Zukin et al., 2022). Des observations chez la levure montrent que Epl1 interagit directement avec H2A via sa queue N-terminale, et avec H4 via son domaine globulaire (Huang & Tan, 2013). De plus, la troncation de EPC1 a le même effet sur l'acétylation de H4 tout en 'épargnant' H2A que les mutations du chromodomaine présentées dans cette thèse (Lalonde et al., 2013). Ainsi il semble clair qu'il existe deux mécanismes distincts pour l'acétylation de H4 et de H2A par NuA4/TIP60, le premier dépendant du chromodomaine et de l'extrémité N-terminale de EPC1.

KAT5/Tip60 n'est pas la seule KAT de la famille des MYST à posséder un chromodomaine, puisque c'est le cas également de KAT8/MOF (Hilfiker et al., 1997), dont il a été suggéré que le chromodomaine puisse reconnaître l'ARN (Akhtar et al., 2000). Un regard à la structure de ce domaine indique une certaine similarité avec celui de KAT5/Tip60, notamment par la présence d'une cage aromatique 'réduite' (Figure C.2). Nous travaillons actuellement sur ce domaine pour déterminer si à l'instar du chromodomaine de KAT5/Tip60 il affecte l'activité acétyltransférase de MOF indépendamment des marques d'histones.

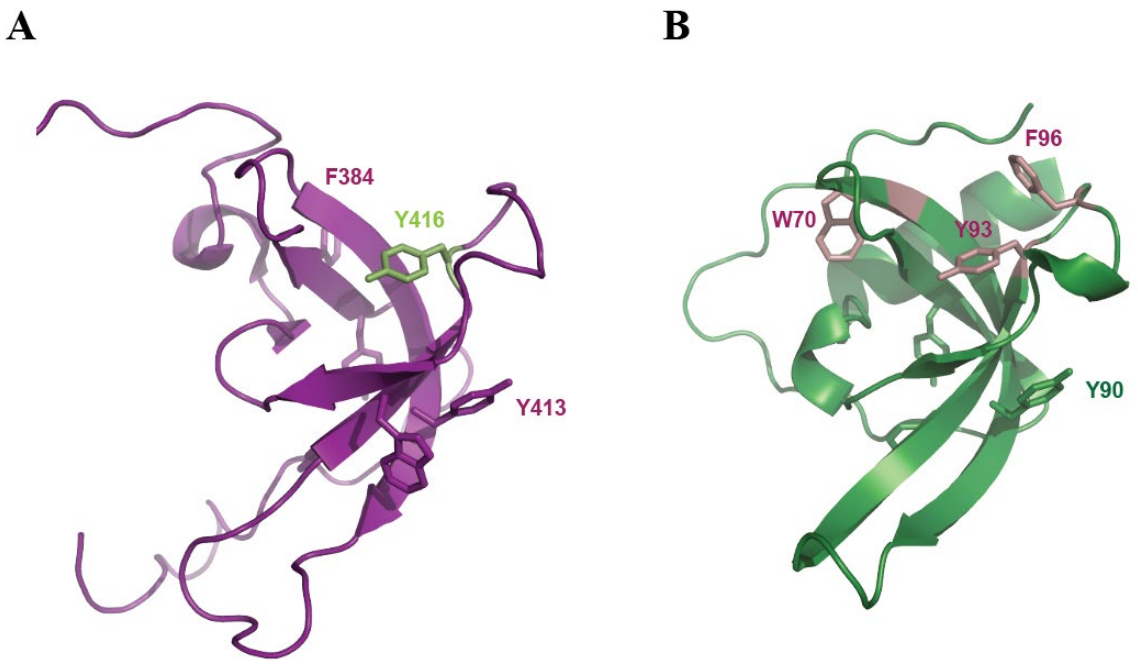


Figure C.2. A. Structure du chromodomaine de KAT8/MOF chez D. melanogaster (PDB ID 2BUD, Nielsen et al., 2005). B. Structure prédite du chromodomaine de KAT8/MOF chez l'humain (AlphaFold, Jumper et al., 2021; Varadi et al., 2022)

C.2 Chromatine et pathologies du cerveau

Au chapitre 2, nous présentions la caractérisation de mutations de KAT5/Tip60 (dont une dans le chromodomaine, illustrant plus encore son importance fonctionnelle) liées à un syndrome neurodéveloppemental.

Il semble qu'il s'agisse d'une tendance importante, de nombreux facteurs épigénétiques étant impliqués dans des pathologies au niveau du cerveau : d'autres KATs (Cogné et al., 2019; Li et al., 2019; Yan et al., 2020), des KDACs (Latypova et al., 2021; Wakeling et al., 2021), des remodeleurs (Snijders Blok et al., 2018), des ubiquitine-ligases (C. Li et al., 2021), ou encore la méthylation de l'ADN (Aref-Eshghi et al., 2020) ont été associés à des syndromes du même type. On peut également citer les mutations de SRCAP qui causent le syndrome de Floating-Harbor (Hood et al., 2012) et d'autres (Nogueira et al., 2021; Rots et al., 2021) et les oncohistones, très étudiées au cours des dernières années, liées à des cancers pédiatriques du cerveau (Chen et al., 2020; Papillon-Cavanagh et al., 2017; Pathania et al., 2017). En parallèle, même s'il ne s'agit pas d'une pathologie liée au développement,

KAT5/Tip60 et d'autres KATs de la même famille semblent jouer un rôle dans la maladie d'Alzheimer (Li & Huang, 2021).

À l'heure actuelle, il n'existe pas d'explication définitive à cette forte association, explication qui sera nécessairement multifactorielle, mais certaines pistes ont été avancées. En premier lieu, les neurones et les tissus qu'ils forment font partie des systèmes les plus plastiques de l'organisme et plus particulièrement lors du développement, les réseaux de neurones et de synapses étant largement modifiés en réponse à des stimuli extérieurs, et de l'établissement de la mémoire (Fagiolini et al., 2009). En modifiant les propriétés du génome sans modification de sa séquence, les facteurs épigénétiques représentent les acteurs de choix pour cette régulation en réponse aux signaux notamment environnementaux, et plusieurs régulateurs chromatiniens régulent des gènes importants pour la plasticité neuronale (Fagiolini et al., 2009; Hwang et al., 2017).

Autre piste intéressante, le développement embryonnaire se déroule en conditions hypoxique, et une variation du niveau d'oxygène (vers encore plus d'hypoxie ou vers la normoxie) peut entraîner une large gamme de syndromes neurodéveloppementaux (Cristancho & Marsh, 2020). Plusieurs régulateurs de la chromatine, dont certains font partie de la liste de mutations identifiées dans de tels syndromes, sont régulés par les quantités d'oxygène dans le milieu ou régulent eux-mêmes la réponse hypoxique (Cristancho & Marsh, 2020; Perez-Perri et al., 2016). Le taux d'oxygène a également une influence directe sur de nombreuses voies métaboliques importantes et, comme évoqué en introduction de cette thèse, il existe une forte interdépendance entre certaines de ces voies métaboliques et les facteurs régulant l'organisation de la chromatine.

C.3 Incorporation de H2A.Z par NuA4/TIP60 et SRCAP

La question de la répartition de l'incorporation de H2A.Z entre NuA4/TIP60 et SRCAP est l'un des intérêts de notre laboratoire pour les années à venir. J'ai pu approcher cette question par deux de mes projets de doctorat, celui présenté au

chapitre 3 sur les paralogues de H2A.Z, et celui qui fait l'objet de l'annexe 1 en lien avec la protéine DMAP1.

La question principale dans ce contexte, à savoir la contribution et l'importance respective de ces deux complexes dans l'incorporation de H2A.Z, n'a pas encore été tranchée, et est compliquée par le nombre de processus régulés par NuA4/TIP60 et, dans une moindre mesure, SRCAP (Bowman et al., 2011; Scacchetti & Becker, 2021) ainsi que par les sous-unités partagées par les deux complexes. Il est ainsi difficile de perturber spécifiquement l'activité d'incorporation de EP400 sans affecter l'activité acétyltransférase de NuA4/TIP60, et le reste du module d'incorporation de H2A.Z est partagé avec SRCAP, empêchant de découpler les observations effectuées suite à leur déplétion. À ce titre, certains résultats préliminaires concernant DMAP1 (annexe 1) pourraient se révéler très intéressants. Il semble en effet que le domaine SANT de DMAP1 soit impliqué dans l'interaction de la protéine avec le reste du complexe, et l'un des mutants du domaine SANT que j'ai testés pourrait potentiellement, si ces résultats sont confirmés, nous permettre de dissocier DMAP1 spécifiquement d'un complexe sans affecter sa présence dans l'autre. Un tel outil s'avèrerait très utile pour distinguer les activités de NuA4/TIP60 et SRCAP en ce qui concerne l'incorporation de H2A.Z. Nous disposons aussi à présent d'outils permettant le déplétion rapide et réversible de protéines endogènes, comme le système dTAG qui consiste à fusionner la protéine FKBP12 à une protéine d'intérêt, puis à l'adresser au protéasome pour dégradation à l'aide d'une drogue (Nabet et al., 2018). Ces techniques, combinées à nos approches d'expression de transgènes à des niveaux physiologiques, aideront à poser ces questions dans les prochains mois/années.

Lors de notre étude des partenaires des deux paralogues de H2A.Z (chapitre 3), nous avons retrouvé, comme attendu, toutes les sous-unités de NuA4/TIP60 et SRCAP. Mais de manière intrigante, il semble que les deux complexes n'interagissent pas avec les deux paralogues de façon similaire : en effet les sous-unités de NuA4/TIP60 sont particulièrement enrichies parmi les partenaires de H2A.Z.2 par rapport à H2A.Z.1, alors que SRCAP ne semble pas favoriser l'un ou

l'autre des paralogues. La signification fonctionnelle de cette différence n'est pas encore connue, mais il pourrait s'agir de l'un des paramètres distinguant l'action de NuA4/TIP60 et SRCAP vis-à-vis de H2A.Z. En parallèle, il a été montré que la surexpression de H2A.Z.2 pouvait s'opposer à l'effet de mutations de SRCAP dans le syndrome de Floating-Harbor (Greenberg et al., 2019), illustrant un autre exemple de phénomène au cours duquel les deux paralogues de H2A.Z ainsi que les deux complexes qui les incorporent pourraient être amenés à jouer des rôles bien spécifiques. De façon surprenante, nous n'avons pas détecté de différences importantes dans la localisation génomique de H2A.Z.1 et H2A.Z.2. Peut-être de telles différences existent-elles dans des contextes cellulaires différents de lignées cancéreuses; il restera dans tous les cas à déterminer si NuA4/TIP60 et SRCAP peuvent incorporer un paralogue spécifique au niveau de régions précises pour conférer des propriétés différentes aux nucléosomes.

En plus d'une différence dans les interactions avec NuA4/TIP60, nous avons également identifié des partenaires exclusifs à chaque isoforme, le complexe PHF14/RAI1 pour H2A.Z.1, et la KDAC SIRT1 pour H2A.Z.2. Le premier est particulièrement intéressant puisqu'il s'agit d'un complexe régulateur de la chromatine encore peu caractérisé. On peut noter que certaines de ses sous-unités, RAI1 et TCF20, ont été elles aussi impliquées dans des pathologies neurodéveloppementales (Babbs et al., 2014; Carmona-Mora & Walz, 2010; Garay et al., 2016). Fonctionnellement, il a été suggéré que PHF14/RAI1 jouerait un rôle opposé au complexe répresseur CoREST dans la régulation de l'expression des gènes (Garay et al., 2016). PHF14 avait précédemment été identifié comme un partenaire de H2A.Z (Draker et al., 2012), mais la découverte de son interaction spécifique avec H2A.Z.1 pourrait ouvrir des perspectives intéressantes quant à sa fonction, et constituer l'un des paramètres expliquant les fonctions différentes jouées par les deux paralogues de H2A.Z. Des travaux sont en cours pour déterminer les bases biochimiques d'une telle spécificité d'interaction avec deux protéines (H2A.Z.1 et H2A.Z.2) différant de seulement trois acides aminés. De même, il reste à déterminer si la préférence de NuA4/TIP60 pour H2A.Z.2 est liée à ces différences

de séquences protéiques, ou si elle relève d’implications plus mécanistiques quant aux rôles spécifiques de ce paralogue.

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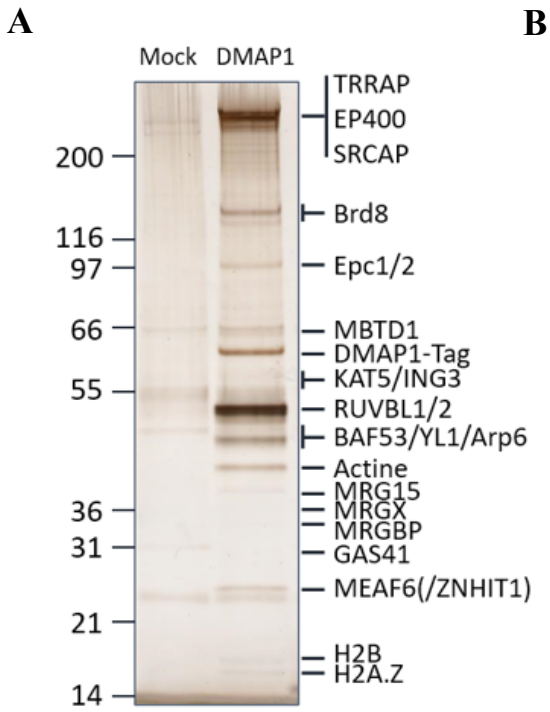
Annexe A : Le domaine SANT de DMAP1 est requis pour l'interaction avec NuA4/TIP60 et SRCAP



B

		DMAP1
TIP60	TRRAP	135
	EP400	102
	EPC1	25
	EPC2	38
	BRD8	31
	KAT5/Tip60	18
	ING3	24
	hEAF6	10
	MBTD1	21
	MRG15	18
	MRGX	13
	MRGBP	9
SRCAP	DMAP1	46
	GAS41	19
	BAF53a	22
	Actin	24
	VPS72/YL1	12
	RUVB1	36
	RUVB2	42
	SRCAP	83
	ARP6	10

Figure A.1. Purification des complexes natifs associés à DMAP1 endogène. A. Après tagging de DMAP1 endogène par CRISPR/Cas9, une double purification par affinité a été réalisée et déposée sur gel SDS-PAGE coloré à l'argent (cf chapitre 3). B. Analyse par spectrométrie de masse des protéines associées à DMAP1 endogène après purification. Le tableau indique le nombre de peptides uniques détectés pour les protéines indiquées. La très grande majorité des partenaires identifiés sont des sous-unités des complexes NuA4/TIP60 et/ou SRCAP, ce qui confirme que DMAP1 est essentiellement retrouvée comme sous-unité de ces deux complexes au sein des cellules.

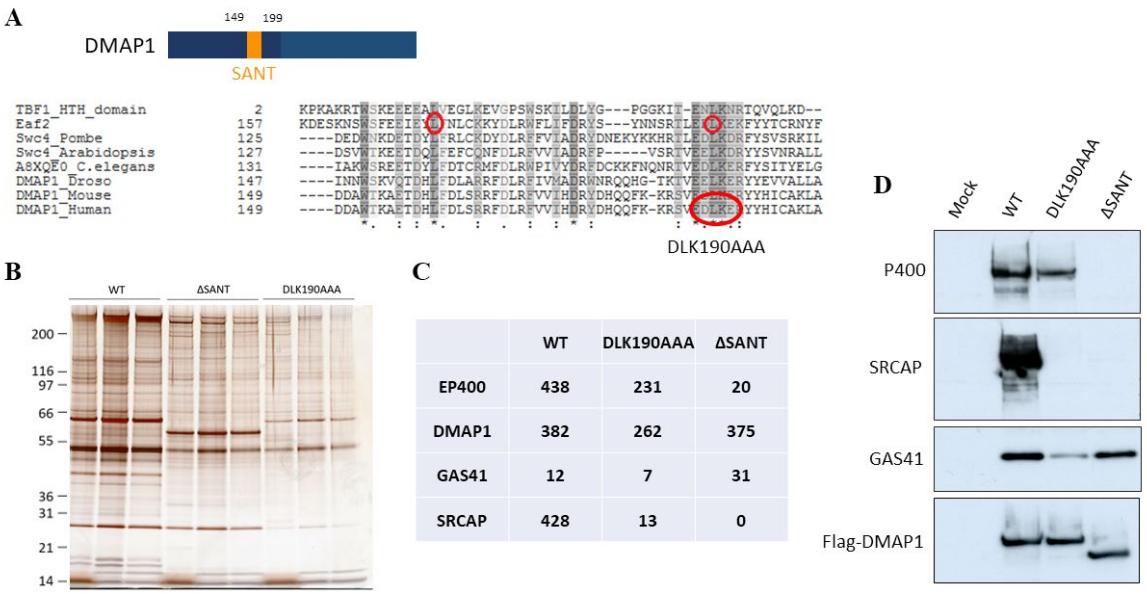


Figure A.2. Implication du domaine SANT de DMAP1 dans l'interaction avec NuA4/TIP60 et SRCAP. A. Représentation schématique de la protéine DMAP1 illustrant la position de son domaine SANT (en haut). Alignement des séquences peptidiques des domaines SANT des orthologues de DMAP1 de la levure (Eaf2) à l'humain ainsi que du domaine HTH de liaison à l'ADN de la protéine télomérique TBF1. Sont entourés en rouge les deux résidus correspondant aux mutants thermosensibles de Eaf2 ainsi que le triplet DLK190 de DMAP1. B. Des transgènes de DMAP1 WT, DMAP1 dépourvu de domaine SANT, ou DMAP1 muté sur le triplet DLK190 ont été intégrés au site AAVS1 dans des cellules K562 et les complexes associés ont été purifiés et déposés sur un gel SDS-PAGE coloré à l'argent (cf chapitres 1 et 2). C. Les complexes purifiés en B ont été analysés par spectrométrie de masse. Le tableau présente le nombre de peptides obtenus pour des sous-unités représentatives de NuA4/TIP60 et SRCAP. D. Analyse par Western blot des complexes purifiés en B confirmant la perte de l'interaction de DMAP1 avec les complexes NuA4/TIP60 et SRCAP en absence du domaine SANT (piste 4) et uniquement avec SRCAP en présence de la mutation DLK190AAA (piste 3).