Epigenetic mechanism of CENP-A loading to centromeres

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MECANISME ÉPIGENETIQUE IMPLIQUÉ DANS LA DEPOSITION DE CENP-A AUX CENTROMERES

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TO MY FAMILY AND TEACHERS

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Epigenetic mechanism of CENP-A loading to centromeres

Summary

Centromere is a specialized chromosomal locus, where kinetochore assembles, which is required for correct chromosome segregation during cell division. Defects in chromosome segregation process can lead to aneuploidy, cell death and promote the development of cancer. The vital role of centromere in chromosome segregation is conserved, and critical for cell and organism life, yet, there is no conservation of centromeric DNA sequence between species. Lack of conservation of centromeric DNA sequence and the presence of neocentromeres suggest that centromeric DNAs are neither necessary and nor sufficient for formation of functional centromeres. Instead, centromeres are specified epigenetically by the presence of a unique nucleosome that contains a centromeric-specific histone H3 variant called CENP-A (Centromere Protein A). The epigenetic mark generated by CENP-A nucleosome, is required for the assembly and maintenance of both active centromere and kinetochore at a single locus on every chromosome for every cell division in the lifetime of an organism. Thus, understanding the mechanism that governs the specific deposition of CENP-A at centromeres is central to understanding chromosome segregation mechanisms.

A fundamental question in centromere biology is that how CENP-A is specifically delivered to and maintained on centromeres. Despite the identification of CENP-A associated proteins, little is known about specific factors in human that could bind CENP-A and assist its specific deposition at centromeres. The objective of my thesis was to identify specific chaperone in human, responsible for CENP-A loading to centromeres, by using biochemical and proteomic strategies.

To identify CENP-A deposition machinery, I purified the prenucleosomal CENP-A complex from HeLa cells stably expressing FLAG-HA epitope tagged CENP-A (e-CENP-A). By mass spectrometry analysis of proteins present in CENP-A and H3.1 complex, I found HJURP (Holliday Junction Recognition Protein) uniquely in CENP-A but not in H3.1 prenucleosomal complex. Down regulation of HJURP by specific siRNA strongly diminished centromeric localization of CENP-A. I showed that bacterially expressed HJURP binds at a stoichiometric ratio to the CENP-A/H4 but not to the H3/H4 polypeptides. By using different deletion mutants of HJURP, I identified a conserved short N-terminal domain in HJURP protein, which is necessary and sufficient for the interaction with CENP-A/H4, and thus, we named it CBD (CENP-A Binding Domain) of HJURP. Domain swap experiments revealed that the centromere-targeting domain (CATD) in CENP-A, which is absent in H3, is the recognition motif by which HJURP distinguishes CENP-A from H3. Finally, I showed that HJURP is able to facilitate the efficient deposition of CENP-A/H4 tetramer on naked DNA, *in vitro*. Taken together, my data demonstrate that HJURP is a key chaperone responsible for the targeting and deposition of newly synthesized CENP-A at centromeres.

Thesis in French

5.1. Résumé de la thèse

Durant la division cellulaire, la bonne répartition du génome répliqué aux cellules filles est essentielle pour maintenir la stabilité génomique et donc critique pour la viabilité de la cellule et de l'organisme. La ségrégation fidèle des chromosomes est dirigée par le centromère, un locus chromosomique spécialisé qui est requis pour l'assemblage des kinetochores actifs. Les défauts dans le processus de ségrégation des chromosomes peut entrainer une aneuploïdie, la mort cellulaire et promouvoir un développement cancéreux. Le processus de ségrégation des chromosomes et la fonction des centromères sont conservés au cours de l'évolution et essentiel, pourtant il n'existe pas de conservation des séquences centromérique entre les espèces. L'absence de conservation des séquences centromériques et la présence de néocentromère suggèrent que l'ADN centromérique sont ni nécessaire ni suffisant pour la formation des centromères. Pourtant, les centromères sont marqués épigénétiquement par la présence d'un nucléosome unique qui contient un variant centromérique de l'histone H3 appelé Centromere protein A (CENP-A).

CENP-A est retrouvé chez tous les eucaryotes et est requis pour l'assemblage et la maintenance des centromères actifs sur tous les chromosomes pour toutes les divisions cellulaires tout au long de la vie de l'organisme. La protéine CENP-A humaine est composée de 140 acides aminés. Elle montre plus de 60% d'identités avec le domaine C-terminale (histone fold domain) de l 'histone H3, par contre sa queue N-terminal est très divergente. Un domaine particulier dans le « histone fold » de CENP-A appelé CATD est requis pour délivrer le CENP-A néo-synthétisé aux centromères. L'"incorporation du domaine CATD dans le domaine Histone fold de l'histone H3 est suffisante pour emmener l'histone H3 aux centromères. Cette donnée suggère qu'une chaperone spécifique de CENP-A reconnaîtrait le téramère (CENP-A/H4) via le domaine CATD et le délivrerait aux centromères.

Une question fondamentale est comment CENP-A est spécifiquement déposé et maintenu aux centromères. En dépit de l'identification de protéines qui sont

impliquées dans l'assemblage des centromères, on sait peu de choses sur les facteurs humains impliqués dans la déposition de CENP-A aux centromères. De plus, on sait peu chose à propos des modifications post-traductionnelles de CENP-A et de leur éventuel rôle dans la fonction des centromères.

L'objectif de ma thèse a été d'identifier les facteurs spécifiques de la déposition de CENP-A.

Pour identifier les facteurs spécifiques impliqués dans la déposition de CENP-A aux centromères, j'ai utilisé la méthode de purification TAP-TAG à partir d'une fraction nucléaire soluble de cellules HeLa exprimant stablement une copie ectopique de CENP-A (e CENP-A). J'ai ainsi pu identifié la protéine holliday Junction Recognition protein (HJURP). En utilisant un siRNA spécifique de HJURP, j'ai montré que la localisation et la déposition de CENP-A étaient fortement affectées. De plus, j'ai pu mettre en évidence in vitro que HJURP facilitait la déposition du tétramère CENP-A/H4 sur de l'ADN satellite. La protéine recombinante HJURP lie de manière stoechiométrique le tétramère CENP-A/H4 mais il ne lie pas le tétramère H3/H4. La liaison se fait grâce à un petit domaine conservé en position N-terminal de HJURP, dénommé CBD (CENP-A binding domain). Au sein du domaine CBD, j'ai identifié une boîte spécifique des vertébrés appelée TLTY box qui est essentielle pour la reconnaissance du tétramère CENP-A/H4. L'ensemble de mes résultats démontre très clairement que HJURP est la principale chaperone responsable de la déposition de CENP-A aux centromères.

5.2. Introduction

La duplication et la transmission de l'information génétique aux cellules filles au cours de la division cellulaire sont des étapes essentielles au développement des organismes vivants. Une transmission aberrante des chromosomes se traduit le plus souvent par un état d'aneuploïdie pouvant avoir de sérieuses répercussions dans le domaine de la santé publique. Des études statistiques ont montré que de telles anomalies de répartition des chromosomes se retrouvaient dans 7 % de toutes les conceptions et dans 45 % des avortements spontanés. Cet état d'aneuploïdie se retrouve également dans des cellules cancéreuses de diverses origines suggérant qu'une mauvaise ségrégation des chromosomes puisse aussi jouer un rôle dans les

processus de cancérogenèse [1]. Il apparaît donc important d'étudier plus en détail les mécanismes qui aboutissent à une dérégulation de cette ségrégation au cours de la mitose. Le centromère est requis pour au moins trois fonctions essentielles durant la division cellulaire. (I) il sert de site d'attachement aux microtubules du fuseau achromatique qui assurent une ségrégation correcte des chromatides sœurs nouvellement répliquées. (II) Il sert à empêcher une séparation précoce des chromatides sœurs durant le processus de ségrégation [2]. (III) Il sert à contrôler l'attachement et l'intégrité des microtubules en activant les protéines du "checkpoint" mitotique qui bloquent la progression du cycle cellulaire en cas d'endommagement des microtubules [3].

Pour comprendre le processus de ségrégation des chromosomes, il est donc indispensable d'identifier les protéines du centromère et de définir leurs fonctions exactes. Le centromère humain est formé d'ADN satellite hautement répété qui peut atteindre plusieurs méga bases associées à une structure nucléoprotéique complexe. La découverte d'un variant de l'histone H3 (CENP-A) localisé au niveau du centromère et capable de se fixer sur cet ADN satellite a laissé supposer que l'identité du centromère serait donnée par ce variant centromérique qui remplacerait l'histone H3 en formant un pseudo-nucléosome spécialisé [4]. En effet, il y a une corrélation très forte entre un centromère humain fonctionnel et la présence de l'histone CENP-A [5]. Par ailleurs, l'histone centromérique CENP-A est à elle seul capable de recruter les nucléoprotéines formant le kinetochore [6]. L'histone CENP-A a été conservée au cours de l'évolution, son homologue chez la levure Cse4p est aussi localisé au niveau du centromère. CENP-A présente 50 % d'homologie avec l'histone H3, elle est constituée d'une région C-terminal avec une forte homologie avec l'histone H3 (histone fold domain) et d'une région N-terminal spécifique à CENP-A. Cette partie N-terminal de CENP-A est très peut conserver entre les autres espèces.

Nous souhaitons, au travers de l'histone centromérique CENP-A, étudier la fonction du centromère humain, pour lequel très peu de choses sont connues. Comme chez la levure, l'histone centromérique humain CENP-A est capable de remplacer l'histone H3 dans des nucléosomes reconstitués in vitro. CENP-A est

amplifié dans plusieurs cancers colo-rectaux, impliquant directement ce variant de l'histone H3 dans la tumorgénèse [7].

Notre laboratoire s'intéresse à la structure et la fonction du centromère. Comprendre la structure et la fonction du centromère est un problème fondamental en biologie cellulaire. L'identification des complexes protéiques associés a CENP-A ainsi que l'élucidation de la fonction de chacune des sous-unités permettra de mieux comprendre la nature des mécanismes mis en jeu dans la ségrégation des chromosomes au cours de la mitose. Une meilleure connaissance de la structure, de la composition et du mécanisme moléculaire d'action du centromère est un enjeu majeur en biologie centromère.

5.3 Objectifs

Comprendre le mécanisme par lequel CENP-A est spécifiquement déposé et maintenu aux centromères, est d'une importance primordiale pour l'établissement et la propagation de l'identité épigénétique du centromère. L'objectif global de mon projet est d'identifier la chaperone spécifique de CENP-A chez l'homme, qui est responsable de la déposition de CENP-A aux centromères, en utilisant des stratégies biochimiques et protéomiques.

5.4. Résultats et Discussion

HJURP est un chaperon spécifique pour CENP-A

La purification par affinité et l'analyse par spectrométrie de masse des complexes de protéines associées avec pré-déposés CENP-A, nous a permis d'identifie HJURP uniquement dans le complexe de CENP-A (Figure 1). Les chaperones responsables de la déposition de H3.1 et H3.3, CAF-1 et HIRA / DAXX respectivement, n'ont pas été détectés dans le complexe de pré-deposition de CENP-A. De même, HJURP n'a pas été détecté dans les complexes responsables de la deposition de H3.1 et H3.3. En plus, la purification par affinité et analyse par spectrométrie de masse du complexe HJURP a révélé CENP-A, mais pas d'autres d'histones H3. Cela indique clairement que tous les variantes d'histone H3 sont

déposés par différentes chaperones d'histones au niveau des régions de chromatine distincte.

En plus de HJURP, nous avons également identifié NPM1 (nucléophosmine) comme un partenaire spécifique du complexe CENP-A (Figure 1). NPM1 est une phosphoprotéine nucléolaire, qui agit comme une chaperone [8] des histones H3, H2A et H2B, il joue également un rôle dans d'autres processus cellulaires importants comme la ségrégation des chromosomes et la réparation de l'ADN [9, 10]. Toutefois, NPM1 ne peut pas compenser l'effet dominant de l'appauvrissement de HJURP et sa régulation à la baisse n'a pas affecté significativement la localisation centromérique de CENP-A [11, 12]. En fait, notre étude d'interaction in vitro montre que NPM1 lie aussi bien les dimers d'histones CENP-A/H4 et H3/H4. Ces observations suggèrent fortement que NPM1 ne peut pas être une chaperone spécifique pour CENP-A, mais il peut jouer des rôles complémentaires au cours de la deposition de CENP-A.

En outre, nous avons également isolé les autres chaperones générale RbAp46 et RbAp48 dans les complexes H3.1 et CENP-A (Figure 1). RbAp48 est la sous-unité du complexe CAF-1 et est également présente dans plusieurs autres complexes impliqués dans la dynamique de la chromatine et l'inhibition de la transcription. Chez la Drosophile, RbAp48 se lie directement l'homologue de CENP-A, CID [358], mais chez l'homme, une interaction directe des protéines RbAp46/RbAp48 avec CENP-A n'a pas été signalée. Toutefois, RbAp46/RbAp48 et hMIS18α/β sont nécessaires a l'amorçage de la déposition de CENP-A [362, 363]. Il intéressant de noter que le complexe HJURP contient également RbAp46/RbAp48 et Dunleavy et al [11] ont observé que la régulation à la baisse de ces protéines a considérablement réduit le niveau de HJURP dans les cellules HeLa. Cette observation suggère que RbAp46/48 pouvait la localisation de CENP-A au niveau du centromère indirectement par l'intermédiaire de stabilisation des HJURP par un mécanisme encore inconnu. RbAp46/RbAp48 interagissent également avec l'histone H4 [13], ce qui soulève une autre explication qui serait que l'appauvrissement des deux protéines RbAp46 et RbAp48 affecterait la localisation de CENP-A indirectement via l'interaction avec H4. Ainsi, nous concluons que HJURP est une chaperone spécifique pour la déposition de CENP-A aux centromères, alors que les

chaperones NPM1 et RbAp46/48 jouent un rôle complémentaire lors de la deposition de CENP-A.

HJURP interagit directement avec CENP-A et son niveau augmente au moment de la synthèse de CENP-A et de sa déposition dans des cellules HeLa. Nos résultats d'immunoprécipitation, montrent que HJURP est associée à la chromatine CENP-A d'une manière dépendante du cycle cellulaire, concomitante avec la déposition de CENP-A. La régulation de la distribution de HJURP au cours du cycle cellulaire, atteste en outre son exigence spécifique pour la déposition de CENP-A.

Le mécanisme par lequel HJURP distingue CENP-A partir de histones en vrac, est la première étape critique pour CENP-A dépôt sur centromères par HJURP. Notre in vitro co-expression de dosage à l'aide de différents mutants de délétion de HJURP a montré que la partie N-terminale de la protéine correspondant aux acides aminés 1 à 80 aa est nécessaire et suffisante pour l'interaction avec CENP-A/H4 recombinant et nous l'avons appelé la CDB (CENP-A Binding Domain) de HJURP. Ce domaine N-terminal de HJURP montre une similarité à une courte région de Scm3 [374], ce qui est nécessaire pour CENP-A^{Cse4} dépôt chez les levures [364-368]. En outre, HJURP (CDB) reconnaît spécifiquement et se lie au domaine préalablement identifié CATD de CENP-A [372].

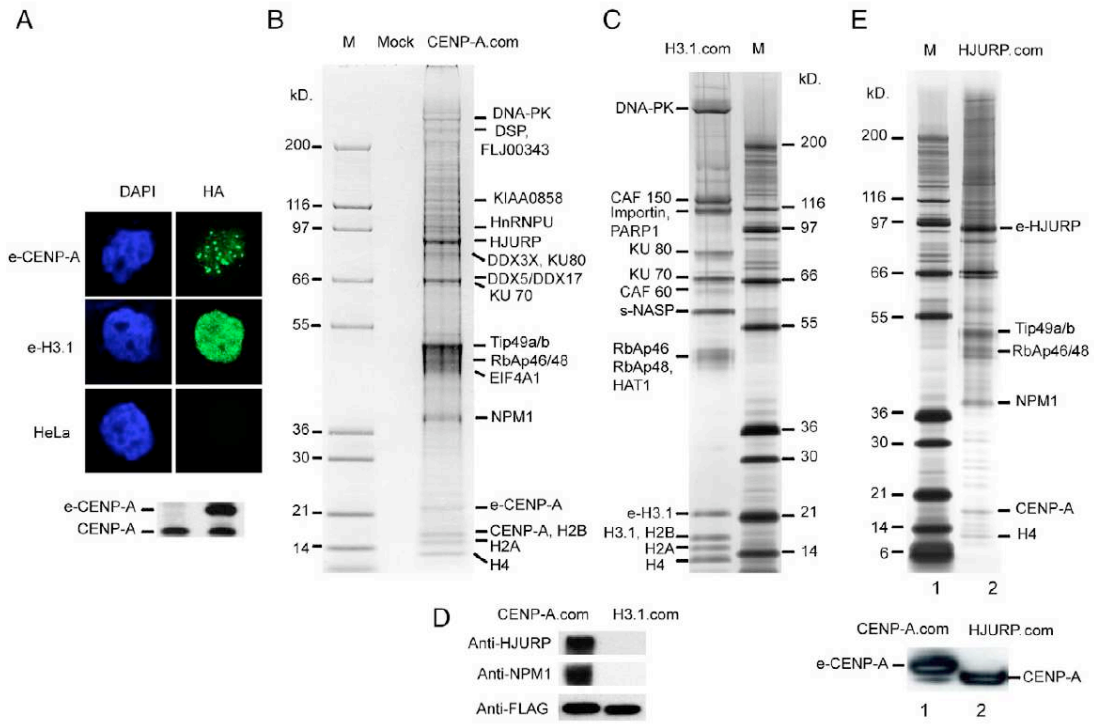

Figure 1. Purification du complexe de pré-assemblage CENP-A. **(A)** Localisation cellulaire de e-CENP-A et e-H3.1. Des lignés cellulaires stables exprimant soit e-CENP-A soit e-H3.1 ont été immunomarquées avec un anticorps anti-HA **(vert)** pour détecter les protéines étiquetées produits. Une coloration au DAPI permet de visualiser l' ADN **(bleu)**. **(Partie Basse)** Western blot de l'extrait cellulaire total à partir de cellules HeLa de contrôle (**Piste 2**) et de cellules HeLa exprimant e-CENP-A (**Piste 2**) Un anticorp anti-CENP-A a été utilisé pour révéler la présence de CENP-A par western blot. **(B)** Coloration à l'argent des protéines associées à e-CENP-A. Le complexe e-CENP-A (CENP-A.com) a été purifié par immuno-affinité et les polypeptides associés ont été identifiés par spectrométrie de masse. La Piste **M** correspond à un marqueur de masse moléculaire de protéine. La Piste **Mock**, correspond à une purification mock d'une ligne de cellules HeLa sauvage. **(C)** Coloration à l'argent des protéines associées à e-H3.1. Le complexe prenucléosomal e-H3.1 (H3.1.com) a été purifié par immunoaffinité tandem et les polypeptides associés (**à gauche**) ont été identifiés par spectrométrie de masse. Piste **M** correspond à un marqueur de masse moléculaire de protéine. **(D)** Détection par Western blot des protéines HJURP et NPM1 dans le complexe e-CENP-A de pré-assemblage. Les protéines des complexes e-CENP-A et e-H3.1 ont été séparées sur gel SDS-PAGE 4-12% et après transfert sur membrane de nitrocellulose, les protéines ont été révélées avec un anticorps anti-HJURP, un anti-NPM1, et anti-FLAG (pour détecter les e-CENP -A et e-H3.1). **(E)** Coloration à l'argent des protéines associées à e-HJURP. Les partenaires spécifiques de e-HJURP ont été purifiés par immunoaffinité tandem et identifié par des analyses de spectrométrie de masse **(Piste 2, Partie Haute)**. Les protéines identifiées sont indiquées a droite. La Piste **M** correspond à un marqueur de masse moléculaire de protéine. **(Partie Basse)** Détection par Western blot de CENP-A présent dans les complexes de prédéposition e-CENP-A (**Piste 1**) et e-HJURP (**Piste 2**). Les protéines des deux complexes ont été séparées sur gel SDS-PAGE 4-12% et après le transfert, le blot a

été révélé avec un anticorps anti-CENP-A. La masse moléculaire de l'e-CENP-A est plus élevée cause de la présence du peptide HA-FLAG fusionné à CENP-A.

Selon notre analyse dans le dépôt in vitro, HJURP facilité le dépôt efficace de CENP-A/H4 tétramère sur l'ADN nu pour faire tetrasome (CENP-A/H4 tétramère enveloppé par l'ADN) (Figure 2). Toutefois, il n'est pas clair si HJURP lie ou dépôts CENP-A-H4 dimères ou tétramères. Les structures cristallines de ces dernières HJURP^{scm3}-CENP-A^{cse4}-H4 complexe a révélé que HJURP/Scm3 lie un hétérodimère CENP-A/Cse4-H4 et empêche la formation de tétramère [380, 382]. Ainsi, les deux structures sont d'une hétérotrimère contenant une copie chacun des HJURP/Scm3, CENP-A/Cse4, et H4. Cela suggère soit un assemblage de deux étapes de CENP-A-H4 dimères par HJURP à centromères suivie par l'incorporation de deux dimères H2A-H2B, ou un dépôt d'une seule étape CENP-A-H4-H2A-H2B tetrasome hetetotypic.

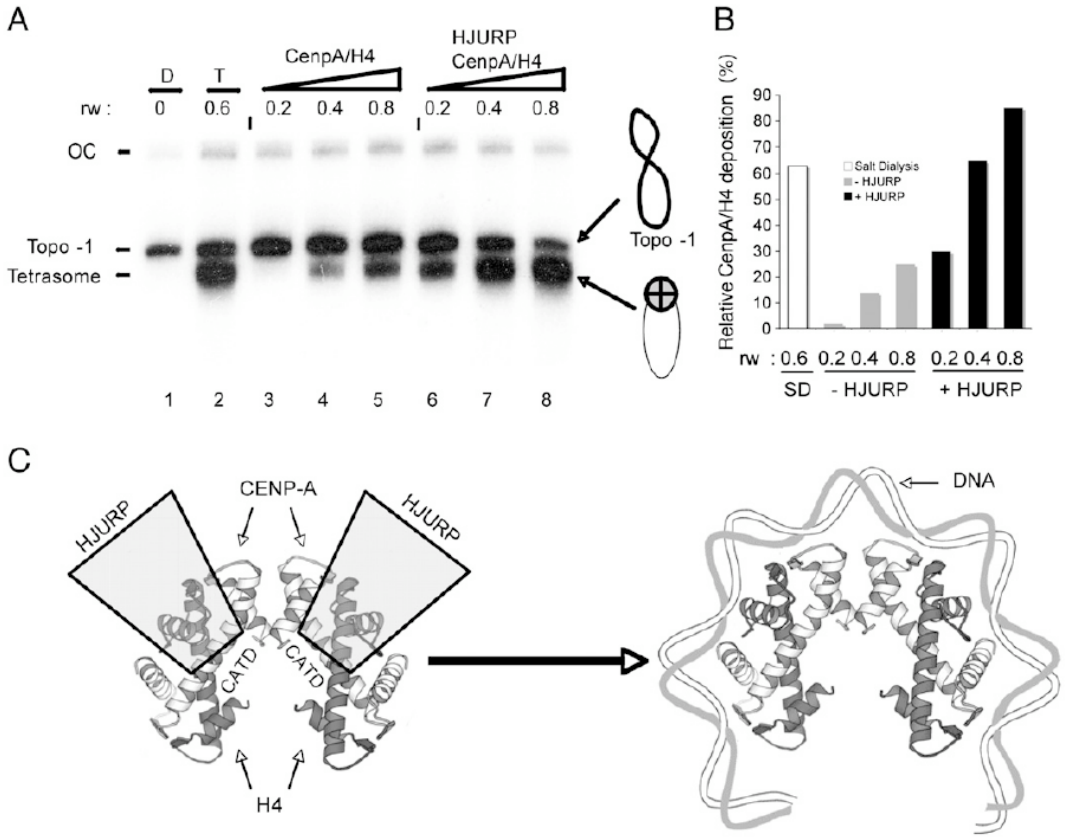

Figure 2. HJURP est capable de déposer efficacement un tétramère CENP-A/H4 sur l'ADN. **(A)** De l'ADN alpha-satellite humaine superenroulé négativement correspondant au topoisomer -1 (**Piste 1**, **D**) a été incubé avec des quantits croissants de CENP-A/H4 (aux rapports histone/ADN indiqué, rw) en absence (**Piste 3-5**) et en présence (**Piste 6-8**) de quantité de HJURP équimolaire au tétramère. La

réaction a été réalisée pendant 30 min à 37 °C. Le s produits de réaction ont ensuite été analysés sur gel de polyacrylamide natif à 4,5%. (**Piste 1**) ADN topoisomer-1 ; (**Piste 2**) Tetrasomes CENP-A/H4 reconstitués sur de l'ADN topoisomer -1 par dialyse saline en utilisant le ratio histone / ADN indiqué (rw). (**Partie droite**) Schéma montrant l'ADN topo-isomérase nu -1 et le tétrasome CENP-A/H4. Les positions des ADN topoisomer-1 nu et du tetrasome reconstitué par dialyse saline sont également indiquées. **(B)** Quantification des tétramères de Cenp-A/H4 déposés par HJURP. Le rapport tetrasome/ADN a été quantifié à l'aide du logiciel ImageJ. S.D. indique les tétramères assemblés par dialyse saline. **(C)** Modèle de la déposition de CENP-A. Deux molécules de HJURP dimérisent par leurs domaines coiled-coil et se lient, par l'intermédiaire de TLTY, au CATD de deux molécules de CENP-A (**à gauche**).

5.5. Conclusion

Ici, nous avons utilisé des stratégies puissantes biochimiques associés à la protéomique, à identifier machinisme dépôt de CENP-A, dans les cellules humaines. Nous montrons que HJURP, un membre de la CENP-A complexe prenucleosomal, est essentiel pour la localisation centromérique de CENP-A, *in vivo*. HJURP reconnaît et se lie spécifiquement au domaine CATD de CENP-A, par une très conservée domaine N-terminal, appelé CDB. L'ensemble de mes résultats démontre très clairement que HJURP est la principale chaperone responsable de la déposition de CENP-A aux centromères.

6.6. Perspectives

En dépit de récentes avancées dans nos connaissances sur la façon dont le centromère est spécifié et se propage d'une génération à l'autre, il y a encore beaucoup de questions qui demeurent sans réponse. L'assemblage et la composition du centromère sont encore très mal connus. Plusieurs questions restent posées. Comment CENP-A est dirigée spécifiquement vers le centromère? Comment une chaperone d'histone comme HJURP dirige spécifiquement CENP-A vers le centromère? Quelles sont les protéines associées directement a CENP-A et comment leur composition varie au cours du cycle cellulaire? Quelles sont les protéines qui assurent la jonction entre CENP-A et les microtubules ? Comment des modifications post-transcriptionelles des histones, telles que la phosphorylation, modulent la structure et la fonction du centromère?

Chez l'homme, la déposition de CENP-A aux centromères se déroule durant la phase G1 et est non couplé à la réplication. Le fait que la déposition de CENP-A ne soit pas couplée à la réplication pourrait résulter d'une dilution de CENP-A sur les centromères des chromosomes sœurs. Cela pose la question suivante : comment CENP-A est distribué sur les centromères sœurs ?

Une autre question qui mérite d'être posée est dans quelle mesure la composition de la chromatine centromérique change durant les différentes phases du cycle cellulaire.

Existe t il d'autres types de modifications post-traductionnelle impliquant CENP-A ? Et quel serait le rôle de ces modifications dans la déposition de CENP-A et dans la maintenance des centromères ?

Une autre importante question est comment le complexe HJURP portant les CENP-A nouvellement synthétisé est spécifiquement recruté au centromères. Existe il des marques épigénétiques spécifiques de la chromatine centromérique.

Quel est le rôle de la transcription et des ARN non codant dans la déposition de CENP-A et dans la fonction du centromère ?

Il est intéressant de comprendre le lien entre la stabilité de CENP-A et son incorporation aux centromères. Ainsi, Chez la levure, l'ubiquitine E3 ligase Psh1 médie la dégradation de Cse4 si Cse4 est mal incorporé. Mais, l'existence d'une telle dégradation de CENP-A chez l'Homme n'est pas connue.

Liste de publication

1. **Shuaib M,** Ouararhni K, Dimitrov S and Hamiche A, (2010). HJURP Binds CENP-A via a highly conserved N-terminal domain and mediates its deposition at centromeres. Proc. Natl. Acad. Sci. USA. Jan 26; 107(4): 1349-54.
2. Dranè P, Ouararhni K, Depaux A, **Shuaib M** and Hamiche A, (2010). The death-associated protein DAXX is the major histone chaperone controlling the replication independent deposition of H3.3. Genes Dev. 24(12): 1253-65.

3. Hamiche A and **Shuaib M** (2011). Chaperoning the histone H3 family. Biochim. Biophys. Acta. Doi:10.1016/j.bbagrm.2011.08.009.

CHAPTER 1
INTRODUCTION

1.1 Chromatin

Human body is composed of approximately 60 trillion cells, each of them carry the genetic information, which is present in its nucleus. Nucleus is the main organelle of the cell, responsible for storage, retrieval, translation and transmission of genetic information. For a long time, the source of this genetic information was unknown. Through pioneering work of Friedrich Miescher, who isolated a substance from the nuclei of white blood cells in 1869 and named nuclein, and similar work by others like Albrecht Kossel, Walther Flemming and Oskar Hertwig chromosomes were suggested to be the carriers of genetic information [14] . Friedrich, who coined the name nuclein, which is now known as deoxyribonucleic acid (DNA), encodes the genetic information of the cells. Watson and Crick elucidated the double helical structure of DNA in 1953 [15] and thus concluded that DNA is the carrier of genetic information.

Eukaryotic cells contain approximately 10 million to 100 billion base pairs DNA in each nucleus. DNA is organized as a double helix, made from two complementary DNA strands. The strands are composed of four different nucleotides, A, T, C and G. The order of the nucleotides on the DNA helix determines the sequence of all proteins.

The DNA molecule that comprises in a single nucleus, when extended goes approximately 2 m in length. These long strands of DNA in each cell need to be packaged into a structure to fit in, an organized manner, in the limited space available in the nucleus. DNA is compacted more than 10, 000 fold to reside in a small nucleus, which is approximately 10 μm in size. DNA has a negatively charged phosphate backbone that produces electrostatic repulsion between adjacent DNA regions, making it difficult for DNA to fold upon itself [16]. Therefore, this compaction is achieved through binding of DNA by histone and non-histone proteins to form a highly compact superstructure known as chromatin (W. Flemming, first used the term chromatin, in 1879).

Chromatin is a highly complex, dynamic, nucleoprotein structure in the cell nucleus. It is composed of genomic DNA, histone and non-histone proteins, and RNA molecules. The non-histone proteins associated with chromatin includes, DNA-binding factors (DBFs), the basal transcription, replication, and repair machineries, and many other factors that interact with any of these components. The basic building block of chromatin, termed the nucleosome, is composed of DNA and histone proteins.

On the first level of compaction, DNA is wrapped around a histone octamer, consisting of two copies of each of the four core histones H2A, H2B, H3 and H4 to form nucleosome. The nucleosomes are connected with linker DNA and the resulting structure is known as 10 nm chromatin filament. The 10nm filament further compacts into the 30nm fiber through interaction with linker histones. The higher order chromatin structures are formed upon folding by itself to form 30 nm fibers [17]. Chromatin is further compacted into chromosomes, which are dispersed in the cell nucleus during interphase when the cell is not dividing, and become highly condensed during cell division (Figure 1.1).

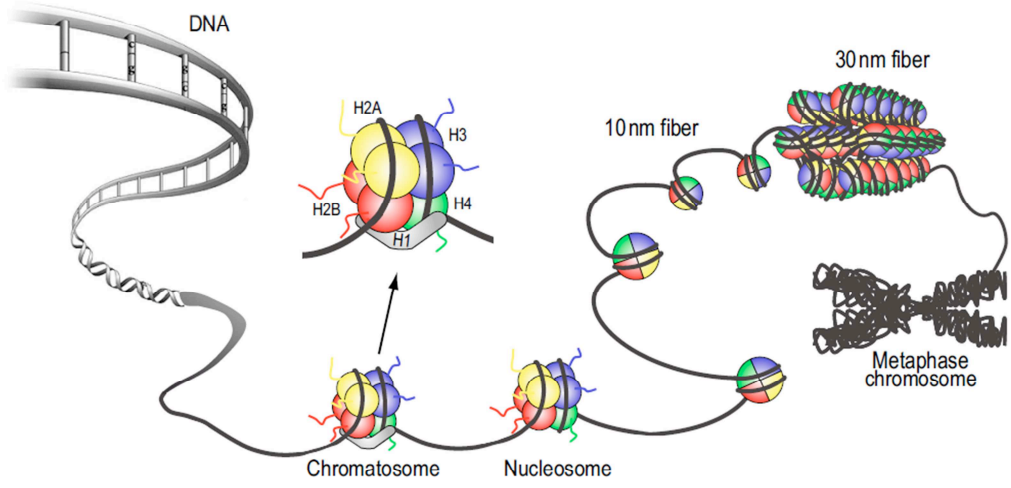

Figure 1.1. Overview of Chromatin: composition and levels *of organization*. The fundamental unit of chromatin is the nucleosome, consisting of DNA, wrapped around an octamer of two copies of each of the four core histones H2A (yellow), H2B (red), H3 (blue) and H4 (green). For further compaction, one molecule of the linker histone H1 (gray) can be bound, resulting in a chromatosome. Nucleosomes with linker DNA constitutes the 10 nm fibers, which can be further compacted by stacking of nucleosomes, leading to the 30 nm fiber. Little is known about higher-order chromatin structures finally leading to a condensation of the genetic material to the level of metaphase chromosomes. Image adapted from Qiu, 2006 [18] (modified).

Despite this high level of compaction, eukaryotic chromatin is highly dynamic and allows access to the DNA template during various essential cellular processes such as DNA replication, DNA repair, transcription and recombination. This dynamic nature of chromatin structure is regulated by different protein factors, including histone chaperones, ATP-dependent chromatin remodeling factors, histone variants, histone post-translational modifications (acetylation, methylation and phosphorylation) [19] and still many other unknown factors.

1.1.1 Chromatin organization

Eukaryotic nuclei show extensive organization with individual chromosomes occupying their own discreet territory within the nucleus [20]. The chromatin in higher eukaryotes is traditionally divided into structurally and functionally distinct euchromatin and heterochromatin regions. In 1928, Heitz first distinguished heterochromatin from euchromatin on the basis of differential compaction at interphase [21].

1.1.1.1 Euchromatin

Euchromatin represents a de-condensed state of chromatin, where it enriches gene density, stains lightly in GTG banding, replicates early during S-phase, transcriptionally active, and partially or fully uncoiled. It is characterized by high level of global histone acetylation and methylation of histone H3 (H3K4 and H3K36) and low level of repressive marks.

1.1.1.2 Heterochromatin

Heterochromatin is generally more condensed, inaccessible, transcriptionally inactive, replicates late in S-phase, and has the ability to suppress the transcription of a euchromatic gene placed adjacent to these domains [21, 22]. The formation of heterochromatin domains is mostly mediated by histone H3K9 methylation that recruits heterochromatin proteins (HP1). RNAi machinery is also a major player in heterochromatinization [23]. Heterochromatin regions are further distinguished as constitutive heterochromatin and facultative heterochromatin.

A. Constitutive heterochromatin

Constitutive heterochromatin remains condensed throughout the cell cycle, contains repetitive elements of the genome, has high level of H3K9 methylation and low level of histone modifications associated with active genes, such as acetylation and methylation of lysines K4 and K36 on histone H3. Constitutive heterochromatin regions are present throughout the chromosomes, but especially at or adjacent to the centromeres. Heterochromatin domains adjacent to centromeres are called pericentric heterochromatin.

B. Facultative heterochromatin

Facultative heterochromatin contains loci that are silent only in certain contexts, such as cell type, stage of the cell cycle, or in time of development. The facultative heterochromatin is enriched with H3K9me, H3K27me, and H3K20me in higher eukaryotes. The repressive polycomb complexes PCR1 and PCR2 are involved in specification of facultative heterochromatin.

1.1.2 The Nucleosome

Traditionally, the periodic nature of chromatin was revealed by biochemical and electron microscopic analyses. The basic subunit structure of chromatin was first identified by micrococcal nuclease digestion of DNA associated with chromatin. Initially, the partial digestion of chromatin resulted in fragments of 180-200 base pairs in length, which were resolved by electrophoretic migration [24, 25]. Further treatment with nuclease trims linker DNA (the DNA between the adjacent nucleosome core particles) generates a particle with approximately 166 bp of DNA, an octamer of core histones and a H1 histone [26]. Additional digestion, led to the production of nucleosome core particles, with 146 bp of DNA and the core-histone octamer [26, 27]. The repeating nature of chromatin structure was confirmed by electron microscopic analysis that revealed chromatin as regularly spaced particles or "beads on a string" [28, 29]. In parallel, chemical cross-linking experiments determined the precise stoichiometry of DNA and histones in the nucleosome to be 1/1 based on their mass [30]. Together these studies led to the proposition that the nucleosome was the fundamental unit of chromatin. Later, the group of Pierre

Chambon proposed the term, nucleosome [31].

Nucleosome is the basic structural and functional unit of the chromatin, which is composed of a core particle and a linker region (or inter-nucleosomal region) that joins adjacent core particles. The nucleosome core particle (NCP), contains 147 bp of DNA wrapped around an octamer of the four core histones (H2A, H2B, H3 and H4) in ~1.7 left-handed super helical turns [32]. The nucleosome core particle is highly conserved between species, however, the length of the linker region containing the linker histones, varies between species and cell type. Thus, the total length of DNA in the nucleosome can vary with species from 160 to 241 base pairs [33-37]. Nucleosomes are connected with one another to form nucleosomal arrays which further fold into less understood 30nm fiber and higher order chromatin structures.

1.1.2.1 Nucleosome Structure

Over the last three decades enormous efforts have been directed at understanding the structure and function of the nucleosome, and how these structures influence important nuclear processes.

Initially in 1977, a low-resolution picture of the nucleosome core was obtained using a combination of X-ray diffraction and electron microscopy [38]. Since then, in 1984, the structure of the nucleosome core particle was solved for the first time at 7Å resolution [39], and later in 1991, the structure of the histone octamer without DNA in the presence of high salt, was determined at 3.1Å [32]. These structures publicized for the first time a basic tripartite assembly of the octamer, reflecting its two H2A/H2B heterodimer and one $(H3/H4)_2$ tetramer components. The tetramer itself is a stable complex of two H3/H4 heterodimers, which have a 'handshake motif' interlocking protein fold very similar in structure to that of the H2A/H2B heterodimers. This protein architectural motif is now referred to as the "histone fold" [40].

However, the detailed and high-resolution structural features of the nucleosome were revealed by X-ray crystallography at 2.8Å, in 1997 [41] (Figure 1.2). The 2.8Å crystal structure of the nucleosome core particle illustrates the importance of the histone fold domains, in histone–histone and histone–DNA interactions within the nucleosome. The histone fold domains (HFDs) organize the

central 121 bp of DNA, with the additional 13 bp at each end organized by an N-terminal alpha-helical extension to the histone fold of H3 and preceding residues from the tail domain. Each histone dimer organizes 27–28 bp, with 4 bp stretches between them. Overall, this structure shows that 146bp of DNA is wrapped around the histone octamer in 1.65 superhelical turns, and portions of the histone tails are extending across the DNA double helix. The crystal structure reveals that internal portions of the charged N-terminal tails of H3 and H2B pass through channels in the DNA superhelix formed by aligned minor grooves, whereas similar portions of the N-terminal tails of H4 and H2A pass over the gyres of the DNA superhelix. Interestingly, the H4 residues from 16–25 extend into the adjacent nucleosome in the crystallized nucleosome array to interact with a highly negatively charged face of the H2A–H2B complex.

In the nucleosome (and in the histone octamer crystallized in the absence of DNA at high salt), each H2A-H2B dimer has two contact points with the (H3-H4)2 tetramer. A four-helix bundle (4HB) arrangement, similar to that formed by two H3 molecules within the (H3-H4)2 tetramer, connects H2B and H4; a second interaction interface is formed between the H2A docking domain and the other arm of the (H3-H4)2 tetramer (Figure 1.2).

Later in 2003, the extension of the diffraction limits of nucleosome crystals to 1.9Å revealed the precise structural parameters of the DNA [42]. After eight years of the first publication on nucleosome structure at 2.8Å, the X-ray crystallographic structure of a tetranucleosome was determined, in 2005 [43].

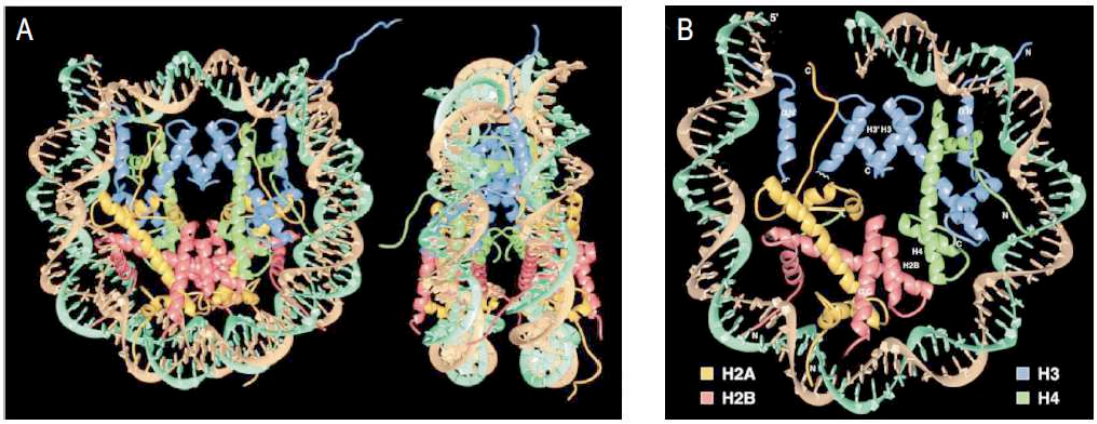

Figure 1.2. Nucleosome Structure. Depicted are ribbon traces of the 146 bp DNA molecules (brown and turquoise) and the core histones H2A (yellow), H2B (red), H3 (blue) and H4 (green). (A)

The complete nucleosome core particle is shown with the view down the DNA superhelix axis (left) and perpendicular to it (right). In both cases the pseudo-twofold axis is aligned vertically with the DNA center at the top. (B) The 73-bp half of the nucleosome core particle is shown with the view down the superhelix axis and the pseudodyad axis aligned vertically. Histone proteins primarily associated with the 73-bp superhelix half are depicted (without interparticle tail regions). The two copies of each histone pair are distinguished as unprimed and primed (e.g. H3 and H3'). 4HBs are labeled as H3' H3 and H2B H4; HFD extensions of H3 and H2B are labeled as αN', αN and αC, respectively; N- and C-terminal tail regions as N or C. Images adapted from Luger et al., 1997 [41](modified).

1.1.2.2 Nucleosome Assembly

The proper *in vivo* assembly of nucleosome occurs in a sequential manner through deposition of two H3-H4 dimer pairs or a single H3-H4 tetramer onto DNA, followed by the cooperative addition of two H2A-H2B dimers to organize the peripheral regions of the DNA [44]. This step-wise assembly of nucleosome is described schematically in Figure 1.3. The complex of H3-H4 tetramer with two H2A-H2B dimers is called histone octamer, around which 146 base pairs (bp) of DNA is wrapped to make the complete nucleosome (Figure 1.3). The ordered assembly of histones into nucleosome is assisted by histone chaperones and chromatin assembly factors, which are discussed in more detail later in section 1.4, of this chapter. Disassembly of the nucleosome is more likely follows the reverse pathway.

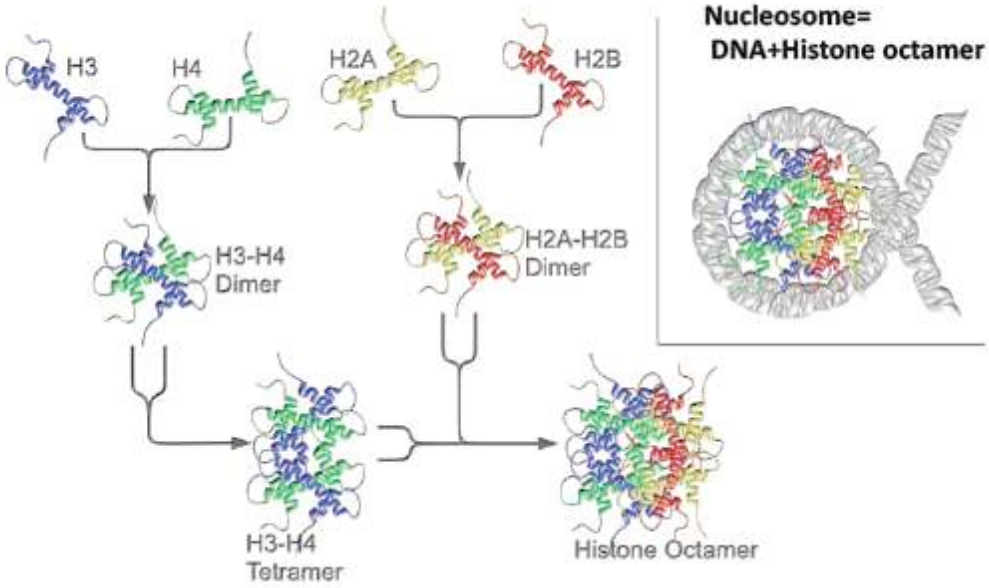

Figure 1.3. Schematic illustration of histones assembly into Nucleosome. Two molecules of each of the four core histone proteins (H2A, H2B, H3 and H4) form the histone octamer via formation of one tetramer of H3 and H4 and two dimers of H2A and H2B. These entities are held together by a so-called hand-shake motif of protein structure. The histone octamer is wrapped by 146bp of DNA to complete nucleosome formation. Image adapted from [45].

1.1.3 Histone Proteins

Histone proteins were first discovered and purified by Albrecht Kossel from bird erythrocytes and sperm in 1884 [46]. As they were further characterized during the last century, five histone families were distinguished which are now universally designated H1, H2A, H2B, H3 and H4. Histones are highly conserved small basic structural proteins in eukaryotes, spanning a molecular weight range of 10-32 kDa. High content of positive lysine and arginine residues make histones excellent DNA binding proteins and contribute to their tight interaction with negatively charged DNA. Histones are divided into two main groups; core histones and linker histones.

The genes encoding canonical histone proteins are intron-free, replication-dependently expressed, organized in multi-copy clusters and their mRNAs have a conserved stem-loop structure at the 3' end instead of a poly (A) tail. This unique 3' structure, together with the stem loop binding protein (SLBP), is responsible for the processing, translation and degradation of canonical histone mRNAs. Thus, they ensure high expression levels throughout S-phase, when large amounts of histones are needed for replication, followed by a rapid degradation at the end. Histone gene clusters typically contain multiple copies of each of the genes encoding for the five different histone proteins.

1.1.3.1 Core Histones

Among the five histone families, H2A, H2B, H3, and H4, are the core histones, characterized by a highly conserved central domain known as the "histone fold domain". This fold domain is consisting of three alpha helices, one long and two short, which are separated by two loop regions [32]. Through the histone fold domain, the core histones interact with each other's and with the nucleosomal DNA. Moreover, the histone fold domains confer interactions with other nuclear factors and nucleosome-nucleosome contacts, which explain their extreme degree of amino acid sequence conservation.

In contrast to the histone fold domain, the N-terminal and C-terminal tails of histones are highly variable in sequence and lengths, depending on the type of

histone. The tails are particularly rich in lysine and arginine residues making them extremely basic. These tail regions contain sites for different histone post-translational modifications that are proposed to modify its charge and thereby alter DNA accessibility and protein/protein interactions with the nucleosome [47]. The tails protrude from the nucleosome; the four H3 and H2B N-terminal tails exit through the minor groove of the DNA superhelix and contribute to chromatin compaction by attaching to the entering and exiting linker DNA [48-50]. Histone H2A is unique among the core histones in having both an N-and a C-terminal basic tail. The H2A C-terminal tail binds the DNA around the dyad axis [51] whereas the N-terminal tails of H2A contact DNA towards the periphery of the nucleosome [52]. Among all histone tails, H4 tails mediate the most internucleosomal interactions, followed by the H3, H2A, and H2B tails in decreasing order [53].

1.1.3.2 Linker Histones

As the name indicates, linker histones associate with the linker region of DNA between two nucleosome cores. Unlike the core histones, they are not well conserved between species. Structurally, the H1 histones are composed of three domains: a globular, non-polar central domain essential for interactions with DNA and two non-structured N- and C- terminal tails that are highly basic and proposed to be the site of post translational modifications [54]. The linker histones are important for further compaction into the 30 nm fiber and higher order chromatin structures.

1.2 Epigenetic Regulations

The term “epigenetics” was originally used to describe the gradual changes during the development of animals, or more specifically the question of how the same genotype can give rise to different phenotypes. Conrad Waddington was the first, who coined the word epigentics in 1940s [55]. An adult multicellular organism contains hundreds of different cell types; each of them carries the same genotype, but displaying different gene expression profiles and phenotypic characteristics. The maintenance of cell-type specific expression profile in differentiated cells and the transmission of these expression profiles to their daughter cells rely mainly on

epigenetic mechanisms. In 1994, Robin Holliday defined epigenetics as “nuclear inheritance which is not based on differences in DNA sequence [56]. Today, epigenetics is generally defined as “the study of changes in gene function that are mitotically and/or meiotically heritable, and that occur without changes in the DNA sequence [57].

Epigenetics, in a broad sense, is the process by which a gene’s activity is modulated through covalent modifications to the DNA, the histones around which it is wrapped, or the physical packaging of the chromatin in which it is embedded. These modifications on the DNA allow regions of the genome to be specified as active or inactive and influence their localizations within the nucleus. Moreover, the epigenetic regulation is involved in specifying and maintaining the function of specific genomic regions such as telomeres and centromeres.

The epigenetic regulation, involving the chromatin structure is executed via different mechanisms like DNA methylation, histone post-translational modifications, non-coding RNAs, nucleosome remodeling, and incorporation/exchange of histone variants by histone chaperones (Figure 1.4).

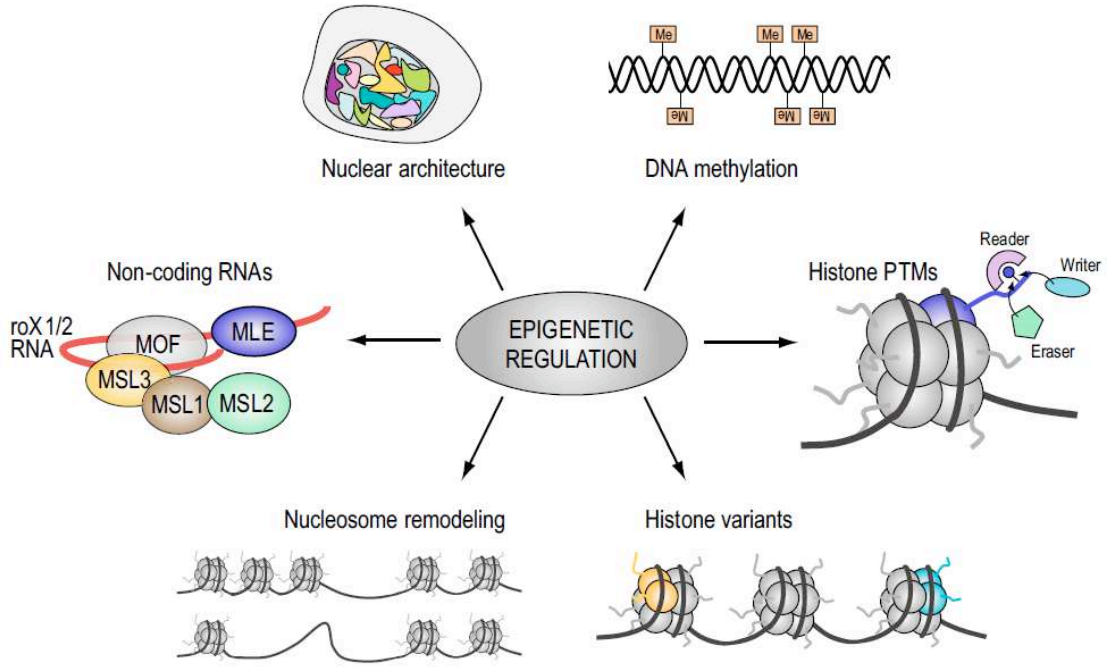

Figure 1.4. Epigenetic regulations.
Schematic overview depicting major epigenetic regulatory processes, which act on different, levels. DNA is methylated on specific cytosines. Histones, which organize DNA into nucleosomes, can be posttranslationally modified by “writer” enzymes, thereby changing the charge and/or the binding site

for specific “reader” molecules. PTMs can also be removed by “eraser” enzymes. In addition, histones can be exchanged by histone variants with different sequences, resulting in a change of structural properties and PTM sites. Accessibility of chromatinized DNA can be regulated by ATP-dependent nucleosome remodeling, for example shifting or evicting nucleosomes. NcRNAs are implicated in diverse processes. Examples are the roX1/2 RNAs in the Drosophila dosage compensation complex and Xist RNA, implicated in X chromosome inactivation in mammals. Not only is the packaging of DNA important for DNA-related processes, but also the localization within the nucleus. All these processes do not act alone but are interconnected. See text for details. Image adapted from [58].

1.2.1 DNA Methylation

DNA methylation is the best-characterized chemical modification of chromatin, in eukaryotes. The process of DNA methylation involves the transfer of methyl group from S-adenosyl methionine (SAM) to the C-5 position of cytosine modifying it to 5-methyl cytosine (5meC), by the action of DNA methyltransferases (DNMTs). This modification is both mitotically and meiotically inheritable, and is implicated in long term chromatin silencing. In mammals, nearly all DNA methylation occurs in the context of CpG dinucleotides, while in plants the cytosine can be methylated at CpG, CpNpG, and CpNpN sites, where N denotes any nucleotide other than guanine. Genomic distributions of cytosine methylation in mammals play a critical role in gene regulation and chromatin organization during embryogenesis and gametogenesis [59]. During early development, after all methyl marks have been erased, methylation patterns are established *de novo*, marking different sites in the maternal and the paternal genome (imprinting). In somatic mammalian cells, methylation occurs symmetrically at CpG dinucleotides, enabling the maintenance DNA methyltransferase (Dnmt1) to copy the marks after replication, thereby maintaining the silenced state of the underlying DNA sequence [60, 61]. Overall, DNA methylation at cytosine plays important roles in many cellular processes including silencing of transposons and centromeric sequences from fungi to mammals; X chromosome inactivation in female mammals; and mammalian genomic imprinting, all of which can be stably maintained.

1.2.2 Histone Post-translational Modifications

Histones are subjected to a variety of post-translational modifications (PTMs), which can alter chromatin structure and thereby influence the major genomic processes such as gene regulation, DNA repair and replication. The residues of the

N-termini of histones H3 and H4 and the amino and C-termini of histones H2A, and H2B, are particularly marked by different PTMs (Figure 1.5). Studies based on genetic as well as biochemical approaches have shown the important role of the core histone tails in chromatin structure and gene regulation. Even though tailless core histones can assemble to form nucleosomes *in vitro*, the N-terminal tails of histones H3 and H4 were shown to be indispensable *in vivo* for the repression of the silent mating-type loci, telomeres in yeast [62], and enhancer-dependent activation of some genes in yeast required these N-terminal sequences as well [63, 64]. Deletion of both the H3 and H4 N-terminal tails in yeast is lethal [65] and mutation analysis has confirmed that certain point mutations in the N-terminus of the H4 tail could inhibit gene silencing [66]. It is known that the tails of the core histones can be differentially modified by different mechanisms.

Until now, acetylation, phosphorylation, and methylation have been described extensively [67], while relatively little is known about ubiquitination, sumoylation, ADP ribosylation, glycosylation, citrullination, biotinylation, carbonylation [68-72] and lysine butyrylation and propionylation [73] (Figure 1.5). These modifications act by either changing the charge of a residue, thereby influencing its affinity for the DNA or adjacent nucleosomes, or they establish high-affinity sites for specific “reader” molecules, which directly or indirectly contribute to regulation of gene expression. The reader molecules contain domains that recognize the specific PTM mark and bind to the histone carrying it. For example, the chromodomains bind methylated lysine residues, and bromodomains bind acetylated lysine residues. Some of these modifications take place immediately after the synthesis of histones in the cytoplasm, for example, acetylation on the lysine 5 and 12 of histone H4, whereas others modifications occur mainly in the cell nucleus.

Specific post-translational modification patterns have been shown closely linked to different chromatin activities, for example, acetylation is associated with transcription, and phosphorylation is associated with chromosome condensation or DNA repair [74, 75]. Interestingly, the differential patterns of modifications at K9/S10/K14 in histone H3 appear to be linked with local gene activity. An inactive state is often characterized by histone deacetylation at Lys14 (K14), which then promotes methylation at Lys9 (K9) [76]. However, acetylation at Lys14 is preceded

by, and depends on, phosphorylation at Ser10. The relationship between distinct modification patterns and physiological functions has raised the concept of "histone code". According to the "histone code hypothesis", single or combined marks on histones store and transmit information on the gene expression status through mitosis and subsequent cell generations [47, 77].

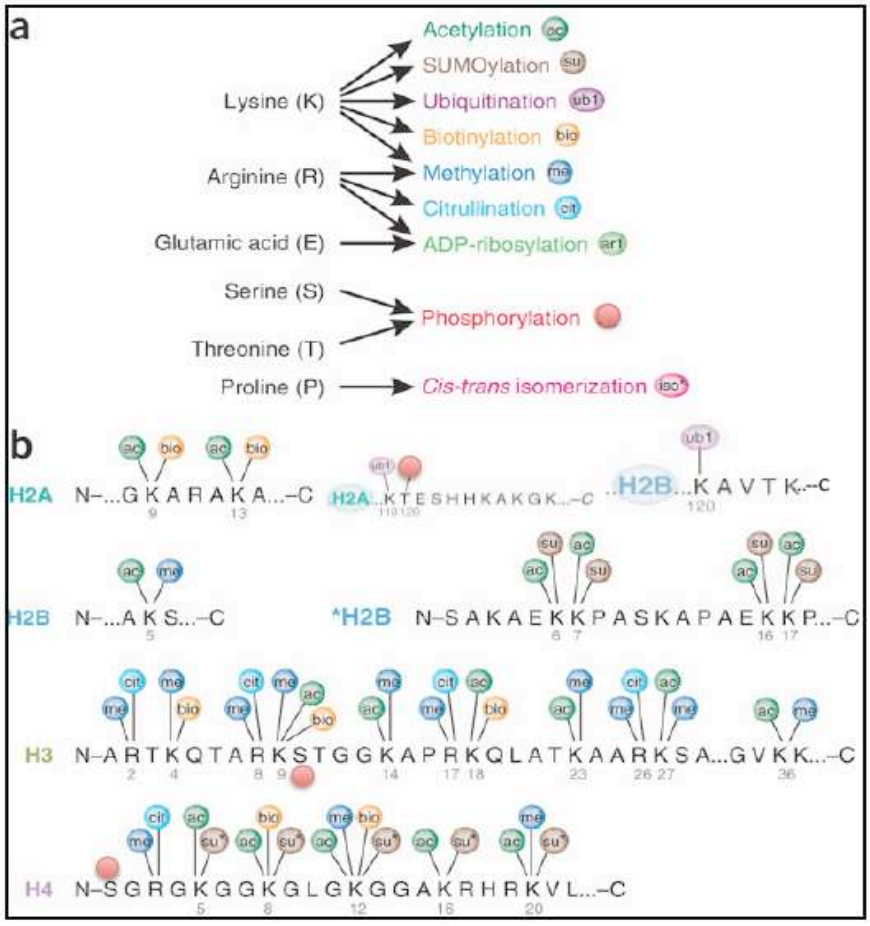

Figure 1.5. Histone Post-translational Modifications.

(a) Post-translational modifications and the amino acid residues they modify. (b) Residues that can undergo several different forms of post-translational modification or cross talk *in situ*. The asterisk indicates that either the histone amino acid sequence or the modification is from *S. cerevisiae*. Image adapted from [78] (modified).

1.2.2.1 Histone Acetylation

The post-translational acetylation of the core histones was first documented in 1964 [79]. Since then, increasing amount of studies has been extended to identify the mechanisms of histone acetylation and its significant roles in chromatin based regulation of gene expression.

Acetylation of lysine residues in histone tails is mediated by acetyltransferases, which catalyze the addition of acetyl groups to either the ε- (side chain acetylation) or the α-amino group of specific lysines in histones and other proteins [80]. The histone acetyltransferases are classified into four families the GNAT (GCN5-related N-terminal acetyltransferases), MYST superfamily includes enzymes that catalyze the transfer of an acetyl group from acetyl-CoA to a primary amine of non-histone proteins and small molecules [81]; the p300/CBP proteins, another protein family such as p270, which is distinct but related to proteins p300 and CBP, the general transcription factors HATs, which include TAF250, the largest of the TATA binding protein-associated factors (TAFs) within the transcription factor complex TFIID, can acetylates lysine residues in the N-terminal tails of histones H3 and H4 *in vitro* [82]. The acetylation of histones is reversible and rapidly turned over. Histone deacetylase (HDACs) family of enzymes mediates the removal of acetyl group from histones. The major groups of HDACs include the RPD3/HDA1 superfamily, the Silent Information Regulator 2 (SIR2) family and the HD2 family. RPD3/HDA1-like HDACs are found in all eukaryotic genomes and are further divided into two classes: class I HDACs (HDAC-1, -2, -3 and -8) are similar to the yeast RPD3 protein; class II HDACs [HDAC-4, -5, -6, -7) are homologous to yeast HDAC1 protein.

The accurate mechanism by which histone acetylation regulates gene expression is not clear. However, there are two general views according to which histone acetylation contributes to the formation of a transcriptionally active environment by 'opening' chromatin and allowing general transcription factors to gain access to the promoter regions and, initiate transcription. According to the first proposal the histone acetylation acts by neutralizing the positive charge on histone tails and thus serves to dissociate the tails from the DNA, making the chromatin more labile [83]. Additionally, histone acetylation may recruit bromodomain proteins that bind directly to the acetylated histones [84]. In contrast to histone acetylation, the deacetylation of histones contributes to the formation of a 'closed' chromatin state and transcriptional silencing. Condensed heterchromatin regions are generally hypoacetylated, whereas euchromatin active domains are associated with hyperacetylated histones. Highly acetylated histones are not limited to the coding

region, they are also found along the entire loop domain, but they are never found close to repressive heterochromatic structures in nuclei [85].

1.2.2.2 Histone phosphorylation

Histone phosphorylation was first discovered in 1967 [86] and since then, many of the kinases that are responsible for histone phosphorylation have been characterized [87, 88]. Phosphorylation occurs on core histones and linker histone H1 at the side chains of serine, threonine and, rarely, tyrosine, residues by substituting a phosphate for a hydroxyl group to give an O-phosphate linkage. Histone phosphorylation is catalyzed by different protein kinases using nucleotide triphosphates (ATP, GTP, cyclic AMP) as PO4 donors, and reversed by specific phosphatases.

Histone phosphorylation plays important roles in a wide range of cellular processes, including transcriptional regulation, apoptosis, cell cycle progression, DNA repair, chromosome condensation, enzyme activation/ inhibition, protein degradation and developmental gene regulation [89-93]. The posttranslational phosphorylation of histone H3 and linker H1 histone has been most extensively studied.

Core histone H3 variants have conserved residues within their N-terminal tail, namely Thr3, Ser10, Thr11 and Ser28, which are phosphorylated in a wide range of organisms during mitosis. Mostly, histone tails phosphorylation at serine and threonine residues appears to be involved in chromatin condensation during mitosis and meiosis; for example, C-terminal phosphorylation of Thr119 in histone H2A is linked to regulation of chromatin structure and function during mitosis [94], and H3S10 phosphorylation is related to chromatin compaction during mitosis. While, phosphorylation of Ser10 and Ser28 (H3S10P and H3S28P) appear to have an additional role in the transcriptional activation of genes in interphase nuclei. The kinases responsible for phosphorylation histone H3 at S10 include IpL1 and Snf-1 in yeast [95, 96], whereas Aurora B, IKK, Rsk2, and AKT have been implicated in mammals [88, 97-99]. The list of core histone phosphorylation and their corresponding functions are summarized in table 1.1.

Table1.1. Histone phosphorylation sites, their kinases, recognizing proteins and functions.

Histones	Sites	Organism	Kinase involved	Protein Recognition	Related Functions
H2A	Serine 139 (H2AX)	Mammals	ATR, ATM, DNAPK, RSK2,MSK1	DSB sensing proteins (MRN, MDC1, P53BP1), AP1	DNA repair, Decreased EGF-mediated cellular transformation, Apoptosis
	Tyrosine 142(H2AX)	Mammals	WSTF	Not Known	Decision between cell survival or apoptosis
	Serine 1	Mammals	MSK	Not Known	Inhibition of transcription
	Threonine 119	Mammals	NHK-1,Aurora B	PTB-domain containing protein Fe65	Regulates chromatin structure and function during mitosis
	Serine 121	Fission yeast	Bub1	Shugoshin	Maintains chromosomal homeostasis by recruiting Sugoshin at sister kinetochore
	Serine 129	Yeast	ATM-related kinase Mec1 &Tel1	NuA4, SWR1,INO80, Cohesin	DSB repair
H2B	Serine 10	Yeast	Ste20	Not Known	Apoptosis
	Serine 14	Number of Vertebrates	Mst1	Not Known	Apoptosis
	Serine 32	Mammals	Protein kinase C	Not Known	Possibly involved in apoptosis-related nucleosomal DNA fragmentation
	Serine 33	Drosophila	CTK-TAF1	Not Known	Transcriptional regulation
	Serine 36	Mammals	AMPK	Not Known	Direct transcriptional and chromatin regulatory pathways leading to cellular response to stress
H3	Threonine 3	Mammals	Haspin	Survivin	Correct localization of CPC at the centromere
	Threonine 6	Mammals	PKCβ1	Not Known	Androgen dependent H3T6 phosphorylation prevents LSD1 mediated H3K4 demethylation thereby maintaining hormone dependent gene activation
	Serine 10	Yeast, mammals	Snf1 ,IpL1(yeast), Aurora B(mammals),MSK1/2,IKKα, PKB/Akt,Rsk2,PIM1	HP1, SRp20, ASF/SF2, 14-3-3	Helps in chromosome condensation during mitosis and meiosis; roles in transcription of certain genes
	Threonine 11	Mammals	Chk1, PRK1, Dlk/Zip kinase	GCN5	Transcriptional activation of certain genes; probable role at centromeres during mitosis
	Serine 28	Mammals	Aurora B,MSK1/2	Polycomb silencing complex	Helps in chromosome condensation during mitosis and meiosis; roles in transcription of certain genes
	Tyrosine 41	Mammals	JAK2	HP1α	Role in differentiation related to Hematopoiesis
	Threonine 45	Mammals, budding yeast	Protein kinase C,S-phase kinase Cdc7-Dbf4	Not Known	Apoptosis, Role in DNA damaged cells when the DNA is nicked, replication of DNA
H4	Serine 1	Yeast, Mammals	CKII	Not Known	DNA damage repair, mitosis and chromatin assembly
	Histidine 18 & 75	Mammals	Unknown	Not Known	Facilitates DNA replication by destabilizing histone octamer

1.2.2.3 Histone Methylation

Histones, especially H3 and H4 have long been known to be methylated on either lysine (K) or arginine (R) residues. The lysine side chains may be mono-, di- or

tri-methylated, and this differential methylation provides further functional diversity to each site of Lys methylation. While, the arginine side chain may be mono-methylated or (symmetrically or asymmetrically) di-methylated [100, 101]. Histone methyltransferases (HMTs) catalyze the addition of a methyl group from S-adenosyl methionine (SAM) to corresponding histone residues. The histone methytransferases (HMTs) display fine substrate specificity, which modify specific lysine residues of free histones or within nucleosomes. For example, Dot1, Set2 and PR-Set7/Set8 can only methylate histone tails presented in the context of nucleosomes [47, 102, 103] while other HMTs prefer free histones or can methylate tails from both free histones and nucleosomes.

At present, there are 24 known sites of methylation on histones (17 are lysine residues and 7 are arginine residues). The major sites of Lys-methylation on histones identified so far are: Lys4, Lys9, Lys27, Lys36, Lys79 on H3 and Lys20 on H4 [104, 105]. Lysine histone methyltransferases usually contain a SET catalytic domain (Suppressor of variegation, Enhancer of zeste and Trithorax). Methylation of lysine residues does not significantly change the positive charge, but progressively increases the bulk and hydrophobicity, thus disrupting intra- or inter-molecular hydrogen-bond interactions of the ε-amino group, or creating new binding sites for other modulators. Histone H3 can be mono or di-methylated on arginine residues 2, 8, 17, 26 and H4 at arginine 3. Arginine methylation is catalyzed by CARM1/PRMT arginine histone methyltransferases.

Arginine methylation has been associated with transcriptional activation whereas lysine methylation has been linked to both activation and repression. For instance, methylated H3K4, H3K36 and H3K79 are considered to be marks for transcriptionally potentiated chromatin structures while methylated H3K9, H3K27 and H4K20 mark silent chromatin [106]. Histone methylation and in particular trimethylation, was long regarded as irreversible because of the high thermodynamic stability of the N–CH3 bond. The recent identification of several demethylases showed that methylation could also be reversed without the exchange of bulk histones [107].

1.2.2.4 Histone ubiquitination

Histone ubiquitination was first reported in 1970 [108]. The core histones H2A, H2B, H3 and their variant forms are known to be ubiquitinated. Histone ubiquitination represents the most bulky structural change to histones. Ubiquitin is a small (76 amino acid) polypeptide, which is attached as a polymer to the ε-amino group of lysine residues in polypeptides targeted for proteasomal degradation. Generally, the addition of an ubiquitin moiety to a protein involves the sequential action of E1, E2, and E3 enzymes. In contrast, removing of ubiquitin moiety is achieved through the action of enzyme called isopeptidases [109].

Histone H2A was the first protein shown to be ubiquitinated at a highly conserved lysine residue K119 [108, 110]. This ubiquitination affects about 5-15% of histone H2A in most eukaryotic cells. However, ubiquitinated H2A has not been reported in the budding yeast *Saccharomyces cerevisiae* [111]. The majority of H2A is in monoubiquitinated form; however, polyubiquitinated H2A has also been detected in many tissues and cell types [112]. Ubiquitinated H2B is another most abundant ubiquitin conjugates in eukaryotes and has been identified in many eukaryotic organisms except *S. pomber* and *Arabidopsis thaliana*. In addition to H2A and H2B, ubiquitination on H3 and H1 have also been reported [113, 114]. Although the exact role of histone ubiquitination on transcription activation is, so far, still controversial, it has been suggested that histone ubiquitination most likely regulates gene transcription both in a positive and negative fashion, depending on its genomic location.

1.2.2.5 Histone Sumoylation

Sumoylation involves the addition of a “Small Ubiquitin like Modifier” (SUMO) protein, of 100 amino acids to the lysine residues in the target proteins. Histone sumoylation was first reported in 2003, when Shiio *et al*. found that H4 can be modified by SUMO and they suggested that this modification was linked to transcription repression by recruitment of HDACs and HP1 proteins [115]. The reported sumoylation sites include lysine 126, on H2A, lysines 6, 7, 16 and 17 on histone H2B and lysines 5, 8, 12, 16 and 20 on histone H4 [116]. Histone

sumoylation has a role in transcription silencing by opposing other active marks such as acetylation and ubiquitination.

1.2.2.6 Histone ADP-Ribosylation

ADP-ribosylation is a post-translational modification of proteins, catalyzed by ADP -ribosyltransferases [ART, mono(ADP-ribosyl)transferases]. The process involves the transfer of an ADP-ribose moiety of NAD to a specific amino acid residue on the target protein via N- or S-glycosidic linkages, and at the same time, release nicotinamide. Another separate class of enzymes, namely poly-(ADP-ribose)-polymerase [PARP, poly(ADP-ribosyl)transferase], yields chains of ADP-ribose units linked to each other by O-glycosidic linkages [117]. All core histones and linker histone H1 are subject to mono (ADP-ribosyl)ation either in response to genotoxic stress or in physiological conditions defending on the cell cycle stage, proliferation activity or degree of terminal differentiation. Poly ADP-ribosylation (PARation) can also be detected on the majority of histone types.

1.2.2.7 Histone Citrullination

Citrullination has been detected at histone H3R17 and H4R3. This modification is catalyzed by peptidylarginine deiminase 4 (PAD4), which converts methylated arginine to citrullinated arginine [118].

1.2.2.8 Histone Biotinylation

Biotinylation on lysine residues is catalysed by 'lysine biotinase' enzyme. These biotinylation marks are commonly abundant in repressed regions of the chromatin. A single biotin moiety contributes ~244 Dalton. Histone biotinylation is often enriched at heterochromatin and participates in gene silencing.

1.2.3 Non-coding RNA

Generally the term non-coding RNA (ncRNA) is used for RNA that does not

encode a protein, but this does not imply that such RNAs do not contain information nor have function. Recently, it has become evident that RNA, particularly noncoding RNAs, plays important roles in various epigenetic phenomena in all kingdoms of life [119]. The non-coding RNAs (ncRNAs), such as ribosomal RNAs (rRNA), transfer RNAs (tRNA), small nuclear RNAs (snRNA), small nucleolar RNAs (snoRNA), and small interfering RNAs (siRNA), can serve catalytic and scaffolding functions in transcription, messenger RNA processing, translation, and RNA degradation.

The non-coding RNAs often act in concert with various components of the cell's chromatin and DNA methylation machinery to achieve stable silencing. The transcriptional gene silencing (TGS) evoking RNAs (e.g., repeat-associated siRNAs, Xist RNA, and small RNAs in *S. pombe*) are more clearly epigenetic in nature, as they can induce long-term silencing effects that can be inherited through cell division [119]. In addition, there exist significant crosstalks between different epigenetic pathways. For example the silencing of the inactive X chromosome is one of the well-known non-coding RNA based epigenetic regulation, Xist RNA, together with DNA methylation, histone modifications, and their writers and readers all play a role.

1.2.4 Chromatin Remodeling

As the name indicates "chromatin remodeling" involves ATP-dependent changes in the structure of chromatin brought about by dedicated nuclear enzymes, and thereby affect DNA accessibility to regulatory proteins [120, 121].

All DNA-dependent processes required chromatin to be in a state of 'plasticity' or 'fluidity'. This dynamic balance between genome packaging and genome access is regulated by the tight interplay between histone modifying enzymes (discussed earlier) with "ATP-dependent chromatin remodeling factors". In contrast to histone covalent modifications, ATP-dependent chromatin remodeling factors physically alter the position or/and structure of nucleosomes, by utilizing the energy of ATP hydrolysis. Chromatin remodeling and histone modifying machineries provide chromatin with dynamic properties and making it suitable substrate for the execution of the DNA templated processes, such as transcription, DNA replication, chromosome recombination, and DNA repair.

In vivo, the action of ATP-dependent chromatin remodeling machinaries may lead to a variety of phenomena, ranging from the complete absence of nucleosomes at regulatory sites [122] to shifting nucleosome positions [123-127] and increasing the access of DNA on the surface of positioned nucleosomes [128] and also the exchange of H2A variants [129, 130] (Figure 1.6). *In vitro*, remodeling factors can facilitate replication from viral origins [131], site-specific recombination [132-134], and nucleotide excision repair [133]. An involvement of nucleosome remodeling in the repair of DNA damage is also suggested from the observation of patients with mutations in the nucleosome remodeling ATPase involved in Cockayne syndrome B suffer from increased UV sensitivity and neurodevelopmental abnormalities [135].

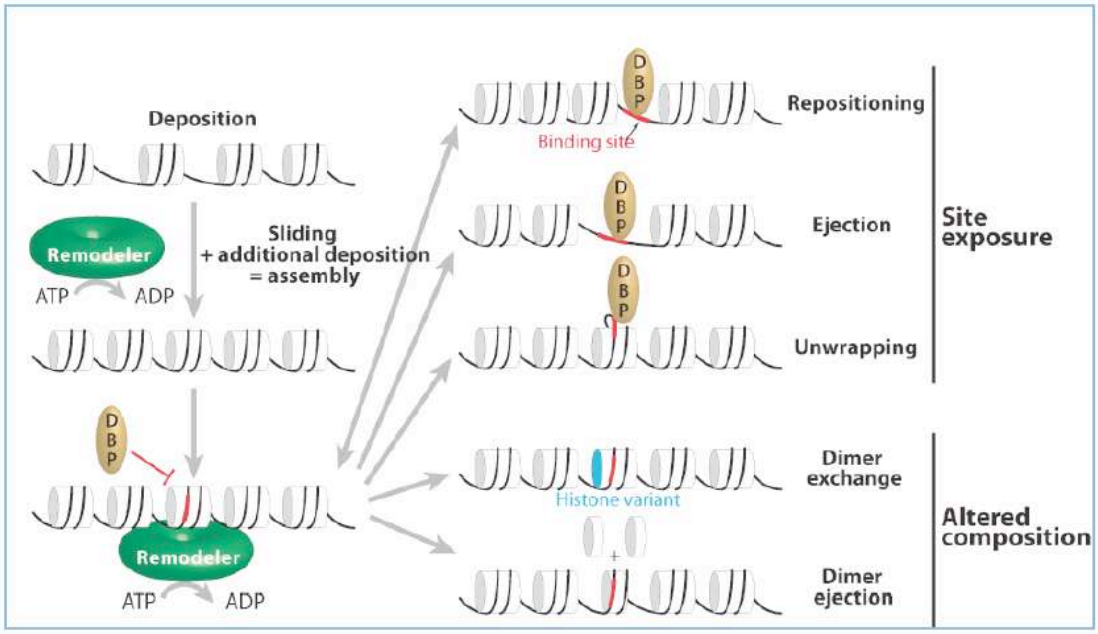

Figure 1.6. Reactions catalyzed by ATP-dependent chromatin remodeling factors. Schematic representation of different reactions catalyzed by chromatin remodeling factors. Remodelers (green) assist in chromatin assembly by moving already deposited histone octamers. The remodeling activity on a nucleosome array results in various products; DNA-binding protein (DBP) (red) becomes accessible by "nucleosomal sliding" (repositioning), or "nucleosomal eviction" (ejection), or local unwrapping, and altered histone composition, in which the nucleosome content is modified by dimer replacement [exchange of H2A-H2B dimer with a histone variant (blue)] or through dimer ejection. Image adapted from [136] (modified).

Chromatin remodeling factors have been found to act in multi-subunit protein complexes, each of them containing a core enzyme with ATPase activity. All chromatin remodeling ATPases belong to the helicase superfamily 2 (SF2 helicase), and is evolutionarily conserved from yeast [137] to drosophila [138], mouse, and

human cells [139, 140]. The different chromatin remodelers share some features like having affinity for the nucleosome, possess some histone modification sensor domains, similar catalytic DNA dependent ATPase domain and possess domains and/or protein necessary for regulation of ATPase action and interaction with other chromatin or transcription factors [136].

Chromatin remodeling complexes are classified into four main subfamilies based upon their associated ATPase, including SWI2/SNF2 family, ISWI family, CHD1 (Mi-2) family and INO80 family (Figure 1.7).

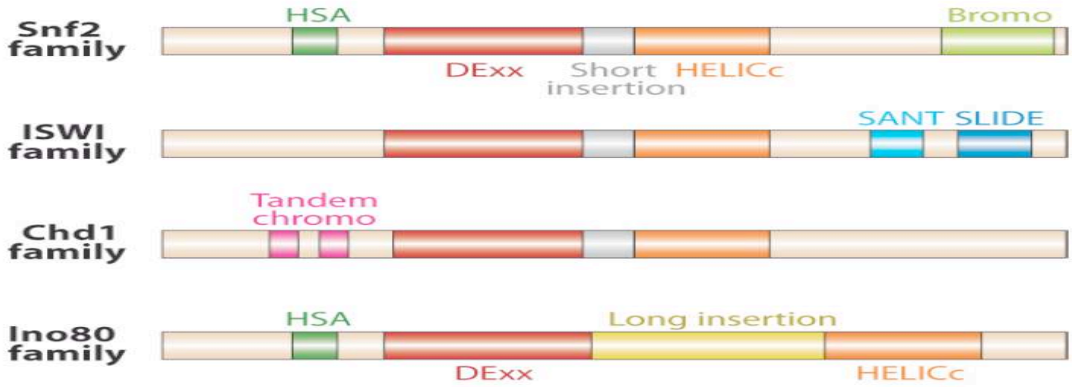

Figure 1.7. Chromatin remodeller subfamilies.

Different classes of remodeling factors on the basis of their ATPase domain. All remodeler families share a SNF2-family ATPase subunit characterized by a split ATPase domain: 1. DExx (red) and HELICc (orange). Each family differs in the unique domains residing within, or adjacent to, the ATPase domain. Remodelers of the Snf2 (SWI/SNF), ISWI, and Chd1 families each have a specific short insertion (grey) within the ATPase domain, whereas remodelers of Ino80 family harbor a long insertion (yellow). Further definition is achieved by the presence of distinct combinations of flanking domains: 1. Bromodomain (light green) and 2. HSA (helicase-SANT) domain (dark green) for Snf2 family; 1. SANT-SLIDE module (blue) for ISWI family; 1. tandem chromodomains (pink) for the Chd family; and 1. HAS domain (green) for the Ino80 family (adapted from [136]).

1.2.4.1 The SWI/SNF family

The first ATP-dependent remodeling complex, SWI/SNF, was discovered through a genetic screen in yeast for mutations interfering with mating type switching (SWI) and sucrose non-fermentation (SNF) [141]. The function of SWI/SNF is conserved in eukaryotes, as related members have been identified in yeast, Drosophila and Human. The different members of SWI2/SNF2 family include yeast SWI/SNF (ySWI/SNF) [137], RSC complexes [142], Drosophila BRM-containing complexes [143], and human SWI/SNF complexes (hSWI/SNF) that contain either BRG1 or hBRM as the catalytic subunit [139, 140]. In addition to a highly

homologous ATPase domain, each catalytic subunit of SWI/SNF also contains a C-terminal bromodomain, which might bind to acetylated histone tails [144]. Another distinguishing feature of this family of ATP-dependent chromatin remodeling enzymes is that each enzyme is purified as a multiprotein complex that contains 8-15 stoichiometric subunits. In yeast, the biochemical purification identified 11-subunits of SWI/SNF complex [141].

The members of SWI/SNF family from yeast play essential roles both in transcriptional activation and repression of selected genes [145, 146]. It often cooperates with histone acetyltransferase complexes to activate transcription. The interaction with specific transcription factors targets it to specific genes. The highly related RSC complex (Remodels the Structure of Chromatin) is involved in chromosome segregation [147] and can facilitate the loading of cohesins onto chromosomes [148]. In addition, RSC has been shown to regulate many genes, including genes for RNA polymerase III promoters, small nucleolar RNAs and RNA polymerase II promoters [149]. Drosophila BRM and human BRG1 are more abundant and essential for cell viability and development. Mammalian SWI/SNF also plays an essential role in regulating nuclear receptor function and cell growth, and mutations in BRG1, hBRM and hSNF5/INI1 have been implicated in cancer development [150-155].

1.2.4.2 The ISWI family

The founding member of ISWI (Imitation SWItch) family ATPase was first identified in Drosophila based on the homology of its ATPase domain to that of brahma, a SWI2/SNF2 homolog [156]. In addition to the ATPase domain, two other domains characterize the ISWI chromatin remodeling subunit, a SANT domain (Switching-defective protein 3, Adaptor 2, Nuclear receptor co-repressor, Transcription factor TF-IIIB), which is essential for histone binding, and a SLIDE domain (SANT-like ISWI domain), which is required for both DNA binding and complete ATPase activity [157-160].

Since the discovery of first ISWI, several ISWI related complexes have been identified in drosophila, yeast, xenopus, mice, and human cells [161]. In Drosophila,

there are three ISWI complexes including ACF (ATP-dependent chromatin assembly and remodeling factor), NURF (Nucleosome remodeling factor), and CHRAC (Chromatin accessibility complex), which are differentiated by the existence of other subunits. The components of NURF are ISWI, the large regulatory subunit NURF301, the pyrophosphatase NURF38 and the WD40 protein NURF55 [162, 163]. CHRAC and ACF contain ISWI and ACF1, but CHRAC additionally contain two small histone fold subunits, CHRAC14 and CHRAC16. Yeast has two ISWI-related genes, ISWI1 and ISWI2, which encode the ATPase subunits of at least four different complexes, including ISW1a, ISW1b, ISW2, and yCHRAC. Xenopus ISWI is present in at least four ISWI complexes including xACF, xWICH, xCHRAC, and xISWI-A. Human ISWI-like ATPases, SNF2H and SNF2L share 86% sequence homology, and hSNF2H protein has 73% of its amino acids identical to that of Drosophila ISWI [164]. Human ISWI-like complexes include SNF2H-containing hACF, WICH (WSTF-ISWI chromatin remodeling complex), hCHRAC, RSF, and SNF2H/NURD/cohesion, and SNF2L-containg hNURF complex [161].

The ISWI complexes from different organisms are involved in a variety of functions including activation and repression of transcription, replication and chromatin assembly [165]. In Drosophila, ISWI-containing NURF complex regulate transcription at the promoter of heat shock protein 70 (Hsp70), NURF301, is essential for the expression of homeotic genes [162, 163]. In addition, ISWI is also required for maintenance of X-chromosome structure [166]. Yeast ISWI-containing complexes are mainly involved in transcriptional repression. ISW1a complex appears to repress transcription at the initiation stage, whereas ISW1b may play a role in transcriptional elongation and termination by delaying RNA polymerase II release [167, 168]. Human WICH complex is recruited to the replication foci by the DNA clamp PCNA to maintain chromatin structure after DNA replication. RNAi-mediated depletion of WSTF or SNF2H caused abnormal heterochromatin formation on newly synthesized DNA [169]. Recently, it has been shown that human SNF2H forms a nucleolar-remodeling complex (NoRC) with bromodomain-containing protein Tip5 (TTF-I-interacting protein 5), and plays an important role in repressing the rDNA promoter through heterochromatin formation [170, 171]. Like dNURF, human NURF is also involved in transcriptional activation of genes, especially those involved in neuronal development [172].

1.2.4.3 The CHD1 family

The CHD (Chromodomain, Helicase, DNA binding) protein was first isolated from *Xenopus laevis*. The members of the CHD subfamily are characterized by the presence of a pair of chromodomains (Chromatin organization modifier) on the N-terminus of the protein, in addition to an SNF2-related ATPase domain [173, 174]. Chromodomains can bind to methylated histone tails, but functional analyses revealed a variety of possible interacting partners, not only histones, but also DNA and RNA [175]. Generally, this subfamily of ATP-dependent chromatin remodeling enzymes possesses both ATPase and histone deacetylase enzymatic activities [176].

The most extensively studied member is the ATPase Mi-2, which resides in NuRD ("Nucleosome Remodeling and Deacetylation") complexes that have histone deacetylase activity [177]. The members of the CHD family are also conserved in different organisms. Xenopus Mi-2 complex contains the deacetylase subunits Rpd3 and RbAp48/RbAp46, and a substoichiometric amount of Sin3 [178], suggesting that this complex may play a role in transcriptional repression. Human Mi-2 complex NuRD contains Mi-2α and Mi-2β (CHD3 and CHD4 respectively), and histone deacetylases HDAC1 and HDAC2. Mi-2β is an autoantigen associated with dermatomyositis, and MTA-2 that may be involved in cancer metastasis [179]. Both human and xenopus NURD complex is associated with other subunits, such as the methyl DNA binding proteins MeCP2 and MBD3 (methyl-binding domain 3), which target the complex to methylated DNA and couples ATP-dependent remodeling to histone deacetylation, resulting in gene silencing [180]. Drosophila Mi-2 complex has the same composition as, that of vertebrate NuRD complexes, and interacts with several transcriptional repressors. In fission yeast (*Saccharomyces pombe*) Chd1 homolog, Hrp1, is required for transcriptional termination either alone or in redundancy with Iswi1 and Iswi2 [181].

1.2.4.4 The INO80 family

The INO80 (INOsitol requiring 80) and SWR1 subclass of ATP-dependent chromatin remodeling complexes are characterized by split ATPase domains, which

contain an insertion of a large spacer region between the DExx and HELICc region. This insertion also serves as a binding platform for the helicase-related Rvb1/2 proteins (RuvB), separating DNA strands, and one actin-related ARP protein [182, 183].

So far, 15 subunits of INO80 complex have been isolated from yeast, which are involved in DNA repair, recombination and transcription [130, 183, 184]. Recently, a similar multi-subunits complex has been purified from mammalian cells that share 8 subunits with the yeast INO80 complex [182]. The INO80 and SWR1complex share several subunits, including the Rvb1p and Rvb2p enzymes, histones, actin and actin-related proteins (ARPs) [185].

In addition to the role in transcriptional regulation, both the INO80 and SWR1 complexes have been implicated in DNA double strand break (DSB) repair, although the precise role of both protein complexes remain elusive and need to be investigated [186, 187]. The SWR complex may play role in exchange of canonical histone H2A within nucleosomes for H2A variants [130]. Although the SWR complex has so far only been purified from yeast, orthologs of the SWR1 gene are known throughout the eukaryotic kingdom and hence similar complexes may be widespread.

1.3 Histone Variants

Histone variants are non-allelic isoforms of the conventional histones. The core histones (H2A, H2B, and H3) and the linker histone H1, except histone H4, possess histone variants, which show variation to a different extent. The reason for H4 variant inexistency is presumably that H4 interacts with all other histones in the octamer and evolutionally it is highly conserved, leaving little room for structural changes [41]. Histone variants are evolved from the corresponding canonical histones and differ from their canonical paralogs in primary protein sequence, gene organization, expression timing, and deposition mechanism. These variants are able to replace the canonical histones, and alter the composition and functional properties of the individual nucleosomes, thereby play essential role in gene expression, anti-silencing, heterochromatinization, epigenetic inheritance of chromatin markings, and

specification of distinct chromatin regions [44, 188-193]. Since different histone variants exist in different species, the following overview focuses on mammalian and in particular human histone variants.

Histone proteins can be classified into two groups, canonical histones and replacement histone variants. In human the canonical core histones are encoded by intronless multicopy genes, which are transcribed into non-polyadenylated mRNAs. In contrast, the variant histones are encoded by genes, which are located outside the canonical histone gene cluster. They are mostly present as single or few gene copies contain introns and their mRNAs are polyadenylated. Canonical histones are expressed during S-phase of the cell cycle and the cell uses them for chromatin assembly during replication. In contrast, histone variants are expressed throughout the cell cycle and are used for deposition and exchange independent of DNA replication. On the basis of their incorporation into chromatin, these variants are called replication dependent (RD) canonical histones and replication independent (RI) histone variants. In the following sections, different variants from H1, H2A, H2B and H3 families are introduced and the diverse functions of their members described. The list of core histone variants and their properties are summarized in Table 1.2.

Table 1.2. Human core histone variants

	Variant	Length (AA)	Mass (KDa)	Distribution	Expression	Localization	Function
H2A	H2A	130	14	Widespread	RD	TG	Genome Packaging
	H2A.X	143	16	Universal	RI	TG *	DNA repair; genome integrity
	H2A.Z	128	14	Universal	RI	TG	Gene activation, silencing, chromosome segregation
	H2A.Bbd.1	115	13	Mammals	ND	Xi exclusion	Spermatogenesis, Gene activation?
	H2A.Bbd.2	115	13	Mammals	ND	Xi exclusion	Spermatogenesis, Gene activation?
	MacroH2A1.1	372	40	Animals	ND	Xi	X chr inactivation, gene silencing?
	MacroH2A1.2	372	40	Animals	ND	Xi	X chr inactivation, gene silencing?
	MacroH2A2	372	40	Animals	ND	Xi	X chr inactivation, gene silencing?
H2B	H2B	126	14	Widespread	RD	TG	Genome Packaging
	H2BFWT	175	20	Primates	ND	Telomeres?	Testis specific, Sperm cell no?
	hTSH2B	127	14	Mammals	ND	Basal part of the nucleus	Testis specific, pronuclei formation?, activation of paternal gene?
H3	H3.1	136	15	Mammals	RD	TG	Genome Packaging, DNA replication, repair, chromosome stability
	H3.2	136	15	Widespread	RD	ND	DNA replication,repair, chromosome stability
	H3.3	136	15	Universal	RI	Genes, TFBS, Telomeres, Peri -centromeres,	Transcription, Sperm pronucleus decondensation, Incorporation during mammalian meiotic sex inactivation (MSCI) , Chromosome inactivation, Pericentric transcription,T elomere silencing
	H3t	136	15	Mammals	RD?	ND	Testis specific, ND?
	H3.5	135	15	Mammals	RI?	ND	Associated with active genes
	H3.X	147	16	Mammals	RI?	ND	ND?
	H3.Y	136	15	Mammals	RI?	ND	Response to external stimuli
	CENP-A	140	17	Universal	RI	Centromeres	Assembly of kinetochore, chromosome segregation

ND=not determined, RD=replication dependent, RI=replication independent, TG=throughout genome, Xi=inactive X chromosome,?=hypothesized, TFBS=transcription factor binding sites. *) γ-H2A.X (=H2A.XS139ph) localizes to DNA DSBs and a population of small nuclear foci.

1.3.1 Histone H1 variants

Histone H1 group is composed of highly variable histone proteins, which display differential specificity based on species, tissue and developmental stages. Multiple histone H1 family members have been identified and characterized in animals and plants. For example in mammals, there are at least eleven different subtypes of H1 variants, while Drosophila has only one single type of histone H1. The linker histone variants differ in their expression timing, rate of synthesis, turnover rates, phosphorylation status, and ability to bind and compact chromatin, as well as localization to euchromatic or heterochromatic regions [194, 195].

Histone H1 variants can be classified into three groups on the basis of their mode of expression: (i) S-phase dependant H1 histones (H1.1 to H1.5 in human), (ii) histones H1 with replacement variant mode of expression in somatic cells (H1.0 and H1x in human) and (iii) germ cell specific H1 histones (H1t, H1T2, H1LS1 and H1oo). As the linker histone H1 variants have the ability to bind the 'nucleosome' from outside and facilitate chromatin condensation. Based on this function, these variants are recently classified into strong (H1.0, H1.4, H1.5 and H1x), intermediate (H1.3) and weak (H1.1 and H1.2) condensers of chromatin [196].

The linker histone variants H1.1-H1.5 are expressed initially in different tissues in prenatal conditions. H1.2-H1.4 histones have been shown to present in all somatic cells with H1.2 and H1.4 being the predominant forms in most of the cells. In contrast, H1.0 is mainly expressed in terminally differentiated cells and H1.1 expression is restricted to certain tissues such as thymus, testis, and spleen [196, 197]. H1x is currently the least well characterized of the human H1 variants and its replication independent expression has only been analyzed in a limited number of cell types. The distribution of H1x protein is non random with a preference in the less accessible regions of the genome [198]. The remaining four variants constitute a set of germ cell-specific H1 histones with H1oo being expressed in oocytes and H1t, H1T2 and HILS1 in testis [196, 199].

To determine the biological functions of individual H1 variants, gene knockout experiments in mice have been conducted. Knockout of one of the H1 variants H1.2, H1.3 and H1.4 alone or together with H1.0 did not show any obvious phenotype [200]. However, triple null mice, lacking H1.2, H1.3 and H1.4, die by mid-gestation with a broad range of defects [201]. These studies suggest, that individual H1 variants might be partially redundant, at least in their ability to compact chromatin globally [199]. Nevertheless, H1 variants are differentially associated with repress or active chromatin and thus regulate gene transcription. Beyond their function in gene regulation, H1 variants may also be implicated in other biological processes such as DNA repair [202]. For a short summary of human linker histone H1 variants properties, see Table 1.3.

Table 1.3. Different histone H1 variants in human

H1 variants	Amino acids	Mass (KDa)	Expression	Gene	Chromosomal location
H1.0	194	21	RI	H1F0	22q13.1
H1.1	215	22	RD	HIST1H1A	6p22.1
H1.2	213	22	RD	HIST1H1C	6p21.3
H1.3	221	24	RD	HIST1H1D	6p21.3
H1.4	219	22	RD	HIST1H1E	6p22.1
H1.5	226	24	RD	HIST1H1B	6p22.1
H1oo	346	36	RI	H1FOO	3q21.3
H1t	207	22	RI	HIST1H1T	6p22.1
H1x	213	22	RI	H1FX	3q21.3
Testis-H1	255	28	RI	H1FNT	12q13.11

RD=replication dependent, RI=replication independent

1.3.2 Histone H2A variants

Histone H2A group is the most diverse family containing the greatest number of variants (around 265 members from different species) that differ considerably in term of sequence and size. In mammals, four major H2A variants have been characterized to date: H2A.Z, MacroH2A, H2A.Bbd (Barr-body deficient), and H2A.X (Table 1.2, Figure 1.8). However, most of the vertebrates encode an even higher number of H2A sequence isoforms with unknown functions. In the human genome, 26 genes encode histone H2A isoforms with majority of them present in cluster 1 and 2 and a single gene in cluster 3. Out of these, 9 genes are not part of any cluster and code for atypical histone H2A variants such as macro-H2A, H2A-Bbd etc.

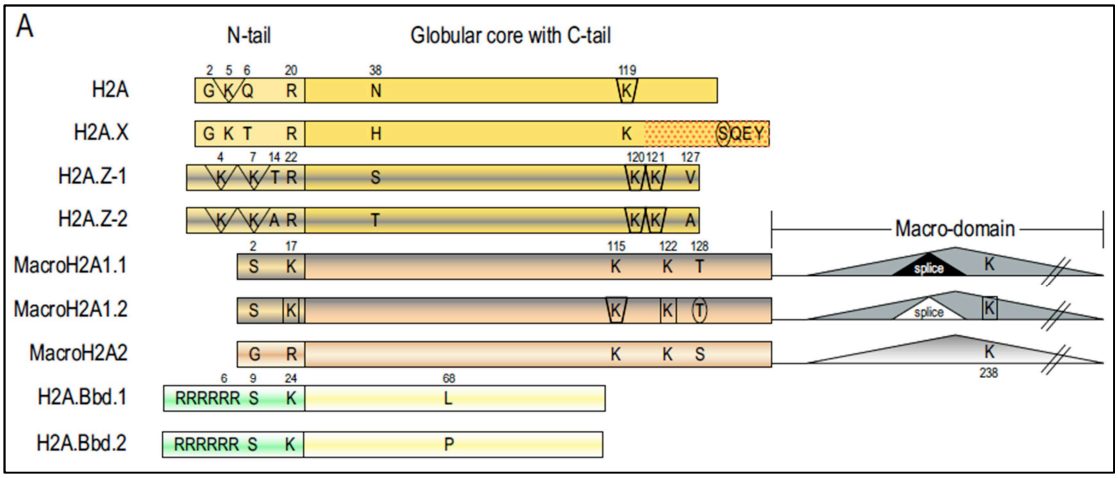

Figure 1.8. Protein sequences of human histone H2A variants.
Schematic representation of human H2A histone variants. Proteins are divided in N-terminal tail and globular domain with C-terminal tail. Highly divergent protein sequences are visualized by different color shades without highlighting sequence differences. PTM sites are marked as follows: ellipse=phosphorylation, square=methylation, triangle=acetylation, trapezoid=ubiquitination. The macrodomains of macroH2A histones are not drawn to MacroH2A1.1 and macroH2A1.2 are splice variants (differentially spliced exon 6), denoted by an internalized white or black triangle in their macrodomain. Image adapted from [203].

1.3.2.1 H2A.Z variant

H2A.Z is one of the universally present H2A variant, which shows ~60% sequence identity to canonical H2A. H2A.Z histones are non-essential for the viability in yeast and essential for survival in a range of species, including Tetrahymena, Drosophila, Xenopus and Mice [204-207], however, the biological role of this variant remains controversial and its functions may also have some species specificity. H2A.Z plays important roles in various biological processes including gene activation, chromosome segregation, heterochromatin silencing, and progression through the cell cycle [208].

H2A.Z has been linked to both transcriptional repression and activation and was found to be partially redundant with chromatin remodeling complexes [209-211]. Recent studies on the genome wide deposition of H2A.Z point towards a function for H2A.Z in the establishment and maintenance of chromatin boundaries that define promoter elements and those that demarcate genes [212]. In humans, H2A.Z enrichment at promoter regions has been reported [208]. In Tetrahymena, H2A.Z localizes to the transcriptionally active macronuclei indicating its role in the activation of gene expression. In addition, post-translational modifications may regulate H2A.Z

function as H2A.Z acetylation levels are higher in euchromatic compared to heterochromatic regions and mono-ubiquitinated H2A.Z, which is found on the Xi chromosome, seems to be important for transcriptional repression [213].

1.3.2.2 H2A.X variant

The second universal H2A variant is H2A.X, which is highly similar to H2A but is distinguished by its conserved C-terminal SQ-motif (Figure 1.8). H2A.X plays an important role in the maintenance of eukaryotic genome integrity by participating in the repair of double stranded DNA-breaks (DSB). The DNA damage signaling kinases [(ATM (ataxia telangectasia mutated), ATR (ATM- and Rad3-related) and DNA-PK (DNA-dependent protein kinase)] phosphorylate serine 139 within the SQ motif of H2A.X yielding a modified form known as gamma-H2AX (γ-H2AX) in response to double-strand DNA damage and apoptosis [214]. This phosphorylated form (γ-H2A.X), is present at sites of DSBs during DSB repair, meiotic recombination [215], apoptotic digestion [216], V (D) J splicing [217], and class switch recombination [218]. Moreover, H2A.X knockout in mice results in infertility in the male but not in the female due to failure of meiotic pairing by X and Y-chromosomes and has been shown to initiate heterochromatinization in the sex body [219].

1.3.2.3 MacroH2A variant

MacroH2A variant was first discovered and characterized by J. Pehrson and C. Costanzi [220, 221]. The macroH2A variants have a distinctive hybrid structure consisting of an N-terminal domain that is closely identical to the full-length canonical histone H2A followed by a C-terminal large nonhistone region (NHR), which resembles a leucine zipper. Among all histone H2A variants, macroH2A is a bulky variant with 327 amino acid in length and ~40 kDa compared to the conventional H2A histone 14 kDa weight (Table 1.2).

In human, there are two macroH2A genes, the first macroH2A1 gene, on chromosome 5, encodes two macroH2A splice variants, macroH2A1.1 and macroH2A1.2, produced by alternate splicing [222]. The second gene on human chromosome 10, encodes macroH2A2 variant [223]. The N-terminal part of

macroH2A1.1 is 64% identical to canonical H2A and its isoform macroH2A1.2; differs from it in a short stretch of amino acids within the non-histone domain. However, the amino acid sequence of human macroH2A2 variant is 68% identical to human macroH2A1.2 [220, 221].

MacroH2A variants play important roles in X chromosome inactivation and transcriptional regulation. MacroH2A1.2 and macroH2A2 have different nuclear localization patterns but are both enriched on the Xi [223, 224], suggesting a role in X inactivation and they are expressed at similar levels in males [222], and maybe as general repressors of transcription. The mechanism of transcriptional repression by histone variant macroH2A has recently been explored. The promoters of numerous genes, particularly the promoters of inducible Hsp70.1 and Hsp70.2 genes, but not that of the constitutively expressed Hsp70.8, were shown to be highly enriched in macroH2A1.1 [225]. The macrodomains are reported to bind ADP-ribose with high affinity [226]. This fact led to the discovery of a PARP-1-macroH2A1.1 nucleosomal interacting complex. This interaction was found to be associated with inactivation of PARP-1 enzymatic activity. Heat shock released both mH2A1.1 and PARP-1 from the Hsp70.1 promoter and activated PARP-1 auto modification activity. These results suggest that mH2A1.1 recruits PARP-1 to the promoter, thereby inactivating it. Upon heat shock, the Hsp70.1 promoter-bound PARP-1 is released to activate transcription through ADP-ribosylation of other Hsp70.1 promoter-bound proteins [225]. In addition to its role in transcriptional regulation, macroH2A also seems to play a role in development as all three variants are differentially expressed in a tissue development dependent manner [223, 224]. Furthermore, macroH2A may also be implicated in cell proliferation, as its association with the Xi is cell cycle dependent and disrupted by the phosphorylation of S137, which is dramatically up-regulated during mitosis [227].

1.3.2.4 H2A.Bbd variant

Histone H2A.Bbd variant was first discovered by Chadwick and Willard in humans and shown to be excluded from the female inactive X chromosome, hence named as “Barr body-deficient H2A” [228]. The protein sequence of the variant H2A.Bbd is highly variable, which is only 48% identical to the conventional H2A and

molecular evolutionary analyses have revealed that H2A.Bbd is a quickly evolving hypervariable mammalian histone variant, in striking contrast to all other histones known to date [229, 230]. In humans, it is encoded by three intronless genes on the X chromosome giving rise to two proteins that differ in only one amino acid (Figure 1.12 and Table 1.2) [228]. H2A.Bbd lacks the typical H2A C-terminus domain containing an acidic patch that directly contacts histone H4 [41]. None of the PTM-carrying residues in H2A is present in H2A.Bbd; instead, it has a characteristic 6 arginine repeat in its N terminal region.

The tagged version of H2A.Bbd is co-localizing with acetylated histone H4 and excluded from the Xi, suggesting its role in transcription activation [228]. H2A.Bbd forms a highly unstable nucleosome in *in-vitro* condition and wraps only 118-130 base pairs of DNA fragment [231, 232]. In addition, H2A.Bbd is recently shown to be involved in the spermiogenesis process and might contribute to the histone-to-protamine exchange in sperms [233].

1.3.3 Histone H2B variants

Histone H2B family includes 214 different isoforms reported from diverse species to date. In human, there are 19 H2B isoforms, which are coded by 23 genes with majority of them present in cluster 1, very few in cluster 2, cluster 3 and some outside of these clusters. However, the variability observed in H2B variants seems to occur exclusively in the male germ line of vertebrates and invertebrate organisms and the pollen of plants with largely unknown functions [234-236]. So far, there are two testes specific H2B variants TSH2B [237], and H2BFW [238] have been described in human.

The testis/sperm-specific variant TSH2B shows 85% similarity to canonical H2B, and is mostly conserved in human, rat and mouse [237]. Human hTSH2B is only expressed in a sub-population of sperm cells (~30 %) where it localizes specifically to the basal part of the nucleus, adjacent to the sperm tail attachment point. Distinct foci in cells expressing lower hTSH2B levels, propose a possible association with specific chromatin domains [237, 239]. Expression of hTSH2B promotes a more rapid and comprehensive chromatin decondensation, suggesting a

potential role in pronuclei formation and the activation of paternal genes following fertilization and during early embryonic development [239].

H2BFWT (H2B family member W, testis-specific) is recently reported as a primate- and testis-specific H2B variant expressed from X chromosome, which shows only 45% sequence identity with the conventional H2B and presumed to be associated with the telomeric DNA [240]. Regarding the function of this novel variant, initial experiments have shown that H2BFWT partially localizes to the interstitial telomeric blocks, suggesting a telomere-associated function [238]. More recent results link H2BFWT to male infertility: A single nucleotide polymorphism in the 5' UTR of H2BFWT has been identified, which extensively reduces translation of H2BFWT, causing reduced sperm count and vitality [241].

1.3.4 Histone H3 variants

The family of H3 histones contains eight isoforms including the conventional histones H3.1, H3.2 and the histone variants H3t, H3.3, CENP-A (Centromere Protein A), H3.X, H3.Y and H3.5 (Table 1.2, and Figure 1.9). These eight histone H3 proteins (H3.1, H3.2, H3t, H3.3, CENP-A, H3.X, H3.Y and H3.5) can be grouped, on the basis of their incorporation into chromatin, into two different categories: (i) canonical, replication dependent H3 histones (H3.1 and H3.2) and (ii) replication independent histone H3 variants (H3t, H3.3, CENP-A, H3.X, H3.Y and H3.5). It is noteworthy that H3.3, CENP-A, H3.X, and H3.Y are somatic histone variants, while H3t and H3.5 are testis specific variants. Interestingly, in yeast occurs a single type of H3, which is equivalent to H3.3 of mammals (what is the name). In fact, H3.3 gene is the common ancestor, which gave rise to the major H3 variants (H3.1, H3.2 and H3t), during the course of evolution in animals [242]. The single H3 isoform of yeast is able to deposit by both replication dependent and replication independent pathways.

Figure 1.9. Sequence alignment of human H3 variants.
Amino acid sequence alignment of different human H3 variants (H3.1, H3.2, H3t, H3.3, H3.5, H3.X, H3.Y and CENP-A). Identical amino acids are represented in black letters and the amino acids differences among human H3 variants are shown in red letters. The residues of H3.3, H3.5, H3.X and H3.Y corresponding to replication independent deposition are highlighted in gray. The position 31 of H3.3 and H3.5 contain serine residue. Differences of CENP-A from other H3 variants are highlighted in green.

1.3.4.1 Canonical H3.1 and H3.2 histones

The two canonical histones H3.1 and H3.2 differed by a single amino acid substitution (S96C) (Figure 1.9). H3.1 is mammalian-specific, whereas H3.2 is present in all eukaryotes except budding yeast [203]. Due to their high similarity, they are often treated as one protein. However, quantitative mass spectrometry analyses have revealed that expression levels and PTM patterns of these variants vary between different mammalian cell lines and tissues, arguing for distinct protein species [243, 244]. H3.2 is enriched in di- and trimethylated K27, these modifications are associated with transcriptional silencing and marks facultative heterochromatin. In contrast, H3.1 shows enrichment in K9 dimethylation, K64 monomethylation and K14 acetylation. K9 dimethylation is found in areas of constitutive heterochromatin [243]. An interesting hypothesiswhich aims to explain the different functions and modes of action of these H3 variants, is the implication of the unique C96 in H3.1 in intermolecular disulfide bonds. In this model, H3.1 promotes higher order chromatin structures and silencing of certain chromatin regions by forming disulfide bonds with

neighboring nucleosomes, lamin B receptor (to retain certain loci at the nuclear periphery) or other yet to be identified factors [243].

1.3.4.2 Testis specific variant H3t

The mammalian testis specific variant H3t has four amino acid substitutions compared with H3.1 (A24V, V71M, A98S, and A111V) [245]. Two of these changes (M71 and V111) have recently been identified to cause lower stability of tH3-containing nucleosomes, which may be important for the replacement of histones with protamines during spermatogenesis [246]. Although tH3 is primarily expressed in testis, low amounts have also been detected in HeLa cells [247], mouse embryos and adult mouse brain and spleen [248]. The function of this somatic tH3 fraction remains to be determined.

1.3.4.3 Histone H3.3

The universal H3.3 variant differs from the canonical H3.1 by five amino acid substitutions (A31S, S87A, V89I, M90G, and C96S). It is well documented that the synthesis of histone H3.3 takes place outside S-phase [249]. The level of H3.3 transcript is constitutively maintained throughout differentiation [250]. This constitutive expression pattern makes H3.3 variants available for deposition and replacement independent of DNA replication. Noteworthy, H3.3 exhibits differences in the primary amino acid sequence and PTMs pattern compared to conventional H3, which conferred distinct properties of H3.3 histones. In fact, the substitution of any one amino acid in H3.1 toward H3.3 identities ($S_{87}/V_{89}/M_{90}$ to $A_{87}/I_{89}/G_{90}$) permits some replication independent deposition [251].

Several studies have shown in detail the localization of H3.3 both at specific regions of the nucleus and as well as genome-wide. The distribution of H3.3 variant in the genome, play important role in the epigenetic marking of specific chromosome regions and regulation of gene expression by altering the local chromatin structure. Deposition of H3.3 variant occurs at highly transcribed regions in flies and mammals [251-255]. Incorporation of H3.3 at regulatory sites of both active and silent genes has been reported [252, 256, 257]. The deposition of H3.3 in germline cells takes

place in a replication independent manner. It has been reported that nucleosome replacement involving the deposition of H3.3 occurs during mammalian meiotic sex chromosome inactivation (MSCI) [258]. Recently enrichment of H3.3 was found at telomeres and pericentric heterochromatin in mouse ES cells and MEF cells [259-262]. These last observations clearly show that H3.3 is not only accumulated at active chromatin but it is also deposited at silent genomic loci. Importantly, the deposition of histone variants in different combinations, to different regions in the genome, could results in quite different consequences for chromatin structure and gene regulation. To this end, the group of Felsenfeld studied the salt stability of H3.3 nucleosomes in combination with either H2A or H2A.Z [263]. They reported that nucleosomes containing the two variants, H3.3 and H2A.Z are less stable than nucleosomes with H3.3 and H2A. Furthermore, nucleosomes containing H3.1 and H2A.Z are as stable as H3.1/H2A nucleosomes [263]. Taken together, these data suggest that H3.3 does not simply promote gene transcription but rather has very distinct functions, which depend largely on its incorporation at specific loci and its interaction partners.

1.3.4.4 CENP-A

The most divergent of the mammalian H3 variants is CENP-A (Centromere Protein A), which is discussed in more detail in section 1.5.3 of this chapter.

1.3.4.5 Other H3 variants (H3.X, H3.Y and H3.5)

Recent studies identified two novel histone H3 variant genes on human chromosome 5, now termed H3.X and H3.Y [58]. Both H3.X and H3.Y constitute primate-specific genes, which have been found, in addition to humans, also in the chimpanzee and the macaque, but not in other mammals or even lower eukaryotes. These variants display interesting changes in amino acids that are known to be modified in H3.1, H3.2, and H3.3. H3.X and especially H3.Y mRNA is expressed at low but significant levels in the human osteosarcoma cell line U2OS and in some human bone, breast, lung and ovary tumor tissues, as well as in testis and certain areas of the brain [58]. Another newly identified histone H3 variant, H3.5 is

specifically expressed in testis and shown to be associated with actively transcribed genes [264].

1.4 Deposition of core histone variants by Chaperones

The deposition of core histones and their variants during chromatin assembly is assisted by different histone chaperones. In the context of chromatin assembly histone chaperones can be defined as histone binding proteins responsible for the safe delivery of histones to DNA without being part of the final reaction product. After their discovery in 1978 [8] by Laskey on nucleosoplasmin (NPM), the first histone chaperone, a variety of histone chaperones have been identified and characterized. Chaperones play a role in histone deposition on DNA in replication dependent and replication independent manner, but are also implicated in their storage, transfer, exchange and removal. Moreover, chaperones prevent the non-specific and deleterious interaction of histones with other factors and DNA. Furthermore, it now appears that chaperones may modulate, directly or indirectly, histone post-translational modifications (PTMs) that are functionally important.

1.4.1 Deposition of H3-H4 family histones

Generally, the deposition of histone H3 variants onto chromatin is classified into two main pathways; (i) replication-coupled deposition (RC) and (ii) replication independent deposition (RI). The RC and RI nucleosome assembly processes occur in both yeast and mammalian cells despite the fact that yeast cells have only one form of histone H3, which is most similar to the mammalian H3 variant H3.3.

In vivo, multiple steps have to precede the productive formation of nucleosomes. In particular, the histones must be synthesized and folded in the cytoplasm, before being imported into the nucleus and recruited to sites of deposition at the DNA. This flow of histones has to be highly facilitated and regulated to meet the supply and demand of DNA-templated processes.

1.4.1.1 Chaperoning histones H3-H4 from the cytoplasm to the nucleus

The first step in the deposition of the newly synthesized histones is the transport from the cytoplasm to the nucleus, a process, which is assisted by distinct chaperones. The chaperone Asf1 (Anti-silencing Function 1) was the first chaperone identified to play a key role in supplying histones H3-H4 to the downstream chaperones, like CAF-1 (Chromatin Assembly Factor 1) and HIRA (Histone Regulatory homolog A) for nucleosome assembly [180, 265]. Structural and biochemical studies show that Asf1 binds only to one H3-H4 dimer [266, 267], thus preventing the formation of H3-H4 tetramer. Recent reports demonstrate that the processing and the transport of newly synthesized H3-H4 are very intricate events requiring both the concerted action of numerous multi-chaperone complexes and the presence of specific post-translational modifications (PTMs) of histones [268]. For example, the acetylation of H4 on lys5 and lys12, a well-studied and highly conserved pre-deposition mark [268], is catalyzed by HAT1-RbAp46 holoenzyme [269].

In two very recent studies, the biochemical purification of the cytoplasmic H3 complex has allowed both the identification of distinct H3 chaperones (HCS70, HSP90, tNASP, sNASP, RbAp46, and Asf1a/b, along with histone H4, importin4 and HAT1) and the suggestion of a comprehensive mechanism(s) for the sequential assembly of H3-H4 dimers [270, 271]. After synthesis, histones H3 and H4 were sheltered from misfolding and aggregation by interaction with chaperones HSC70 and HSP90, respectively [270, 271]. For transport and deposition onto DNA, histones H3-H4 first assembled to form the dimer, a process facilitated by HSP90 and tNASP [270]. Once the H3-H4 dimer was assembled, RbAp46 associates with the H4 carboxyl domain [270] and helps the recruitment of HAT1, which in turn acetylates H4 on lys5 and lys12. Then the acetylated histones are transferred to Asf1a/b and importin4 for nuclear transport [270, 271].

1.4.1.2 Chaperoning histone H3 proteins from nucleus to chromatin

Analysis of the preassembly complexes associated with the different human H3 variants has identified CAF1, Asf1a/b, HIRA, and DAXX as the major histone chaperones controlling their targeting and deposition to specific chromatin loci (Table 1.4). CAF1 is the key chaperone in replication coupled chromatin assembly, while Asf1 plays a role in both replication coupled and replication independent deposition. The deposition of replication independent histone H3 variant H3.3 is assisted by HIRA and DAXX. Chaperones involved in deposition of H3.5, H3t, H3.X and H3.Y histones are not known.

1.4.1.3 Replication coupled deposition (RC)

The canonical histones H3.1 and H3.2 are synthesized and deposited during S-phase of the cell cycle in a replication-dependent manner. During replication the “old” nucleosomes are disassembled and the “new” ones are assembled. There are two sources of histones for the replication-coupled deposition: (i) “old” histones and, (ii) newly synthesized histones. According to the generally accepted view, replication-induced disruption of “old” nucleosomes produces two H2A-H2B dimers and H3-H4 tetramer [272].

CAF-1 is the bona fide histone chaperone for replication coupled chromatin assembly. CAF-1 was first identified in humans and was shown to promote chromatin assembly on replicating SV40 DNA *in vitro* [273]. In mammals, the CAF-1 complex is composed of three highly conserved subunits p150, p60 and p48. The p150 subunit of CAF-1 is recruited to the site of DNA synthesis through direct interaction with proliferating cell nuclear antigen (PCNA) and colocalizes with the replication foci and p60 during S-phase (reviewed in [44]). Importantly, CAF-1 was found associated *in vivo* with the replication dependent H3.1 complex and not with the replication independent H3.3 complex, a key finding further demonstrating the direct implication of CAF-1 in replication coupled deposition [274].

In the nucleus, the newly synthesized H3-H4 dimers appeared to remain initially associated with Asf1. Next Asf1 supplies the newly synthesized H3-H4 dimers to CAF-1. The direct interaction of Asf1 and p60 subunit of CAF-1 both *in vitro* and *in*

vivo facilitates the delivery of histones from Asf1 to CAF-1 for deposition [275]. However, the exact mechanism for the removal of Asf1 from H3-H4 dimer, transferring of the dimer to CAF-1 and formation of H3-H4 tetramer on CAF-1 still remains unclear.

1.4.1.4 Replication Independent Deposition (RI) of H3.3

Unlike canonical histones the expression and deposition of H3.3 variant occurs throughout the cell cycle. Enrichment of H3.3 at different genomic regions as discussed earlier, suggests the existence of distinct deposition factors. Presently two specific chaperones HIRA and DAXX are known for replication independent deposition of H3.3 histone variant (Table 1.4).

A. HIRA mediated deposition of H3.3

HIRA was the first described chaperone responsible for H3.3 deposition. Initially, DNA replication independent chromatin assembly *in vitro* was found to be facilitated by HIRA in Xenopus egg extracts [276] and histones were identified as proteins able to specifically interact with HIRA [277]. The subsequent affinity purification study in human cells identified two distinct chaperones, CAF-1 and HIRA, for replication dependent and replication independent assembly of H3.1 and H3.3, respectively [274]. Asf1, a common partner of both H3.1 and H3.3 complexes, is believed to provide histones to CAF-1 and HIRA through chaperone-chaperone interactions. The exact mechanism for this differential delivery of histones to CAF-1 and HIRA by Asf1 is not clear. Interestingly, HIRA is involved in the deposition of H3.3 during de-condensation of the Drosophila sperm pronucleus but is not required for H3.3 deposition in embryos or adult cells [278]. Recent study shows that enrichment of H3.3 at promoters and in the body of genes in ES cells is dependent on HIRA [259] in agreement with a role of HIRA in H3.3 deposition at these regions.

B. DAXX mediated deposition of H3.3

DAXX was initially linked to FAS-mediated apoptosis [279]. DAXX was found to colocalize with both the promyelocytic leukaemia (PML) nuclear body and the

alpha-thalassemia/mental retardation X-linked syndrome protein (ATRX), which is highly enriched at pericentric heterochromatin [280]. Recently, our group [260] and the group of David Allis [259, 281] showed that DAXX, in complex with ATRX, facilitates H3.3 deposition. DAXX directly and specifically interacts with H3.3 both *in vivo* and *in vitro* and mediates deposition of bacterially purified recombinant H3.3-H4 tetramer on naked DNA *in vitro* [260]. A central acidic domain of DAXX strongly interacts with H3.3-H4 [260] and the motif "AAIG" of H3.3 was found sufficient for specific interaction with DAXX [281].

Interestingly, the DAXX-ATRX complex deposited H3.3 at regions different from the ones that contained H3.3 deposited by HIRA. Genome-wide enrichment study shows HIRA-independent localization of H3.3 at telomeres and transcription factors binding sites [259]. DAXX-ATRX dependent H3.3 deposition at pericentric heterochromatin in mouse embryonic fibroblasts (MEFs) was described, and the presence of H3.3 appeared to regulate transcription of pericentric DNA [281]. In contrast, DAXX-ATRX dependent deposition of H3.3 at telomeres in ES cells is required for transcription repression from telomeric repeats [259]. This suggests that DAXX-ATRX mediated deposition of H3.3 at different genomic regions can play multiple roles. Surprisingly CAF-1 was found associated with H3.3 predeposition complex in the absence of DAXX [281], suggesting that cells can use replication dependent assembly pathway to counterbalance the loss of DAXX. It seems that DAXX prevents the interaction of H3.3 with CAF-1 complex in order to promote replication independent chromatin assembly of H3.3.

1.4.2 Deposition of H2A-H2B family histones

H2A-H2B family histones are incorporated into chromatin by several different proteins. The cytosolic H2A–H2B are bound by NAP1 (Nucleosome Assembly Protein1), which functions as a shuttle for H2A-H2B from the cytoplasm to the nucleus [282]. NAP1 also plays important roles in the nucleus, where the histones are channeled into distinct pathways associated with DNA metabolic events. In addition, it was shown to assemble nucleosomes *in vitro* and to interact with H2A-H2B *in vitro* and *in vivo* [283, 284], suggesting it to be a specific chaperone for H2A-H2B. However, biochemical studies indicate that NAP1 preferentially binds H3-H4

when all core histones are present [285-287]. In budding yeast, it has been shown that Chz1 together with SWR1 complex are involved in Htz (H2A.Z) deposition [288, 289]. The conserved CHZ motif conferring this function is also found in human HIRA-interacting protein 3 (HIRIP3), a factor shown to interact with core histones [277]. In addition, p400/Domino and SRCAP, homologs of the SWR1 complex, have been identified which are able to exchange H2A-H2B with H2A.Z-H2B dimers [290]. It is tempting to speculate that HIRIP3 constitutes the human H2A.Z-specific chaperone, which together with p400/Domino or SRCAP is responsible for the site-specific incorporation of H2A.Z. Although the FACT protein complex can mediate the exchange of γ-H2A.X-H2B with unmodified H2A.X-H2B [291], no specific *de novo* deposition chaperone for H2A.X has been identified. The specific chaperone for the other H2A variants, macroH2A and H2A.Bbd, are not known.

Table1.4: Summary of histone chaperones

Histones	Specific histone chaperones		
	Name	**Mass (KDa)**	**Functions** (In context of chromatin assembly)
H3.1-H4	**CAF-1** **CHAF1A**	105	Replication Coupled (RC) deposition of H3.1-H4
	CAF-1 **CHAF1B**	61	
	CAF-1 **RbAp48**	48	
H3.3-H4	**HIRA**	81	Replication Independent (RI) deposition of H3.3-H4 Genic regions
	DAXX	112	Telomeres, Pericentric heterochromatins
H2AZ-H2B	**CHZ1 (yeast)**	18	Exchange of H2A.Z-H2B and H2A-H2B
	Common histone chaperones		
	Name	**Mass (KDa)**	**Functions** (In context of chromatin assembly)
H3-H4 family	**ASF1A**	23	Supply of H3.1/H3.3-H4 dimers for RC and RI
	ASF1B	22	
	NASP	85	Transport of histones to nucleus, Promotes H4 acetylation, Linker histone deposition
	RbAp46	48	Regulation of chromatin metabolism by assisting different enzymatic activities, Chromatin assembly
	RbAp48	48	
H3-H4 H2A-H2B family	**FACT** **SSRP1**	81	Transcription elongation, Recombination exchange of γ-H2A.X-H2B with unmodified H2A.X-H2B
	FACT **Spt16**	120	
	hDEK	43	Possible chaperone in human Targeted deposition of H3.3 in Dorosophila
	JDP2	19	Transcription, Inhibition of HAT
	ANP32B	29	Deposition of core histones at promoter regions
	NAP1	45	H2A-H2B import to nucleus, exchange of H2A.Z/H2A

1.5 Epigenetic Specification of Centromere by CENP-A

1.5.1 The Centromere

The centromere or primary constriction on the eukaryotic chromosome is a specialized region, where kinetochore is formed that interacts with spindle microtubules to ensure chromosome separation during mitosis and meiosis. It is of vital importance to genetic stability and has many roles during cell division, such as; spindle microtubule attachment, checkpoint control, sister chromatid cohesion and release, chromosome movement and cytokinesis. It is responsible for the bi-orientation of each chromosome so each sister chromatid will attach to microtubules from opposite spindle poles, ensuring correct segregation of newly replicated sister chromatids into daughter cells [2]. This fundamental role of centromere is conserved in eukaryotic organisms from yeast to humans.

Defects in the chromosomes segregation process, including mutations and aberrant incorporation of proteins that have a role in the assembly of the kinetochore, can lead to severe consequences. Aneuploidy is an error in chromosomes segregation that results in the daughter cells with an abnormal chromosomal number. In human, meiotic aneuploidy is a major cause of birth defects (Down's syndrome, Edwards' syndrome and Patau's syndrome), spontaneous abortions and infertility [292]. Mitotic aneuploidy is associated with tumor formation and cancer.

The centromere is organized in three domains; the pairing domain, the central domain and the kinetochore domain [293-295] (figure 1.10). The pairing domain is the region of the inner centromere where the sister chromatids are the closest together. The central domain is the densely packed centromeric heterochromatin that serves as a structural support for the kinetochore. This is typically α-satellite DNA and associated proteins. The kinetochore is a trilaminar structure and is made up of an inner plate, a middle zone and an outer plate, which binds the microtubules.

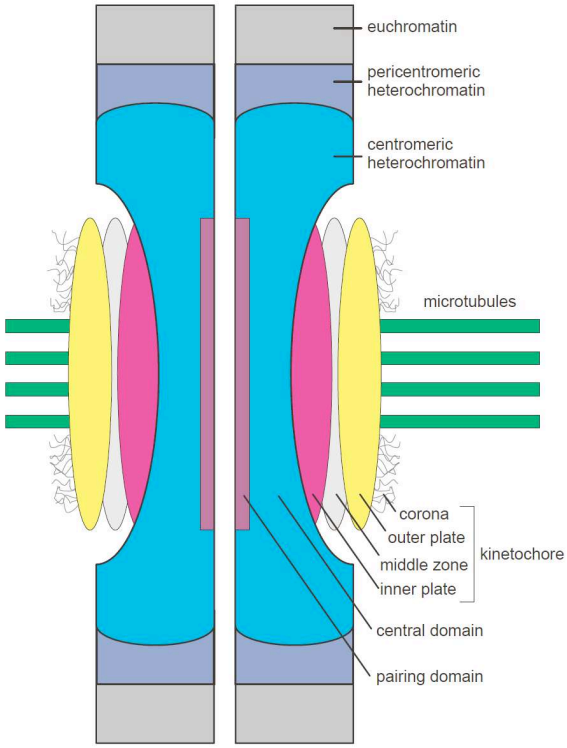

Figure 1.10. Centromere domains. The centromere is comprised of three domains; the pairing domain, the central domain and the kinetochore domain. The kinetochore is made up of the inner and outer plates, a middle zone and an outermost fibrous corona [293-295].

In eukaryotes, three types of centromeres have been described: point centromere, regional centromere [296] and holocentromere [297]. The point centromere is the simplest centromere, present in the budding yeast (*Saccharomyces cerevisiae*), comprises 125 bp of DNA that are sufficient to facilitate kinetochore assembly in a sequence-dependent manner [298]. In contrast to the "point" centromeres of budding yeast, however, most other eukaryotes, including the fission yeast (*Schizosaccharomyces pombe*), possess "regional" centromeres of varying length [296]. Regional centromeres are generally composed of repetitive DNA sequence elements, with centromere size increasing across evolutionary more complex species, ranging from 35-110kb in fission yeast to 0.3-5 Mbp in humans [296, 299, 300]. In holocentric organisms, such as *Caenorhabditis elegans*, the centromere is spread throughout the length of the chromosome.

1.5.1.1 Centromeric DNA

Despite, the function centromere is highly conserved among eukaryotes, yet the centromeric DNAs are highly divergent and rapidly evolving during speciation. The size and sequence of centromeric DNA vary significantly between different eukaryotes. The centromere DNA can range from as little as 125 bp for the budding yeast up to tens of megabases in higher eukaryotes (figure 1.11).

The budding yeast centromeric DNA sequence is the simplest one, spanning only 125bp of DNA, and consists of three elements: centromere DNA element I (CDE I,) 8 bp), the A:T rich CDE II (78–86 bp) and CDE III (26 bp) where the CBF3 protein complex binds [301, 302]. In fission yeast, the centromeric DNA contains a central core element (cnt) of 4-7kb, which is flanked by imr sequences and pericentric outer repeats (otr) with an overall size range of 30-110kb, depending on the chromosome [303]. In contrast to yeast, the centromeric DNA organization in higher eukaryotes is much more complex. The centromeric DNA of higher eukaryotes is characterized by highly repetitive AT-rich tandem sequence repeats (figure 1.11).

In human, centromeric DNA consists of extensive arrays comprising 0.2-7 megabases of a 171bp α-satellite motif repeated in tandem head-to-tail manner [304] (figure 1.13). The α-satellite can be subdivided in two types based on their monomer sequence and composition [304]. Type I (αI-satellite) forms regular higher order arrays, and are flanked by more diverged, monomeric type II satellites (αII-satellite) that are frequently interspersed with other repetitive elements, such as long and short interspersed elements (LINEs and SINEs, respectively) [305-307]. An important structural feature of type I satellite DNA is a 17 bp motif called the CENP-B box, which represents the binding site for the constitutive centromere protein B (CENP-B) [308, 309]. These CENP-B box-containing monomers are found in all human centromeres except the Y centromere.

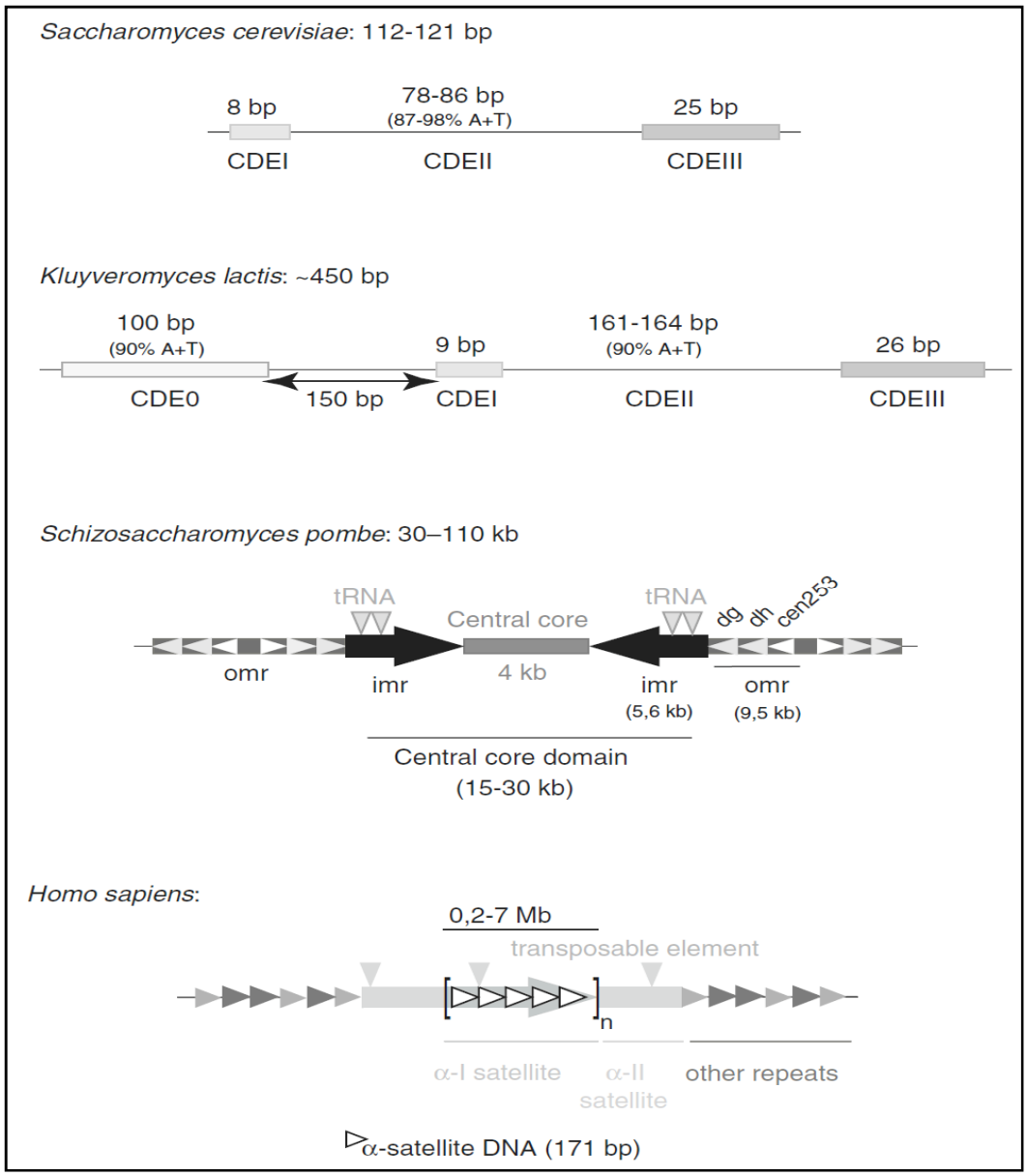

Figure 1.11. Centromeric DNA organization. The centromeric sequence elements of Saccharomyces cerevisiae, Kluyveromyces lactis, Schizosaccharomyces pombe, and human chromosomes. Based on [299]. For detail see text.

1.5.1.2 Centromere Proteins

The proteins associated with centromere can be broadly classified into two groups based on their localization manner to centromeres. The first class named as 'constitutive proteins' that associate with the centromere throughout the cell cycle, and the second class called, 'transient proteins' that transiently localize to the centromere for only a portion of the mitotic cycle. These proteins can be further divided on the basis of their essentiality, that whether or not the given centromere

protein is essential. The essential constitutive centromere proteins include CENP-A [310], CENP-C [311], CENP-H class (CENP-H, CENP-I, CENP-K and CENP-L) [312-314], CENP-M [314], CENP-T CENP-W [315] and Mis12 [316]. These proteins are critical for the recruitment of other constitutive centromere components and/or transient kinetochore components such as those involved in monitoring microtubule attachment. The non-essential constitutive centromere proteins can collectively be defined as a group of proteins that exhibit little or no detrimental chromosome segregation phenotype when their genes are functionally knocked out. These proteins include CENP-B [317] and the CENP-O class (CENPO, CENP-P, CENP-Q, CENP-R and CENP-U (CENP-50)) [314, 315].

The constitutive centromere-associated network (CCAN), also known as the CENP-A NAC/CAD (CENP-A nucleosome associated complex and CENP-A distal complex) comprises 16 proteins including CENP-C, -H, -I, -K to -U, -W, and –X [314, 318-321], that co-purify and co-localize together with CENP-A to centromeres throughout the cell cycle. Within the CCAN, the majority of the proteins are organized into distinct sub-complexes. Together, the CCAN is largely responsible for recruitment and assembly of the KNL1-Mis12-Ndc80 (KMN) network in mitosis, which in turn promotes regulated recruitment of additional outer kinetochore components, and facilitates interaction with spindle microtubules.

The different protein complexes that transiently localize to the centromere during cell cycle include KMN network (KNL1-Mis12-Ndc80), Mis12 complex (Mis12, Nnf1, Nsl1, and Dsn1), chromosomal passenger complex (CPC, include Aurora B kinase, INCENP, Survivin and Borealin), and spindle assembly checkpoint (SAC, the main components are Mad1, Mad2, Bub1, BubR1 and Bub3). A representation of some of the proteins that make up the centromere is depicted in Figure 1.12.

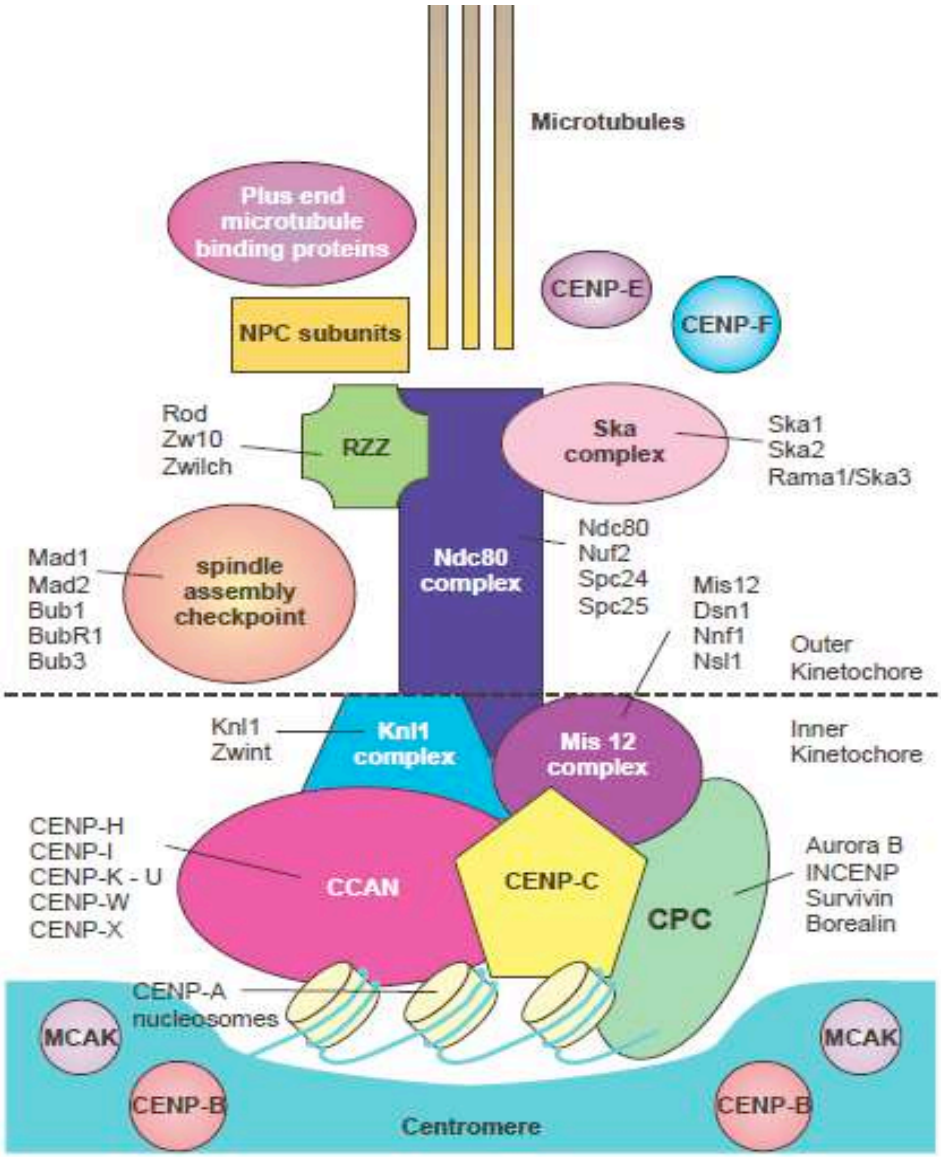

Figure 1.12. Organization of centromere proteins.

The centromere is depicted in blue at the bottom of the figure with nucleosomes containing the centromere histone variant CENP-A directly above. The centromere proteins that associate with the centromere throughout the cell cycle, CENP-B, CENP-C and the constitutive centromere-associated network (CCAN) are shown in the inner kinetochore, below the dashed line. The first proteins to be recruited to the centromere are the Mis12 complex and the Knl1 complex. These are followed by other centromere proteins: the mitotic centromere-associated kinesin (MCAK), the Ndc80 complex, the chromosomal passenger complex (CPC), the spindle assembly checkpoint proteins, the Rod–ZW10–Zwilch (RZZ) complex, the Ska complex, the microtubule associated proteins, the microtubule motor proteins, and the nuclear pore complex (NPC). The precise order of assembly and interactions between all of these centromere/kinetochore proteins is still unclear. Image adapted from [322].

1.5.2 Evidence for Epigenetic Identity of centromere

Does the centromeric DNA sequence or CENP-B specify centromere function? Although in budding yeast centromere DNA alone is sufficient to facilitate centromere formation *de novo* [298], centromeres in fission yeast and metazoan cells don't depend solely on centromeric DNA.

In spite of the fact that higher order α-satellite DNA has been found associated with centromere, shown by genomic [307, 323], biochemical [5] and artificial chromosome assays [307, 324]. There are several lines of evidence show that the simple presence of α-satellite DNA and CENP-B on a chromosome is not sufficient for centromere function. First, centromeric DNA sequences are rapidly evolving and co-evolving with their essential partner CENP-A, and show no obvious sequence conservation between species or even between different chromosomes in the case of Drosophila. Second, chromosomes that are naturally or artificially deleted for much of this α-satellite array can still assemble a kinetochore and segregate normally [325, 326]. In addition, mitotically stable dicentric chromosomes that contain two spatially distinct alpha-satellite DNA regions as a consequence of chromosome fusion or translocation events, CENP-B is present at both alphoid sites, whereas detectable levels of CENP-A and other essential kinetochore components localize only to the "active centromere" site [327, 328]. This is further supported by the lack of CENP-B from the centromere of the endogenous Y chromosome both in human and mouse [309], and perhaps most strikingly illustrated by the viability of CENP-B knock-out mice in the absence of any detectable mitotic defects [317]. Furthermore, neocentromeres can form and assemble fully functional kinetochores in the absence of alphoid DNA, recruiting all kinetochore components except CENP-B [327, 329].

Taken together, these studies argue that centromeric DNA itself is not the dominant determinant of centromere identity and function but, rather, epigenetic mechanisms determine the functional identity of this locus. Those epigenetic determinants must reside in the surroundings, such as the chromatin status, or in the kinetochore composition itself. Increasing experimental evidence suggests that chromatin composition and organization play a major role in centromere specification and propagation.

Despite, the enormous variations in composition, organization and length of centromeres, CENP-A is found to be present at all natural centromeres as well as functional neocentromeres and *de novo* artificial centromeres. CENP-A is a histone H3 variant that replaces canonical histone H3 in the centromeric nucleosomes of all eukaryotes. Inactivation or down regulation of CENP-A in different experimental systems results in chromosome segregation defects and eventually cell death, and its presence is required for assembly of all other centromeric proteins. It has been therefore proposed that CENP-A is the epigenetic mark of the centromere.

1.5.3 The Centromere-specific Histone H3 Variant CENP-A

Historically, autoimmune sera from patients with a variant of systemic sclerosis characterized by the CREST (calcinosis, Raynaud's, esophageal dysmotility, sclerodactyly, telangiectasia) showed certain features and led to the discovery of autoantibodies that recognize specific mammalian centromere proteins about three decades ago [330]. This has led to the identification of the three major constitutive centromeric autoantigens, CENP-A, CENP-B, and CENP-C [308, 331, 332]. Among these CENP-A, was first found to be a histone H3 variant that co-purifies with centromeric nucleosome [333]. Since then, homologues of CENP-A have been identified in other organisms, including budding yeast Cse4 [334], fission yeast Cnp1 [335], *C. elegans* HCP3 [336], Drosophila CID [337] and *Arabidopsis thaliana* HTR12 [338] (Table 1.5). In all organisms examined till date, CENP-A localizes exclusively to active centromeres, including those of human artificial chromosomes, stable dicentric chromosomes and neocentromeres. Genetic studies of CENP-A knockouts in mice [310], DT40 cells [339], C. elegans [340] and by RNAi in human cells [316] reveal that loss of CENP-A results in the failure of centromere formation and kinetochore assembly thus causing mitotic arrest and embryonic lethality. Moreover, CENP-A depletion results in mislocalization of most kinetochore proteins, whereas depletion of most kinetochore proteins has no effect on CENP-A localization. In addition, CENP-A overexpression results in its mislocalization to normally non-centromeric sites and the formation of ectopic kinetochores [341]. These observations attest that the faithful assembly of CENP-A containing chromatin epigenetically maintains centromeres at the same locus, cell division after cell division and generation after generation.

1.5.3.1 CENP-A Structure

The hallmark of centromeric chromatin, CENP-A is a histone H3 variant, which replaces the canonical H3 histone in the centromeric nucleosome core particle. It is one of the most rapidly evolving members of the histone H3 family. From evolutionary point of view, CENP-A was suggested to be the result of convergent evolution, i.e., multiple lineages of histones H3 have converged on a common centromeric function [229]. As the N-terminal tail of CENP-A is highly variable between species, suggesting that CENP-A evolves to adopt with variable centromere loci. Indeed, there is evidence in two different Arabidopsis species and in Drosophila for adaptive evolution of the N-terminal and histone fold domain of CENP-A [342, 343].

From a structural point of view, CENP-A protein consists of a highly variable N-terminal sequence, followed by a globular histone-fold domain, which in the case of human CENP-A, shows 60% sequence identity to the conventional H3 counterpart [344]. The N-terminal tails of CENP-A from different species display variations both in length and amino acid composition. For example, the tails are 120 and 130 amino acids in budding yeast and drosophila CENP-A, respectively, but only 45 amino acids in humans and 20 amino acids in fission yeast. The globular histone-fold domain of CENP-A resembles that of canonical H3 histones, i.e., composed of three alpha helices including two flanking short alpha helices (alpha1 and alpha3) and one central alpha helix (alpha2). The loop 1 region between alpha1 and alpha2 is both necessary and sufficient to target drosophila and human CENP-A to centromeres, where it forms a heterodimer with histone H4 [4, 345, 346]. The part of the histone-fold domain (HFD) consisting of loop L1 and the adjacent alpha2-helix is called the CENP-A targeting domain (CATD), which is required for the targeting of the newly synthesized protein to the centromeres [4, 345, 346] (figure 1.13). Substitution of the CATD into canonical H3 in human is sufficient to target the chimeric $H3^{CATD}$ to centromeres throughout the cell cycle, capable of rescuing the lethality of CENP-A depletions and sustain the assembly of a healthy kinetochore [345, 347]. However, in budding yeast and *A. thaliana*, the CATD is not sufficient to convert H3 to CenH3, suggesting that the N-terminal domain is also required to convert histone H3 into a functional centromeric histone [345, 348].

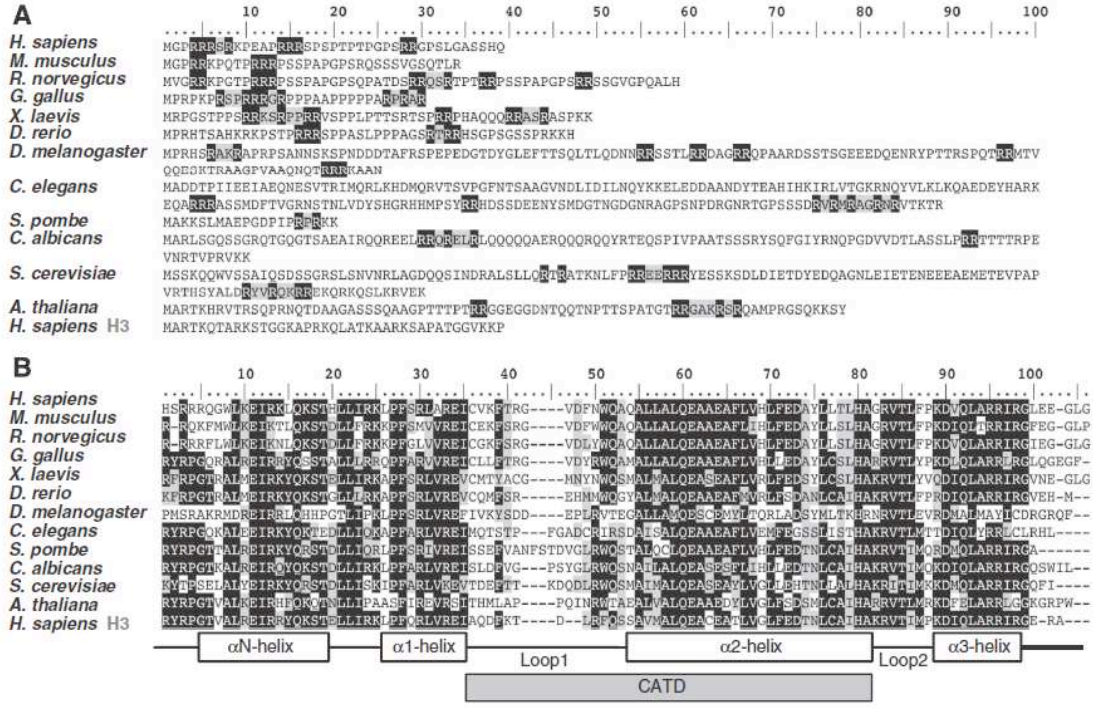

Figure 1.13. Sequence alignment of CENP-A from different species.
CENP-A is a highly divergent histone H3 variant that evolves very rapidly. Sequence comparison of the N-terminal domain (A) and the histone-fold domain (HFD) (B) of CenH3 proteins from different species, ranging from yeast to humans, is shown. The sequence of canonical histone H3 is shown at the bottom for comparison. R-rich motives are indicated in A. Secondary structure of the HFD is indicated in B. The position of the CATD, which mediates centromeric targeting of CENP-A and confers distinct structural properties to CENP-A -nucleosomes is indicated.

Recently, the crystal structure of human DNA-free CENP-A-H4 heterotetramers revealed that CENP-A-H4 dimer pairs are closer mutually by rotation with a difference of 9–14^{o} compared to the corresponding H3–H4 dimer pairs from the H3–H4 heterotetramer [349]. The two residues His104 and Leu112 of the α2-helix that resides at the rotational interface were found to be responsible for this closeness of dimer pairs. The replacement of these two residues with their counterparts from H3 abolished the centromeric localization of the mutant histone [349]. This study indicates that the CENP-A-H4 tetramer may be more compact than H3-H4 tetramer. More recently, the crystal structure of human centromeric nucleosome containing histones (CENP-A, H4, H2A and H2B) and 147bp alpha-satellite DNA revealed that CENP-A nucleosome organized in a left-handed orientation [350]. Overall, the global structure of CENP-A and H3 nucleosomes are quite similar. However, unlike the conventional H3 nucleosome, only the central 121bp are visible but 13bp from both ends of the crystal structure are invisible. There are two extra residues (Arg80 and Gly81) in the loop1 region of CENP-A nucleosome

compared to H3 nucleosome [350]. In fact, the mutation in the loop1 residues affects CENP-A centromeric localization [350]. Therefore, the loop1 region may function by stabilizing the CENP-A chromatin, most probably by providing a binding site for other interacting factors. In the light of these observations, CENP-A nucleosome structure is probably not compacted as was suggested previously.

Table 1.5. Properties of centromeric H3 variant CENP-A

Cent H3s	Organisms	Amino acids	Mass (KDa)	Timing of centromeric deposition
CENP-A	*H. sapiens*	140	16	Late telophase, early G1
CID	*D.melanogaster*	225	26	Anaphase, Metaphase
HCP3	*C. elegan*	288	33	Not determine
CSE4	*S. cerevisiae*	229	27	S-phase
Cnp1	*S. pombe*	120	14	S-Phase, G2-Phase
CenH3	*X. laevis*	150	17	Early-interphase
HTR12	*A. thaliana*	174	19	G2-Phase
CENP-A	*G. gallus*	131	15	Not determine

1.5.3.2 Post-translational modifications of CENP-A

As mentioned earlier, the functional activity of the major core histones is regulated through post-translational modifications of residues within their N-terminal and C-terminal domains. These modifications can act to alter chromatin properties directly or serve as signals for the recruitment of effectors. Moreover, these modifications have an essential contribution to the regulation of chromatin functions; they correlate with different functional states and are involved in chromatin assembly/disassembly processes. Little is known, however, about the posttranslational modification of CENP-A and their possible important roles in centromere function. The only posttranslational modification reported for CENP-A is the phosphorylation of serine7 by Aurora kinases, which also phosphorylates H3 and several other substrates [351-353]. Phosphorylation of CENP-A is required for normal progression of mitosis and cytokinesis [351]. According to these reports the pattern of CENP-A phosphorylation is distinct from that of histone H3 Ser10. Phosphorylation of histone H3 first accumulates in pericentric heterochromatin in late

G2 and later increases throughout the chromosome arms. In contrast, phosphorylation of CENP-A Ser7 begins in mitotic prophase, after centromere duplication but before microtubule attachment [351]. The CENP-A modification appears to modulate gradually throughout mitosis and decreases during anaphase. These results suggest that CENP-A phosphorylation is not required for assembly of the core centromere chromatin, but may participate in the maturation or function of the active kinetochore. However, there is no specific kinase known for CENP-A phosphorylation during G1 phase and its relevance to CENP-A deposition. The N-terminal tails of the CENP-A family are highly variable in sequence, and potential histone H3 Ser10 motifs can be found in some members but not others. Unraveling the range of modifications and their functions in the CENP-A family histones is likely to provide important insights into centromere function.

1.5.3.3 Specific Deposition of CENP-A at centromeres

CENP-A forms dimer with the histone H4, like a canonical histone H3. It could in principle be deposited everywhere on a chromosome. However, except in holocentric organisms (e.g: *C. elegans*), CENP-A is exclusively restricted to centromere region, in all other eukaryotes examine till date. Therefore, special mechanisms must exist in cells for selective localization of CENP-A to centromeres. In theory, there are two possible scenarios: CENP-A could be deposited everywhere and actively removed from noncentromeric regions or CENP-A could be selectively deposited only at centromeres. Overexpression of CENP-A^{CID} in Drosophila results in its mislocalization to normally non-centromeric sites and the formation of functional ectopic kinetochores [341]. Heterochromatin regions did not incorporate CENP-A^{CID} after overexpression, indicating that heterochromatin may limit centromeric chromatin. In addition, overexpression of CENP-A^{CID} caused severe mitotic defects and cell death [341]. This finding suggests that the amount of CENP-A must normally be tightly controlled to avoid such catastrophic effects. In fact, ubiquitin-mediated proteolysis in drosophila and yeast, control levels of CENP-$A^{CID/CSE4}$ and prevent its non-centromeric deposition [354, 355]. Recent studies identified a specific E3 ubiquitin ligase called Psh1 that recognizes CSE4 and reduces its ectopic incorporation [356, 357]. Thus, overexpression experiments with CENP-A suggest that CENP-A levels are strictly regulated by proteolysis indicating its selective

deposition at centromeres.

1.5.3.4 Timing of CENP-A deposition

To understand the mechanism of CENP-A deposition, it is critical to determine the exact cell cycle window during which CENP-A loading to centromeric chromatin takes place. The timing of new CENP-A deposition at centromeres during the cell cycle has been reported to be variable among animal, plant and fungi (Table 1.5).

In human the peak of the synthesis of CENP-A occurs during G2-phase [358] and deposition of CENP-A at centromeric DNA starts late in mitosis and continues to early G1-phase [358, 359]. In the fast cycles of Drosophila syncytial embryos, CENP-A incorporates during anaphase [360]. However, in Drosophila S2 cells, GFP-tagged CENP-A was detected in metaphase cells 2 hrs after induction of its expression, implying that incorporation occurred at some point between the preceding G2 and metaphase [361]. In budding yeast (*S. cerevisiae*), all pre-existing CENP-A is replaced by newly synthesized CENP-A during S phase [362] whereas in *S. pombe*, two pathways of CENP-A deposition have been reported at different times of the cell cycle, S phase and G2 [363, 364]. These observations clearly show that CENP-A loading in animals, particularly in human and Drosophila occur after mitosis (before genome duplication). Whereas, deposition during S and G2 phase in case of fission yeast, would suggest that post-reduction loading mechanism for CENP-A have evolved after divergence of fungi from metazoan. In contrast to human and drosophila, CENP-A deposition takes place during G2 phase in plants and protozoan, by a replication independent mechanism [365-367]. This indicates that in plants deposition of CENP-A occurs before mitosis (after genome duplication). Although, the exact reason for differential loading time of CENP-A is not yet clear, it might indicates the presence of different mechanisms for CENP-A loading between animals and plants.

1.5.3.5 Mechanism of CENP-A deposition

Two key factors may contribute to the centromeric localization of CENP-A, include the properties of CENP-A that are necessary and sufficient to direct

deposition of new CENP-A specifically to centromeres following replication, and the trans-acting CENP-A interacting factors, responsible for delivery of CENP-A to centromeres. As discussed above, a specific domain of CENP-A called CATD (CENP-A Targeting Domain) within the histone fold region is essential and sufficient for centromeric localization of CENP-A [345, 368]. How does CATD determine centromeric localization of CENP-A? It is possible that CATD mediates interaction with specific CENP-A recruiting proteins and/or chromatin assembly factors, which could directly bind to CATD or, alternatively, recognize the distinct structural features of CATD-H4 interface. It is also possible that CATD influences stability, and/or specificity, of the interaction with DNA.

Recent studies have identified several factors in different organisms that affect CENP-A localization but their precise roles in this process requires further investigations. In Drosophila, p55 (RbAp48) was found to be associated with CENP-A/H4 tetramer and to facilitate its deposition to DNA [13]. A genome wide RNAi screen for defects in Drosophila CENP-A localization at centromeres identified CAL1 and CENP-C as essential proteins for assembly of newly synthesized CENP-A [369]. In *S. pombe,* Mis 6 and Ams2 proteins are involved in CENP-A localization [370, 371], Mis16 and Mis18 are required for CENP-A loading and Sim3 might act to escort CENP-A to centromere [364, 372]. The human protein Mis18 and M18BP1, recruited to centromere at telophase-G1, and RbAp46/RbAp48 may act to prime centromere for CENP-A localization [372, 373]. In *S. cerivisiae* and *S. pombe*, Scm3 (Suppressor of chromosome mis-segregation 3) protein was shown to specifically bind the CENP-A-H4 complex and to be required for its assembly into the centromeric chromatin [374-378]. Despite the identification of CENP-A associated proteins little is known about specific histone chaperones in humans that could bind CENP-A and assist its specific deposition to centromeres.

1.6 Aims of this study

Centromeres of higher eukaryotes are specified epigenetically by the presence of a unique nucleosome that contains a centromeric-specific histone H3 variant CENP-A. Understanding the mechanism that how CENP-A is specifically

loaded to and maintained on centromeres, is of paramount importance for establishment and propagation of epigenetic centromere identity. Despite the identification of CENP-A associated proteins in different organisms, that have been reported to be implicated (please avoid repeatiting of words in centromere function, little is known about specific factors in human that could bind CENP-A and assist its specific deposition at centromeres. The overall goal of my project is to identify CENP-A specific chaperone in human, which is responsible for CENP-A loading to centromeres, by using biochemical and proteomic strategies.

More specifically the aims of this study have been:

- To search for molecular factors participating in CENP-A delivery to centromeres by isolating the specific partners of predeposited CENP-A in HeLa cells.
- To find out the most putative and specific histone chaperone among different CENP-A interacting proteins by comparison with H3.1 interacting partners.
- To confirm the direct interaction of the putative chaperone with CENP-A, *in vitro*.
- To examine the mode of interaction by identifying the specific interaction domains in chaperone and CENP-A proteins.
- To investigate the *in vivo* requirement of this chaperone for CENP-A loading by knockdown experiments.
- To study the *in vitro* deposition of CENP-A-H4 tetramer on naked alpha satellite DNA in the presence and absence of identified chaperone.

CHAPTER 2
RESULTS: PAPER 1

Overview of results

CENP-A, a centromere-specific histone H3 variant, is found in all eukaryotes and is required for the assembly and the maintenance of both active centromere and kinetochore. Despite the identification of CENP-A associated proteins, little is known about specific factors in human that could bind CENP-A and assist its specific deposition at centromeres at the right time. To identify the specific factors involved in CENP-A deposition at centromeres, we purified the prenucleosomal CENP-A and H3.1 complexes from soluble nuclear fraction of HeLa cells stably expressing the epitope tagged histones e-CENP-A and e-H3.1. We found Holliday Junction Recognition Protein (HJURP), uniquely in CENP-A prenucleosomal complex. We showed that depletion of HJURP by specific siRNA strongly decreased centromeric associated CENP-A. Bacterially expressed HJURP binds at a stoichiometric ratio to the CENP-A/H4 but not to the H3/H4 polypeptides. The binding occurred through a conserved HJURP short N-terminal domain, termed CBD. Inside the CBD domain we identified a conserved box that we named TLTY box, which is essential for CENP-A binding. HJURP facilitated efficient deposition of CENP-A/H4 tetramers to naked DNA in vitro. Taken together, our data established HJURP as a specific chaperone responsible for CENP-A deposition at centromeres.

HJURP binds CENP-A via a highly conserved N-terminal domain and mediates its deposition at centromeres

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The human histone H3 variant, CENP-A, replaces the conventional histone H3 in centromeric chromatin and, together with centromere-specific DNA-binding factors, directs the assembly of the kinetochore. We purified the prenucelosomal e-CENP-A complex. We found that HJURP, a member of the complex, was required for cell cycle specific targeting of CENP-A to centromeres. HJURP facilitated efficient deposition of CENP-A/H4 tetramers to naked DNA in vitro. Bacterially expressed HJURP binds at a stoichiometric ratio to the CENP-A/H4 tetramer but not to the H3/H4 tetramer. The binding occurred through a conserved HJURP short N-terminal domain, termed CBD. The novel characteristic identified in vertebrates that we named TLTY box of CBD, was essential for formation of the HJURP-CENP-A/H4 complex. Our data identified HJURP as a vertebrate CENP-A chaperone and dissected its mode of interactions with CENP-A.

histone chaperone | histone variant

The centromere is a specialized region on eukaryotic chromosomes required for the assembly of active kinetochore. The centromere is of vital importance for genetic stability. Defects in meiotic chromosomes segregation may lead to aneuploidy and tumor formation (1).

The structure of the centromeres, despite the many efforts invested, remains elusive (2, 3). CENP-A (termed also CenH3, (2, 3), a centromere-specific histone H3 variant, is found in all eukaryotes (4) and is required for the assembly and the maintenance of active centromeres (5–8). Human CENP-A shows >60% sequence identity with the C-terminal histone fold domain of H3, but its N-terminal tail is highly divergent (4, 9, 10). A domain in the histone fold of CENP-A, termed CATD, is required for the targeting of the newly synthesized protein to the centromeres (9, 11–13). Substitution of the CATD into canonical H3 is sufficient to replace the essential function of CENP-A suggesting that any specific CENP-A chaperone would recognize the (CENP-A/H4)2 tetramer via the CATD and deliver it to centromeric chromatin (14).

A fundamental question in centromere biology is how CENP-A is specifically deposited to and maintained on centromeric DNA. Recent studies have identified several factors that affect CENP-A localization but their precise roles in this process remain to be determined. In *Drosophila*, p55 (RbAp48) was found to be associated with CenH3/H4 tetramer and to facilitate its deposition to DNA (15). A genome wide RNAi screen for defects in *Drosophila* CenH3 localization at centromeres identified CAL1 and CENP-C as essential proteins for assembly of newly synthesized CenH3 (16). In *S. pombe* Mis 6 and Ams2 proteins are involved in CenH3 localization (17, 18), Mis16 and Mis18 are required for CenH3 loading and Sim3 might act to escort CENP-A to centromere (19, 20). The human proteins hMis18 and M18BP1, recruited to centromere at telophase-G1, and RbAp46/RbAp48 may act to prime centromere for CENP-A localization (21). In *S. cerivisiae* and *S. pombe*, Scm3 (Suppressor of chromosome mis-segregation 3) protein was shown to specifically bind the CenH3-H4 complex and to be required for its assembly into the centromeric chromatin (22–26). Despite the identification of CENP-A associated proteins little is known about specific histone chaperones in humans that could bind CENP-A and assist its specific deposition to centromeres.

In this study, we purified the prenucleosomal CENP-A complex from soluble nuclear fraction of HeLa cells. We present evidence that HJURP (Holliday Junction Recognition Protein) (27), a member of the CENP-A prenucleosomal complex (28), is essential for the deposition of CENP-A at the centromeres in cell cycle dependent manner. We further analyzed how HJURP interacts with CENP-A and identified the domains of both proteins involved in this interaction.

Results

Purification of Prenucleosomal CENP-A and H3.1 Complexes. To identify proteins interacting specifically with CENP-A, we established stable HeLa cell lines expressing either a C-terminal FLAG-HA epitope tagged CENP-A (e-CENP-A) or a C-terminal FLAG-HA epitope tagged H3.1 (e-H3.1). The immunofluorescence analysis of e-CENP-A and e-H3.1 in these cells revealed that the tagged histones are found in the nucleus (Fig. 1*A*). As expected for a conventional histone, e-H3.1 shows a rather diffuse nuclear staining. In contrast, e-CENP-A was localized in discrete foci, a distribution pattern typical for endogenous CENP-A (7, 10). These data indicate that the presence of the tag epitopes do not interfere with the deposition and association with chromatin of both e-H3.1 and e-CENP-A.

Cell extracts from the tagged cells were prepared and the e-H3.1 and e-CENP-A prenucleosomal complexes were purified by sequential immunoprecipitations with antiFLAG antibody followed by antiHA antibody (29). The proteins associated with e-CENP-A and e-H3.1 were separated in 4–12% gradient PAGE containing SDS and silver stained (Fig. 1*B* and *C*). Mass spectrometry analysis identifies the following proteins as common components of the e-CENP-A and e-H3.1 complexes: Core histones (H2A, H2B, H4), RbAp46/RbAp48 proteins, Ku proteins (Ku70 and Ku80), and DNA-dependent protein kinase (DNA-PK). Two of the three CAF-1 subunits, CAF 150 and CAF 60, were specific to the e-H3.1 complex, whereas the third CAF-1 subunit RbAp48 and RbAp46 was a common component to e-CENP-A and e-H3.1 complexes (Fig. 1*B* and *C*). RbAp46 and RbAp48 are highly homologous histone chaperones found in many chromatin-related complexes (30) and apparently they interact with H4 (31–33). These results are in agreement with the reported data, showing that CAF-1 subcomplex is part of e-H3.1 containing-complex (34).

The e-H3.1 prenucleosomal complex contained also importin, s-NASP, and histone acetyl transferase-1 (HAT1) (Fig. 1*C*). The

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The authors declare no conflict of interest.

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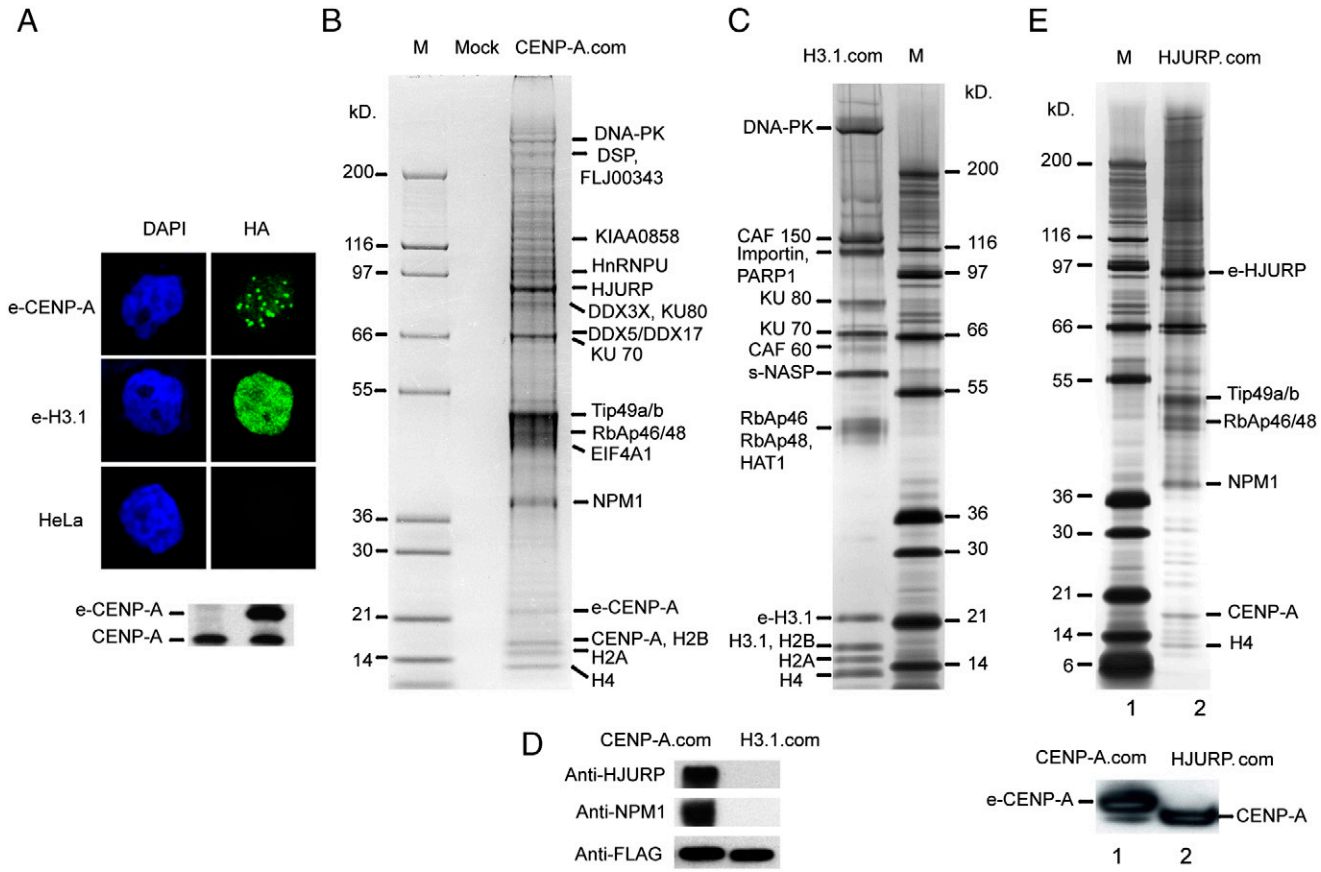

Fig. 1. Purification of CENP-A preassembly complex. (*A*) Localization of e-CENP-A and e-H3.1. Stable cell lines expressing either e-CENP-A or e-H3.1 were immunostained with antiHA antibody (*Green*) to detect the epitope tagged proteins and DAPI staining (*Blue*). (*Lower*) Western blotting of total cell extract from control HeLa cells (*Lane 1*) and stable HeLa cell line (*Lane 2*) expressing e-CENP-A. An anti-CENP-A antibody was used to reveal the blot. (*B*) Silver staining of proteins associated with e-CENP-A. The preassembly e-CENP-A complex (CENP-A.com) was purified by tandem immuno-affinity and the associated polypeptides were identified by mass spectrometry. Lane M corresponds to a protein molecular mass marker. Lane Mock, corresponds to a mock purification from a nontagged HeLa cell line. (*C*) Silver staining of proteins associated with e-H3.1. The prenucelosomal e-H3.1 complex (H3.1.com) was purified by tandem immunoaffinity and the associated polypeptides (*Left*) were identified by mass spectrometry. Lane M corresponds to a protein molecular mass marker. (*D*). Western blot detection of HJURP and NPM1 in the e-CENP-A preassembly complex. e-Cenp-A and e-H3.1 complexes were run on 4–12% SDS PAGE and after transfer, the blot was revealed with an antiHJURP, an anti-NPM1, and antiFLAG (to detect e-CENP-A and e-H3.1). (*E*) Silver staining of proteins associated with e-HJURP. The specific partners of e-HJURP were purified by tandem immunoaffinity and identified by mass spectrometry analyses (*Lane 2, Upper*). The identified proteins are indicated on the right. Lane M corresponds to a protein molecular mass marker. (*Lower*) Western blot detection of CENP-A present in the preassembly e-CENP-A complex (*Lane 1*) and e-HJURP complex (*Lane 2*). Both complexes were run on 4–12% SDS PAGE and after transfer, the blot was revealed with an anti-CENP-A antibody. The higher molecular mass of e-CENP-A is due to the presence of the HA-FLAG peptide fused to CENP-A.

prenucleosomal e-CENP-A complex is associated with Tip49a/Tip49b, DEAD (Asp-Glu-Ala-Asp) box polypeptide (DDX3X, DDX5, and DDX17) and some RNA/DNA binding proteins like HnRNPU (Heterogenous nuclear Ribonucleoprotein U) and EIF4A1 (Fig. 1*B*). None of these proteins would be expected to have histone chaperone properties. With this in mind we focused on the two other specific members of the e-CENP-A complex, HJURP, and nucleophosmin (NPM1), two proteins found associated with the CENP-A nucleosome (28). Immunoblotting of the purified complexes evidence additionally that both proteins were present in the e-CENP-A complex, but not in the e-H3.1 complex (Fig. 1*D*). In vitro experiments showed that NPM1 was able to bind equally well to the CENP-A/H4 and H3/H4 tetramers (see *SI Text*), strongly suggesting that it cannot be a bona fide chaperone specific for CENP-A. Consequently, the best candidate for a specific CENP-A chaperone remained HJURP.

If HJURP was a CENP-A chaperone, it should exhibit a cell cycle dependent association with CENP-A chromatin, because the incorporation of CENP-A is cell cycle dependent and its deposition occurs at G1 (35). And indeed, we found that in G1, in contrast to S and M phases, the quasi-totality of HJURP was tightly associated with CENP-A chromatin (*SI Text*).

We next conducted experiments to further confirm the presence of HJURP in the e-CENP-A prenucleosomal complex. We established a stable HeLa cell line expressing a N-terminal FLAG-HA epitope tagged HJURP. Tandem affinity purification of e-HJURP from soluble HeLa nuclear cell extract followed by mass spectrometry analysis identifies CENP-A, H4, NPM1 Tip49a/Tip49b, and RbAp46/RbAp48 as integral components of the human HJURP complex (Fig. 1*E*). Immunoblotting of the purified complex with an anti-CENP-A antibody further confirmed the presence of CENP-A in this complex (Fig. 1*E Lower*).

HJURP Is Required for Loading CENP-A to Centromeres. The above described data suggest strongly that HJURP is a specific CENP-A chaperone. If this was the case, its depletion would result in impediment of CENP-A delivery to the centromeres. To test this, we used HeLa cells where HJURP was depleted by siRNA treatment. Two distinct siRNAs against HJURP (Si1 and Si2) were used to suppress its expression in HeLa cells. A scrambled (Ssi) sequence was used as a negative control. The suppression of HJURP expression was confirmed 72 hr posttransfection by a specific antibody (Fig. 2*B*). Note that the depletion of HJURP was very efficient, because the cells transfected with

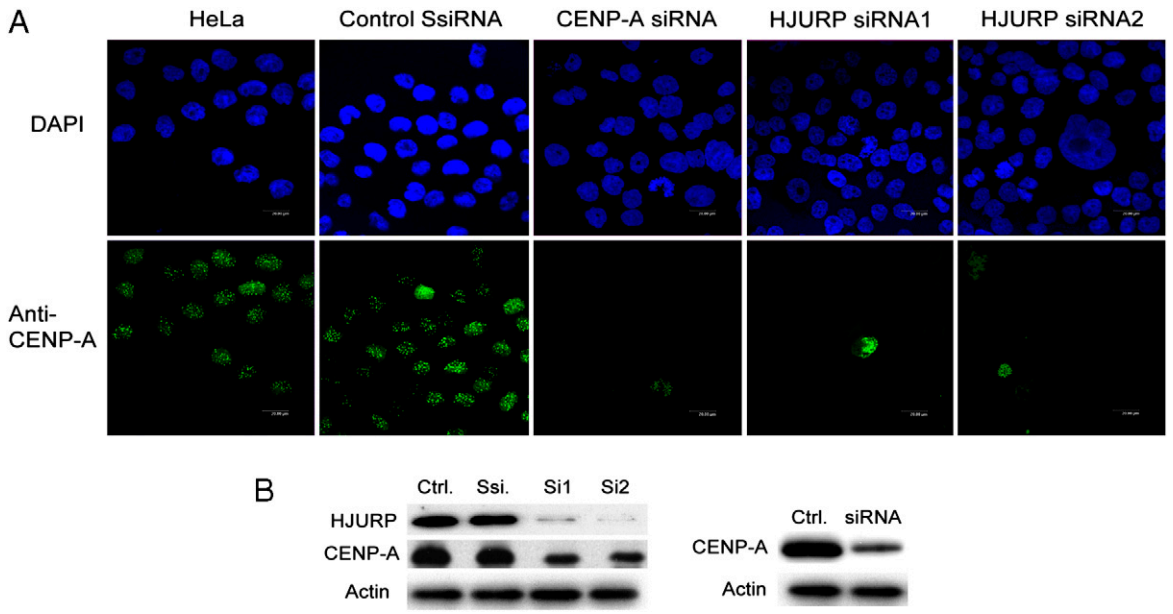

Fig. 2. HJURP is required for CENP-A localization to centromeres. (*A*) The centromeric association of CENP-A is lost in cells depleted of HJURP. HeLa cells were transfected with either scrambled siRNA (SsiRNA) or with CENP-A siRNA or with HJURP siRNA1 and siRNA2. Seventy-two hr posttransfection cells were immunostained (*Green*) with anti-CENP-A antibody and DAPI staining (*Blue*). (*B*) Western blot analysis of the depletion of HJURP and CENP-A upon treatment with siRNA. HeLa cells were transfected with the respective siRNA and 72 hr posttransfection they were harvested, total cell extracts were prepared, and the presence of HJURP and CENP-A was detected by Western blotting using antiHJURP and anti-CENP-A antibodies. The blot was also revealed with an antiactin antibody as a control for equal loading. (*Left*) Depletion of both CENP-A and HJURP upon treatment with siRNA against HJURP. (*Right*) Depletion of CENP-A upon treatment with siRNA against CENP-A. Ctrl, nonsiRNA treated cells; Ssi, cells treated with scrumble siRNA; Si1 and Si2, cells treated with two distinct (Si1 or Si2) siRNAs against HJURP (Si1 and Si2 siRNA were used to suppress the expression of HJURP in the experiments presented in (*A*).

either one of the siRNA probes expressed $\leq 5-10\%$ of the amount of HJURP in the control, treated with scrambled siRNA cells (Fig. 2*B*). Remarkably, immunostaining with anti-CENP-A antibody showed a loss of CENP-A at the centromeres (Fig. 2*A*). Essentially identical results were obtained when CENP-A was depleted by siRNA transfection (Fig. 2*A*). This suggests that in the HJURP depleted cells either the stability of the already incorporated CENP-A or the provision of new CENP-A at centromeres, or both, are compromised. In addition, immunoblotting shows that the depletion of HJURP resulted in decrease of CENP-A, i.e. the amount of CENP-A present in the depleted cell was reduced to at least 50% of its initial level before siRNA treatment (Fig. 2*B*). These data are evidence for a key role of HJURP in the CENP-A loading at the centromeres.

HJURP Recognizes the CENP-A/H4 and Specifically Interacts With It. The unambiguous identification of HJURP within the prenucleosomal CENP-A complex indicates that the two proteins should be closely associated but do not distinguish between direct and indirect binding. If HJURP is a chaperone for CENP-A, a direct interaction between the two proteins should be observed. To address this question, GST-HJURP fusion (GST-HJURP) together with CENP-A/H4 were coexpressed in bacterial cells. Then GST-HJURP, together with the associated proteins, was purified and run on a SDS gel and the gel was stained with coomassie. The data clearly show that GST-HJURP binds stoichiometrically to CENP-A/H4 tetramers (Fig. 3*A*, lane 2). Immunoblotting with an anti-CENP-A antibody confirmed these results (Fig. 3*A Lower*). Note that the GST-HJURP binding to CENP-A/H4 tetramers does not depend on the presence of either DNA or RNA and thus, it involves protein–protein interactions only (*SI Text*).

By using the same assay, we have mapped the specific region of HJURP that interacts with CENP-A. GST fusions of different deletion mutants of HJURP (Fig. 3*B*) were coexpressed with CENP-A/H4 in bacteria and tested for their interactions with CENP-A. Interestingly the N-terminal deletion mutants Δ1 (215–748 AA)

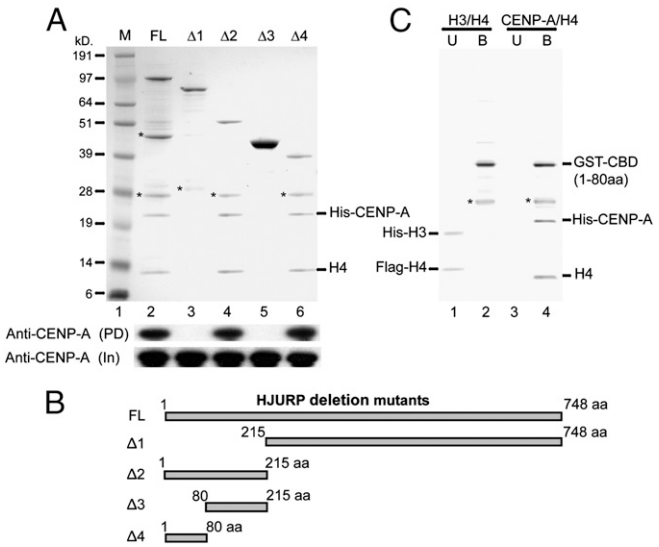

Fig. 3. Identification of a short N-terminal domain of HJURP required for interaction with CENP-A. (*A*) Interaction of full-length and deletion mutants of HJURP with CENP-A. Full-length HJURP and its deletion mutants (Δ1–Δ4) fused to GST, together with CENP-A and H4, were coexpressed and purified from bacteria. The purified material was separated on a SDS-PAGE and stained with coomassie. (*Lower*) Western blot of either the eluted samples (PD) or the input (In) revealed with anti-CENP-A antibody. Note that a short amino acid sequence (1–80 AA) from the N-terminal of the protein recapitulates the main property of the full-length protein and was able to bind stoichiometrically to the CENP-A-H4 tetramer (compare lanes 2, 4, and 6). (*B*) Schematic representation of the different HJURP deletion mutants used as GST-fusions in (*A*). (*C*) The GST-fusion with CBD (CBD of HJURP, 1–80 AA) does not interact with the H3-H4 tetramer. GST-CBD was coexpressed with either H3.1/H4 or with CENP-A/H4. The purified material was run on a SDS-PAGE and stained with coomassie. U, unbound material. B, bound material. The bands designed with stars are degradation products of the fusions.

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and Δ3 (80–215 AA) of HJURP did not interact with CENP-A (Fig. 3*A*), suggesting that the CENP-A binding domain is a part of the N-terminal domain of HJURP. In agreement with this, the two C-terminal deletion mutants Δ2 (1–215 AA) and Δ4 (1–80 AA) showed essentially the same binding capacity as the full-length HJURP (Fig. 3*A*). We conclude that the N-terminal part of the protein corresponding to amino acids 1–80 aa is the CENP-A Binding Domain (CBD) of HJURP.

Using the identified CENP-A binding domain (CBD, 1–80 aa) as a GST-fusion, we next asked whether it interacts also with H3/H4 or it is exclusively specific to CENP-A/H4. Importantly, no binding to histones H3/H4 was detected (Fig. 3*C*). These results evidence that the binding of HJURP to CENP-A/H4 is (*i*) specific, (*ii*) direct, and (*iii*) stoichiometric.

HJURP Interacts with the CATD Domain of CENP-A Through a Highly Conserved TLTY Box. We next aimed to identify the peptide sequence within the CBD of HJURP required for the specific binding to CENP-A. Bioinformatic analysis using SMART (Simple Modular Architecture Research Tool), pBLAST and multiple sequence alignments were conducted. By SMART analysis a coiled-coil (CC) motif consisting of 26 amino acids residues (16–42 AA) was found in the N-terminal part of the protein (Fig. 4*A*). Coiled-coil motifs are known to function as oligomerization domains for a wide variety of proteins and are unlikely to be involved in the interaction with CENP-A (36). By multiple sequence alignment analysis of HJURP, we identified a novel box TLTY that it is highly conserved across vertebrate from human (*Homo sapiens*) to chicken (*Gallus gallus*) (Fig. 4*A*). To explore the importance of this novel TLTY box for CENP-A interaction, we deleted this box from the CENP-A binding domain of HJURP (CBD, 1–80 aa) and coexpressed this mutant with CENP-A/H4 in bacteria. The analysis of the binding was carried out as described above. We could not detect binding of the TLTY deleted mutant CBD (Δ-TLTY) to CENP-A/H4 (Fig. 4*B* compare lane 1 with lanes 2–4) whereas the CBD showed a stoichoimetric interaction (Fig. 4*B*, lane 1). Immunoblotting analysis with an anti-CENP-A antibody confirmed this result and only detected a trace amount of CENP-A interacting with CBD (Δ-TLTY) (Fig. 4*B*, compare lane 1 with lanes 2–4). We concluded that the TLTY box is required for the interaction of HJURP with CENP-A.

The next question we addressed was whether the CBD of HJURP, containing the conserved TLTY box, can specifically interact with the previously dentified CENP-A targeting Domain (CATD) (9). The CATD, consisting of the loop1 and helix 2 of the histone fold domain, is required for centromeric loading of CENP-A (9, 11). The substitution of CATD into H3.1 led to a $H3^{CATD}$ chimera that recapitulated the functional properties of CENP-A (9, 11). These findings suggested that any specific histone chaperone for CENP-A deposition should also bind to the CATD of CENP-A. To test this, GST-CBD fusion together with $H3^{CATD}$/H4 were coexpressed in bacteria and their association analyzed (Fig. 4*C*). GST-CBD was found associated in stoichiometric ratio with $H3^{CATD}$/H4 as it was with CENP-A/H4 (Fig. 4*C*). We conclude that HJURP binds to CENP-A through its CATD domain and this interaction is likely to occur via the TLTY box of CBD.

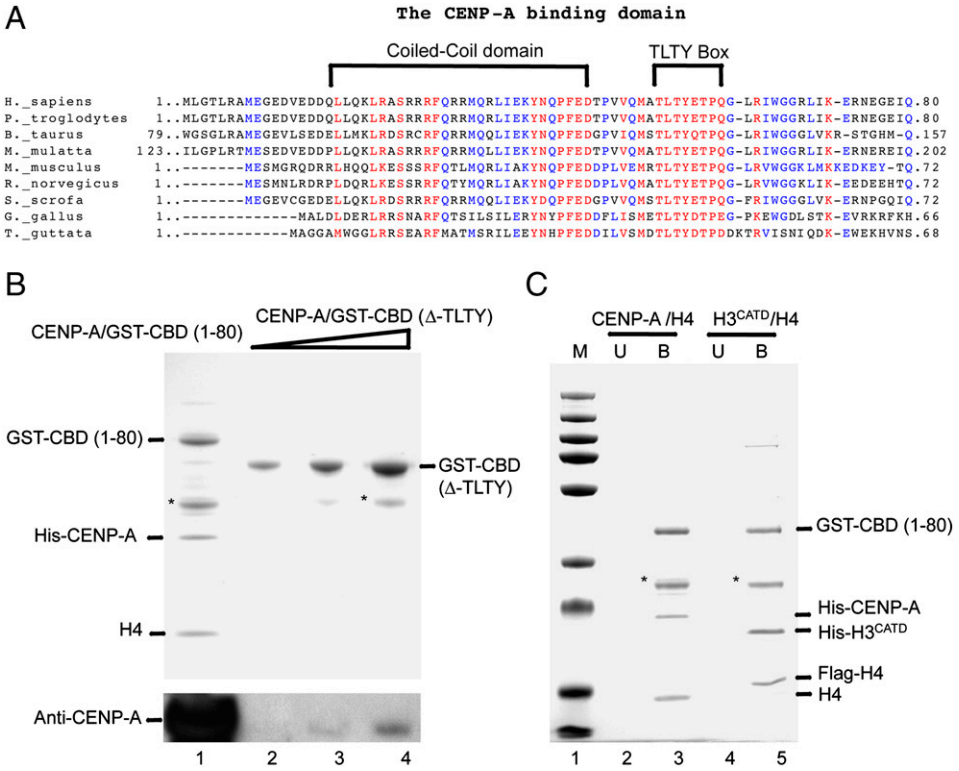

Fig. 4. The identified novel TLTY box within the HJURP vertebrate homologs is essential for the interaction with CENP-A. (*A*) Identification of a conserved coiled-coil domain and a novel TLTY box in higher-eukaryote HJURP homologs. The sequence alignments for the indicated species are shown. Alignments were generated by MultAlin. The brackets highlight the coiled-coil and the TLTY motifs, which are conserved from birds to human. (*B*) The TLTY box is essential for the interaction of HJURP with CENP-A. The TLTY box of HJURP was deleted from the minimal CBD and coexpressed as a GST-fusion [GST-CBD (Δ-TLTY)] in bacteria together with CENP-A and H4. Increasing amounts (*Lanes 2–4*) of the eluted from the gluthatione column GST-CBD (Δ-TLTY) complex was analyzed by SDS PAGE. GST-CBD was used as a positive control (*Lane 1*). (*Lower*) Western blot revealed with anti-CENP-A antibody for the respective samples. (*C*) The CBD of HJURP recognizes and binds to CATD, the CENP-A centromere targeting domain. GST-CBD was coexpressed with either $H3^{CATD}$/H4 or CENP-A/H4 in bacteria, the GST-CBD complexes were purified as described above, run on SDS PAGE, and stained with coomassie. U, unbound material. B, bound material. M, protein molecular mass markers. (*), a degradation product of GST-CBD.

HJURP Stimulates CENP-A Deposition on DNA. If HJURP is a bona fide CENP-A chaperone it should be able to deposit CENP-A/H4 to DNA and to assemble a CENP-A/H4 tetrasome. We have approached this problem as follows. Labeled 360 bp alpha satellite DNA was circularized under conditions that generated one negative supercoil corresponding to topoisomer −1 (37). Negatively supercoiled DNA was then incubated with an increasing amount of CENP-A/H4 histones in the absence or presence of equimolar amount of HJURP and then the deposition of histones onto DNA was analyzed by EMSA (Fig. 5*A*). In the absence of HJURP, very low amount of CENP-A/H4 tetramer deposition was observed (Fig. 5, lanes 3–5). In contrast, the presence of HJURP strongly facilitates the CENP-A/H4 tetramer deposition and enhanced (up to 15-fold at low histone concentration and up to 3-fold at high histone concentration) the assembly of the CENP-A/H4 tetrasome (Fig. 5*A*, lanes 6–8 and Fig. 5*B*). The HJURP-mediated deposition of CENP-A/H4 tetramers on DNA was also at least as efficient as that obtained by the salt dialysis method (Fig. 5, compare lane 2 with lane 8 and Fig. 5*B*). These data illustrate the ability of HJURP to assemble CENP-A variant particles.

Discussion

In this work we have identified by affinity purification and mass spectrometry HJURP as a major partner in the CENP-A nuclear soluble complex. Depletion of HJURP by siRNA affected the expression of CENP-A and impaired its deposition at centromeres. Immunoprecipitation experiments show that HJURP is associated with CENP-A chromatin in a cell cycle dependent manner, concomitant with the new CENP-A deposition. These results are in complete agreement with the recently reported experiments, where very similar approaches were used (38, 39). All these data strongly suggest that HJURP is a specific CENP-A chaperone, required for the cell cycle deposition of CENP-A in chromatin.

In addition to these in vivo experiments, we have performed a series of in vitro studies. This has allowed the identification and characterization of a conserved HJURP short N-terminal domain, responsible for the specific and stoichiometric binding to the CENP-A/H4 complex. We found that a TLTY box within this domain was required for the binding. Interestingly, the TLTY box was found to bind to the previously identified CENP-A targeting Domain (CATD) (9).

The recently identified yeast CenH3 chaperone Scm3 (22–26) is likely to be a distant ortholog of HJURP. Scm3 is required for kinetochore assembly, conserved across fungi, and displays a remarkable variation in protein size (40). Though Scm3 has extensively diversified in course of fungal evolution to make different types of potential DNA contacts via its C-terminal regions, it is likely to mediate a conserved interaction with the CenH3-H4 complex via its N-terminal Scm3 domain. Indeed, recent bioinformatics analysis established some similarity between fungal Scm3 domain and mammalian HJURP N-terminal domain (41). This result is in agreement with our data implicating the N-terminal domain of HJURP in CENP-A binding.

Taken together our and the reported data (38, 39) demonstrate that HJURP is a key chaperone responsible for the targeting and deposition of newly synthesized CENP-A at centromeres. Our in

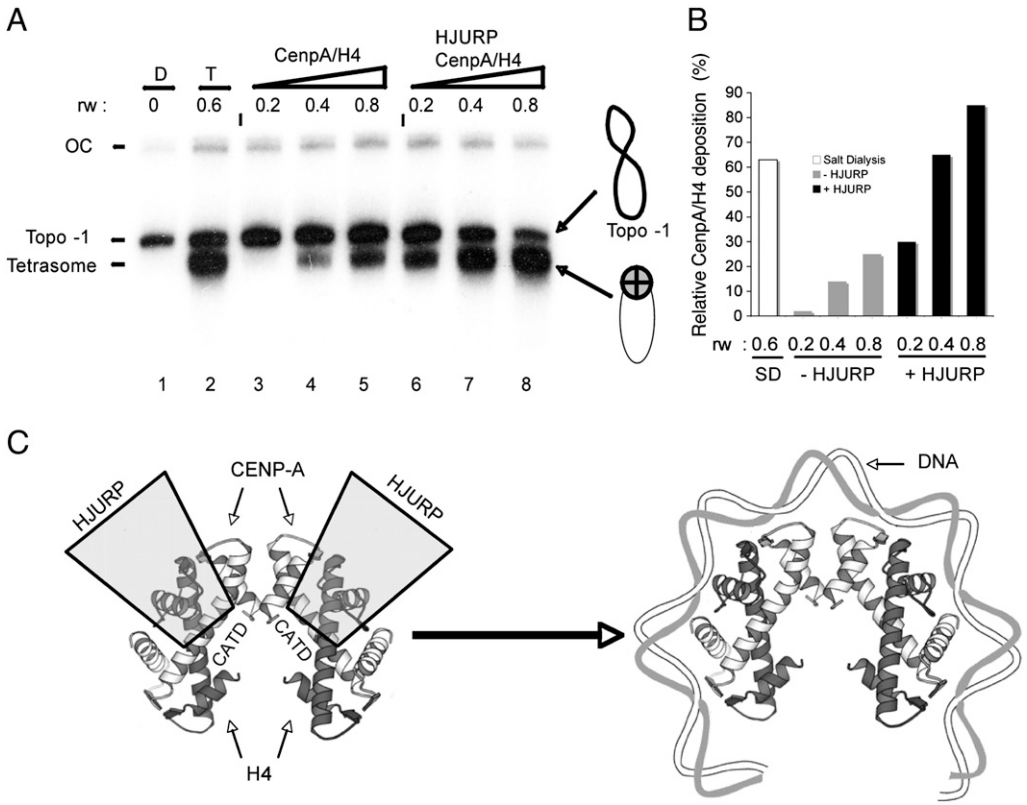

Fig. 5. HJURP is able to deposit efficiently CENP-A/H4 tetramer on DNA. (*A*) Negatively supercoiled human alpha-satellite DNA corresponding to topoisomer −1 (*Lane 1*, *D*) was incubated with increasing amount of CENP-A/H4 (at the indicated histone/DNA ratio, rw) in the absence (*Lanes 3–5*) or presence (*Lanes 6–8*) of equimolar (to the tetramers) amount of HJURP. The reaction was carried out for 30 min at 37 °C. The reaction products were then analyzed on native 4.5% polyacrylamide gel. (*Lane 1*) topoisomer-1 DNA; (*Lane 2*) reconstituted CENP-A/H4 tetrasomes on topoisomer −1 by salt dialysis using the indicated histone/DNA ratio (rw). (*Right*) Drawings showing the naked topoisomer −1 DNA and the CENP-A/H4 tetrasome. The positions of the naked topoisomer −1 DNA and the salt dialysis reconstituted tetrasome are also indicated. (*B*) Quantification of the relative amount of Cenp-A/H4 tetrameres deposited by HJURP in Fig. 5*A*. The tetrasome/DNA ratio was quantified using ImageJ software. S.D. indicates tetramers assembled by salt dialysis. (*C*) Model of CENP-A deposition. Two molecules of HJURP dimerize through their coiled-coil domains and bind, via their TLTY boxes, the CATD of two molecules of CENP-A (*Left*).

vitro experiments suggest a model for HJURP binding to the CENP-A/H4 complex (Fig. 5*C*). According to the model, two molecules of HJURP are supposed to dimerize through their coiled-coil domains and to bind, via the TLTY box, two dimers of CENP-A/H4. This would constrain the CENP-A/H4 tetramer in a specific conformation that facilitates its deposition to DNA and allows the assembly of the CENP-A/H4 tetrasome.

Materials and Methods

Purification of e-CENP-A and e-H3.1 Complexes. Prenucleosomal CENP-A and H3.1 complexes were purified from soluble nuclear extracts prepared from stable HeLa cell lines expressing either CENP-A or H3.1 proteins fused to C-terminal FLAG and HA epitope tags (e-CENP-A/e-H3.1). A tandem affinity purification protocol on antiFlag antibody-conjugated agarose followed by antiHA purification and peptide elution was used (29).

Immunofluorescence. Immunofluorescence was performed using standard procedures. Anti-CENP-A was used at 1:200 dilution, the secondary antibody used is a goat antirabbit IgG coupled to Alexa Fluor 488 (Molecular Probes) at 1:400 dilution. Rat antiHA antibody (Roche) was used at 1:400 dilution; the secondary antibody used is a goat antirat IgG coupled to Alexa Fluor 488 (Molecular Probes) at 1:400 dilution.

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Supporting Information

Shuaib et al. 10.1073/pnas.0913709107

SI Text

Materials and Methods. ***Plasmid construction.*** Full-length human cDNA clones of CENP-A (IMAGE 3626578), HJURP (IMAGE 2820741), and NPM1 (IMAGE 4276604) were purchased from Invitrogen. Human histone H3.1 was PCR amplified by using Vent-DNA polymerase from HeLa cells genomic DNA. The complete coding sequence from each clone was subcloned into the XhoI-NotI sites of the pREV-HTF retroviral vector (29) or pGEX-5X.1 vector (Amersham) using standard techniques.

Mass spectrometry. The peptide mixtures obtained from tryptic digestion of the bands were analyzed by an ion-trap mass spectrometer (Thermo LTQ-XL + ETD) equipped with a nanoelectrospray source. The interpretation of the data was performed with the Proteom Discoverer software package.

Antibodies. Polyclonal antiHJURP produced in rabbit (HPA008436, SIGMA), Polyclonal anti-CENP-A produced in rabbit (07–574, Millipore), and Monoclonal anti-NPM1 antibody produced in mouse (WH0004869M1, SIGMA) were used in the study.

Histones. Human histone H3.1 and H4 DNA sequences were PCR-amplified from HeLa cells genomic DNA. $H3^{CATD}$ is identical to the sequence published in ref. 9 and was constructed by megaprime PCR. All the histones were cloned in a homemade bicistronic pET28b vector. H3.1 and $H3^{CATD}$ were cloned at the NdeI-BamHI sites of pET28b in frame with an N-terminal His tag, whereas FLAG-tagged H4 was cloned at the EcoRI-NotI sites. Human CENP-A, with optimized codon usage for expression in bacteria, was PCR-amplified from pHCE-CENP-A vector [a kind gift of Yoda (42)] and cloned at the Nde1-BamH1 sites of pET28b in frame with an N-terminal His tag. The CENP-A cDNA sequence was immediatelly followed by a ribosome binding site containing a nontagged human histone H4 sequence cloned at EcoRI-NotI sites. Histones were expressed in BL21-CodonPlus-RIL (Stratagene) and purified using standard methods.

Purification of the expressed in bacteria GST-fusion complexes. GST-fusion HJURP and its deletion mutants were coexpressed with the bicistronic CENP-A/H4 or H3/H4 in *Escherichia coli* strain BL21-CodonPlus-RIL-pLysS (Stratagene) at 16 °C. The soluble proteins were purified on glutathione Sepharose 4B beads (Amersham) by standard methods.

siRNA-mediated silencing. HeLa cells in exponential growth were seeded onto six-well plates or Labteks and transfected with 5 nM of HJURP siRNA1 (5′ CUACUGGGCUCAACUGCAA-3′), HJURP siRNA2 (5′ UGGAGUGUCUACAGAUAAA-3′), CENP-A siRNA (5′-CACAGUCGGCGGAGACAAGTT-3′) or control siRNA (5′-CAUGUCAUGUUCACAUCUCTT-3′) using HiPerFect Transfection Reagent (QIAGEN). Forty-eight to seventy-two hr posttransfection, cells were either assayed for HJURP and CENP-A silencing by immunoblotting or for CENP-A deposition at centromeres by immunofluorescence. Synthetic siRNAs were purchased from Dharmacon.

Histone deposition assay. Assay of histone deposition in the presence of the histone chaperone HJURP was performed using a negatively supercoiled DNA topoisomer −1 prepared from the 360-bp human alpha satellite DNA (37). CENP-A/H4 tetramers were mixed or not with equimolar amount of HJURP and incubated with DNA for 30 min at room temperature. Control tetrasomes were assembled on circular DNA according to the "salt jump" method as described in ref. 37.

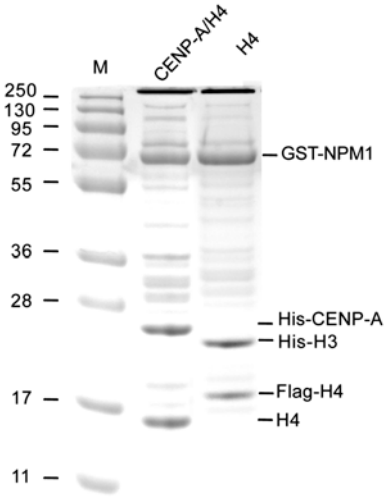

Fig. S1. NPM1 interacts equally well with CENP-A/H4 and H3.1/H4. GST-NPM1 fusion, together with either CENP-A/H4 (CENP-A was histidine tagged at its N terminus) or H3.1/H4 [H3.1 and H4 were either histidine (H3.1) or FLAG (H4) tagged at their N terminus], were coexpressed in bacteria and purified on a glutathione column. The eluted material was then separated on a SDS-PAGE and the gel was Coomassie stained. The positions of GST-NPM1, His-CENP-A, His-H3, FLAG-H4, and H4 are indicated in the right part of the figure. M, molecular mass marker. The masses of the protein markers are indicated.

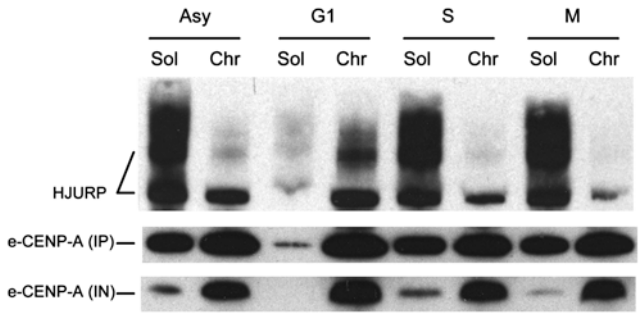

Fig. S2. HJURP centromeric localization is cell cycle regulated. HeLa cell lines stably expressing e-CENP-A were synchronized at different stages of the cell cycle (G1, S, and M phase) by thymidine-nocodazole and double-thymidine treatment. Identical amounts of nuclei originating from either the asynchronous (Asy) or the synchronized cells were used to prepare soluble nuclear extract and chromatin. e-CenpA was then pulled down by an anti-FLAG antibody (IP) from the soluble and the chromatin nuclear fractions and assessed by Western blot analysis for the presence of HJURP (HJURP). Note that the total amount of nonchromatin associated CENP-A in the input (IN) of the soluble nuclear fraction was very low at G1 compared to that from the other phases of the cell cycle.

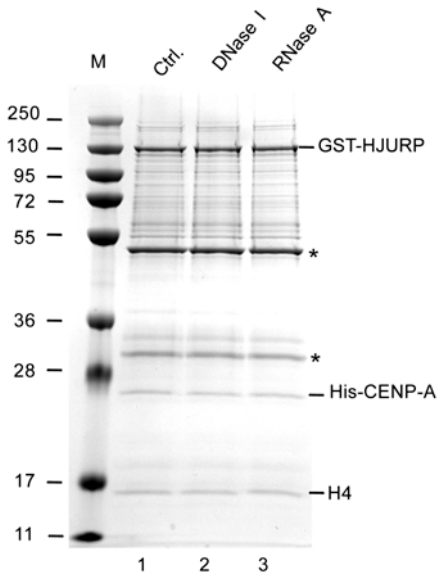

Fig. S3. Interaction of HJURP with CENP-A is direct and not mediated by DNA or RNA components. GST-HJURP together with CENP-A and H4 was coexpressed in bacteria. The bacterial protein extract was incubated with glutathione sepharose beads and the bound material extensively washed and treated with 10 μg/mL of DNase I or RNase A for 30 min at 25 °C. The bound proteins were then extensively washed and eluted with reduced glutathione. The eluted material was then separated on a SDS-PAGE and stained with Coomassie. The positions of GST-HJURP, His-CENP-A, and H4 are indicated in the right part of the figure. M, molecular mass marker. The masses of the protein markers are indicated in the left part of the figure. Ctrl, untreated material. (*), a degradation product of GST-HJURP.

Alpha satellite DNA

```
    |   10      |   20      |   30      |   40      |   50      |   60      |   70      |   80      |   90      |   100

  1 AATTCTGCAA GTGGATATAC AGACATCCTT GAAGCTTTCG TTACAAACGG  GATTTAATCA TATTCTGCTA TACAGAAGAA TTCTCGGAAA CGTCCTTGTG 100
  1 TTAAGACGTT CACCTATATG TCTGTAGGAA CTTCGAAAGC AATGTTTGCC  CTAAATTAGT ATAAGACGAT ATGTCTTCTT AAGAGCCTTT GCAGGAACAC 100

101 TTGTGTGTTT TCAACTCACA GAGTTGAACG ATGCTTTACA CAGAGTAGGC  TTGAAACACT CTTGTTGTGG AATTTGCAAG TGGAGATTTC ATCCGCTTTG 200
101 AACACACAAA AGTTGAGTGT CTCAACTTGC TACGAAATGT GTCTCATCCG  AACTTTGTGA GAACAACACC TTAAACGTTC ACCTCTAAAG TAGGCGAAAC 200

201 AGGTCAATGG TAGAATAGGA AATATCTTCC TATAGAAACT AGACAGAATG  ATTCTCAGAA ACTCCTTTTT GATGTGTGCA TTCAACTCAC AGAGTTTAAC 300
201 TCCAGTTACC ATCTTATCCT TTATAGAAGG ATATCTTTGA TCTGTCTTAC  TAAGAGTCTT TGAGGAAAAA CTACACACGT AAGTTGAGTG TCTCAAATTG 300

301 CTTTCTTTTC ATAGAGCACT TAGGAAACAC TCTGTTTGTA AAGTCTGCAA  GTGGATATTG                                             360
301 GAAAGAAAAG TATCTCGTGA ATCCTTTGTG AGACAAACAT TTCAGACGTT  CACCTATAAC                                             360

    |   10      |   20      |   30      |   40      |   50      |   60      |   70      |   80      |   90      |   100
```

Fig. S4. DNA sequence of the human alpha satellite DNA amplified from HeLa cells and used in Fig. 5*A*.

Figures

CHAPTER 3
DISCUSSION

3.1 HJURP is a specific Chaperone for CENP-A

The maintenance of epigenetic centromere identity in all eukaryotes requires deposition of new CENP-A at centromeric chromatin. In this study, we identified HJURP (Holliday Junction Recognition Protein) as a unique chaperone required for CENP-A deposition at centromeres, by affinity purification and mass spectrometry analysis of protein complexes associated with pre-deposited CENP-A. HJURP is also known as hFLEG1 (Fetal liver-expressing gene 1 protein), FAKTS (14-3-3-associated AKT substrate), or URLC9 (Up-regulated in lung cancer 9).

The chaperones responsible for deposition of H3.1 and H3.3 include CAF-1 and HIRA/DAXX respectively, were not detected in the preassembly CENP-A complex. Similarly, HJURP was not detected in H3.1 and H3.3 deposition complexes. In addition, the affinity purification and mass spectrometry analysis of HJURP complex revealed the occurrence of CENP-A but not other histone H3 variants. This clearly indicates that all histone H3 variants are deposited by different histone chaperones at distinct chromatin regions. HJURP was initially suggested to play an important role in double strand break repair and shown to interact with Holliday junction like DNA structure in vitro [379]. Moreover, mismatch repair proteins hMSH5 and NBS1 were also reported to interact with HJURP and with our preassembly CENP-A complex containing Ku proteins (Ku70 and Ku80), which bind to double strand DNA break. It can be suggested that in addition to centromeric deposition of CENP-A, HJURP may also deposits CENP-A to DNA repair sites. To this end, a recent study in human and mouse cells reported that CENP-A is rapidly recruited to double-stranded DNA breaks and this recruitment depends on CATD domain of CENP-A [380]. This report anticipates the role of CENP-A in DNA repair process, which confirmed the initial observation of HJURP recruitment to damaged DNA [379]. CENP-A accumulation in response to DNA breaks suggests a possible mechanism for establishment of neocentromeres.

HJURP directly interacts with CENP-A and its level rises during the time of CENP-A synthesis and deposition in HeLa cells. Our immunoprecipitation

experiments show that HJURP is associated with CENP-A chromatin in a cell cycle dependent manner, concomitant with the new CENP-A deposition. This cell cycle regulated pattern of HJURP distribution further attests its specific requirement for CENP-A deposition. These results are in complete agreement with the recently reported experiments, where very similar approaches were used [11, 12]. HJURP transiently localizes to centromere during late telophase/early G1 [11, 12] matching to the time when new CENP-A deposition starts at centromeric DNA [358, 359]. In human, incorporation of CENP-A into centromeric chromatin is not coupled with DNA replication [381], resulting in CENP-A dilution on centromeres, before mitosis. The interesting point is that the “dilution” of CENP-A in daughter centromeres during S-phase and its subsequent restoration at the next G1-phase may be required for faithful cell division.

Down regulation of HJURP by specific siRNA resulted in a dramatic decrease of centromere associated CENP-A, which confirmed the central role of HJURP in centromeric localization of CENP-A. However, the loss of HJURP results in some reduction of total cellular CENP-A levels. This observation suggests that the interaction of HJURP with CENP-A prevents its degradation. Previous studies in other organisms showed that the over-expressed/mislocalized CENP-A is degraded by proteolytic pathways [354, 355]. Recently, two independent studies in yeast identified Psh1, an E3 ubiquitin ligase, which specifically recognizes the CATD domain of Cse4 (yeast CENP-A homolog) and reduces its misincorporation at non-centromeric regions [356, 357]. Interestingly, they also show that the binding of Scm3 (a yeast homolog of HJURP) protects Cse4 from degradation [357]. HJURP/Scm3 interacts with the CATD domain of CENP-A/Cse4. The similar interaction mechanism of Scm3 and Psh1 with Cse4 suggests a potential antagonistic relationship between Scm3 and Psh1 in regulating Cse4 stability. Dunleavy et al. [11] showed that the over-expressed GFP-CENP-A in HJURP depleted cells are more stable than endogenous CENP-A but failed to localize to centromeres. This clearly indicates that HJURP not only promotes CENP-A stability but also targets CENP-A to centromeres. In fact, our in vitro deposition assay of purified CENP-A-H4 on naked alpha-satellite DNA in the presence and absence of recombinant HJURP proteins shows that HJURP strongly facilitates CENP-A-H4 deposition on DNA. Collectively these observations suggest that HJURP as a specific deposition factor for CENP-A.

Is the essential functional role of HJURP in CENP-A deposition conserved in other organisms? Sequence analysis of HJURP identified two specific motifs at the N-terminal tail. A coiled-coil motif consisting of 26 amino acids, and a novel motif that we called TLTY box. The N-terminal TLTY box is highly conserved across the vertebrate from human to chicken. This shows that sequence and functional homologs of HJURP may exist in several different organisms. In this regard, a yeast CENP-A^{CenH3} chaperone Scm3 [374-378] is likely to be a distant ortholog of HJURP. Scm3 is required for kinetochore assembly, conserved across fungi and displays a remarkable variation in protein size. Indeed, recent bioinformatics analysis established some similarity between fungal Scm3 domain and mammalian HJURP N-terminal domain [382]. So far, HJURP homologs have been identified in fungi and vertebrates but not in plants or invertebrates. Therefore, the function of HJURP is replaced by other chaperones in other species. For example in flies, p55/RbAp48 [13] and CAL-1 [383], have been shown to be involved in CENP-A deposition.

In addition to HJURP, we also identified NPM1 (nucleophosmin) as a specific partner of CENP-A complex. NPM1 is a nucleolar phosphoprotein, which acts as a histone chaperone [8] for both H3-H4 and H2A-H2B family of histones, as well as it plays role in other important cellular process like chromosome segregation and DNA repair [9, 10]. However, NPM1 cannot compensate the dominant effect of HJURP depletion and its down-regulation, which did not significantly affect CENP-A centromeric localization [11, 12]. In fact, our *in vitro* interaction study shows that NPM1 binds equally well to the CENP-A/H4 and H3/H4 histones. These observations strongly suggest that NPM1 cannot be a specific chaperone for CENP-A, but it may play additional roles during CENP-A assembly. For example, in *Drosophila*, NPM1 like protein assist in the ATP-dependent chromatin assembly *in vitro* [384]. H3.3 deposition in Drosophila, depends on both HIRA and CHD1 ATPase. The CHD1 and other potential ATPases like RuvBL1 and hSNF2H were found associated with CENP-A chromatin in our unpublished data and they were also reported by other studies in CENP-A complex [12]. Moreover, we also isolated the other general histone chaperones RbAp46 and RbAp48 in both H3.1 and CENP-A complexes. RbAp48 is the subunit of CAF-1 complex and is also present in several other complexes that are involved in chromatin dynamics and transcription silencing. In *Drosophila*, CAF-1 subunit RbAp48 directly binds CENP-A homolog CID [13], but in

human, a direct interaction of RbAp46/RbAp48 with CENP-A has not been reported. However, RbAp46/RbAp48 and hMIS18α/β proteins are required for priming CENP-A deposition [372, 373]. Interestingly, HJURP complex also contains RbAp46/RbAp48 and Dunleavy *et al*. [11] observed that the down-regulation of these proteins dramatically reduced the level of HJURP in HeLa cells. This might suggests that RbAp46/48 promote CENP-A localization indirectly through stabilization of HJURP by mechanism, which is unknown till date. RbAp46/RbAp48 also interacts with histone H4 [13], and this raises another possibility that depletion of both RbAp46 and RbAp48 affects CENP-A localization indirectly *via* interaction with H4. Thus, we conclude that HJURP is a specific chaperone for CENP-A deposition at centromeres, whereas NPM1 and RbAp46/48 chaperones play supplementary role during HJURP mediated assembly of CENP-A.

3.2 Mechanism of CENP-A deposition and maintenance at centromeres

The deposition of histones at chromatin involves various steps, including histone synthesis in cytoplasm, modifications in cytoplasm or nucleus, import into the nucleus, and nucleosome assembly, remodeling or exchange. In this context, how after synthesis CENP-A is transported into the nucleus, specifically delivered to centromeric chromatin and maintained on centromeres during cell cycle are the key issues to be discussed. In mammals, CENP-A synthesis occurs during G2-phase [358] and then, it assembles into a prenucleosomal complex with H4 and HJURP (CENP-A/H4-HJURP), but is not deposited on centromeric DNA until G1 phase. The centromeric DNA replicates during S phase, thus diluting the amount of previously loaded CENP-A to half. Cells progress through mitosis with half CENP-A on daughter chromosomes. New CENP-A deposition starts late in mitosis and continues to early G1-phase [358, 359]. Thus, the process of establishment of new CENP-A chromatin at centromeres is regulated by different cell cycle dependent mechanisms.

3.2.1 Priming centromeric chromatin for CENP-A loading

The factors responsible for priming centromeres before CENP-A deposition include hMis18α/β, Mis18BP1 (KNL-2 is the *C. elegans* homolog of Mis18BP1) and RbAp46/48 [372, 373]. The Mis18 complex (Mis18 and Mis18BP1) is transiently localized to centromeres during late mitosis just before CENP-A loading. In addition to this, the down-regulation of Mis18 in human cells prevented incorporation of new CENP-A into centromeres [373]. Human chromatin assembly proteins RbAp46/48 (Mis16 yeast homolog), which are the interacting partners of CENP-A and HJURP prenucleosomal complexes, they are also required for centromeric localization of CENP-A [372]. Recent study in Xenopus egg, has reported that CENP-C can bind and recruit Mis18BP1 (and thus Mis18) to centromeres for CENP-A assembly. Interestingly, the Mis18 complex is not highly conserved, *Drosophila* lacks both Mis18 and Mis18BP1. *C. elegans* contains only Mis18BP1/KNL-2 but not Mis18, and Mis18BP1 is absent in fission yeast [385]. The exact mechanism of centromere priming is not yet clear. The direct interaction of CENP-A with priming proteins (hMis18α/β, Mis18BP1 and RbAp46/48) has not been reported. While there is some evidence that these proteins prime centromeres through regulation of centromeric nucleosome acetylating status. Further investigations are needed for understanding the mechanisms of centromere priming and changes in chromatin status at centromeres by Mis18 complex.

3.2.2 HJURP mediated deposition of CENP-A

As described above, a specific chaperone required for CENP-A deposition and stability is the Holliday Junction Recognition Protein (HJURP) in mammals, and Scm3 (a distant relative of HJURP) in yeast.

The mechanism by which HJURP distinguishes CENP-A from bulk histones is the first critical step for HJURP mediated deposition of CENP-A at centromeres. Our *in vitro* co-expression assay using different deletion mutants of HJURP showed that the N-terminal part of the protein corresponding to amino acids 1-80 aa is necessary and sufficient for the interaction with recombinant CENP-A/H4 and we named it CBD (CENP-A Binding Domain) of HJURP. This N-terminal domain of HJURP shows

similarity to a short region in Scm3 [382], which is required for CENP-A^{Cse4} deposition in budding and fission yeasts [374-378]. Moreover, HJURP (CBD) specifically recognizes and binds to the previously identified CATD domain of CENP-A [12]. Three recent structural analyses of CBD domains of HJURP [386], Scm3 (*Saccharomyces cerevisiae*) [387] and Scm3 (*Kluyveromyces lactis*) [388] with CENP-A-H4 and Cse4-H4 complexes further confirmed the recognition of CENP-A (CATD) by CBD. According to these structures the critical recognition of CENP-A/Cse4 occurs in the α2 helix of CATD region. The CENP-A specific residues (Q89, H104, L112) in the α2 helix and part of α1-helix interact with the residues of HJURP [386]. In contrast, Cse4 recognition residues (M181, M184, A189, S190) are present in the N-terminal region of α2-helix, which are sufficient and necessary for Scm3 interaction [387, 388]. The proposed recognition residues in the yeast Cse4 are different from mammalian CENP-A residues. Surprisingly, Ser68 residue which is located outside the CATD domain of CENP-A, was proposed to provide specificity for HJURP interaction, while the corresponding residue Gln68 in H3 prevent HJURP binding [386]. They showed that CENP-A substituted with S68Q does not bind HJURP, while H3.1 with the Q68S mutation can interact with CENP-A [386]. However, *in vivo* experiments are necessary to be performed to validate this finding. A very recent study using cell-based, biochemical and biophysical strategies showed that Ser68 is neither necessary nor sufficient for HJURP recognition and subsequent deposition into chromatin [389]. Interestingly, they identified six exposed residues within the CATD of CENP-A that are important for HJURP recognition. These residues include one on L1 (Asn85), three on the N-terminal portion of the α2-helix (Ala88, Gln89 and Leu92), and two on the C-terminal part of the α2-helix (His104 and Leu112) [389].

According to our *in vitro* deposition assay, HJURP is able to facilitate the efficient deposition of CENP-A/H4 tetramer on naked DNA to make tetrasome (CENP-A/H4 tetramer wrapped by DNA). However, it is not clear whether HJURP binds/deposits CENP-A-H4 dimer or tetramer. The recent crystal structures of HJURP^{scm3}-CENP-A^{cse4}-H4 complex revealed that HJURP/Scm3 binds a CENP-A/Cse4-H4 heterodimer and prevents tetramer formation [386, 388]. Thus, both structures are of a heterotrimer containing one copy each of HJURP/Scm3, CENP-A/Cse4, and H4. This suggests either a stepwise assembly of two CENP-A-H4

dimers by HJURP at centromeres followed by the incorporation of two H2A-H2B dimers, or a one step assembly of a single CENP-A-H4-H2A-H2B hetetotypic tetrasome. Indeed, heterotypic tetrasomes have been reported to exist in *Drosophila* and human interphase cells [390, 391] and we also found in HeLa cells, that CENP-A reside in a soluble complex containing HJURP and H2A-H2B. The presence of H2A and H2B in the preassembly soluble complex of CENP-A, suggests that the deposition of H2A-H2B unit occurs simultaneously with CENP-A deposition at centromeres. In contrast, the purification of soluble CENP-A complex by other studies revealed the presence of only H4 and HJURP, but not H2A and H2B [11, 12]. The difference of these results from ours might be due to the differences in the purification conditions, used by the other studies. As we know that CENP-A/H4 exists as a heterotetramer in solution with two copies of CENP-A [345] and they can also form DNA free octameric complex with H2A-H2B histones. Thus, we cannot exclude the possibility that immunoprecipitation of CENP-A might contain two pools of histones “HJURP bound (CENP-A/H4 dimer)” and “free tetrameric complex (CENP-A-H4/H2A-H2B)”. In fact, the immunoprecipitation of HJURP contains only H4 and CENP-A, which is consistent with the current structural data. However, the exact composition of centromeric nucleosomes is still under debate.

To get more insight into the mechanism of CENP-A deposition it is important to understand how HJURP transfers CENP-A to DNA. HJURP and its yeast ortholog Scm3 compete with DNA for non-specific binding to the histone complex [386, 388] and thereby, promote the nucleosome assembly. Interestingly, the retention of Scm3 on centromeric DNA is mediated by distinct DNA binding domain of Scm3 and doesn’t depend on Scm3 and Cse4-H4 interaction [387]. Whether the same is true for HJURP is not known.

Presently it is not well clear how the HJURP complex, carrying newly synthesized CENP-A, is specifically recruited to centromeres. The specific targeting of CENP-A-H4/HJURP complex is most likely occurred by an interaction with other molecular factors (proteins or RNAs) that recognize the centromeric regions. A recent study, using synthetic human artificial chromosome, stresses the importance of alpha-satellite DNA transcription for HJURP recruitment and centromeric CENP-A assembly [392]. It can be suggested that the centromeric transcripts may guide

HJURP-CENP-A complex to centromeres. Further studies are needed to test this hypothesis.

3.2.3 Maturation/ Stabilization of CENP-A chromatin

The next step after deposition is the stablization of CENP-A containing chromatin on centromeres. The candidate factors involved in maintenance of CENP-A chromatin include ATP-dependent remodeling and spacing factor (RSF) complex subunits Rsf-1 and SNF2h, along with MgcRac-GAP and small GTPase Cdc42 [393, 394]. Obuse *et al* [395] first identified the RSF complex in CENP-A immunoprecipitates from HeLa cells. Subsequently, it was confirmed that RSF is only transiently associated with centromeres, accumulating on CENP-A chromatin in mid-G1 [393], after CENP-A loading. Morover, RSF complex interacts with CENP-A oligonucleosomes in human cells [393] as well as in chicken cells. The knockdown of RSF in HeLa cells decreased the level of centromeric associated CENP-A by washing with high salt buffer [393]. Based on these observations, it was suggested that CENP-A loading takes place in two steps during the G1 phase of the cell cycle. Firstly a weak association of CENP-A to the centromeric DNA is mediated by HJURP, followed by RSF remodeling to enhance CENP-A stability at the centromeres. The remodeling factors can also contribute to CENP-A assembly by evicting H3 histones.

In addition to RSF, the other ATP-dependent remodeling factors such as Chd1 and both subunits of the FACT complex namely SSRP1 and SPT16 have also been reported to be involved in centromeric assembly of CENP-A [319, 396]. The functional association of FACT with CENP-A assembly is not clear. FACT may play important role in CENP-A maintenance, either by facilitating centromeric transcription or regulating nucleosome dynamics. It can be suggested that the combined action of RSF1 and FACT may mediate stable assembly of CENP-A chromatin, following the initial deposition of CENP-A into non-nucleosomal complex by HJURP.

Recently, Lagana *et al* [394] found MgcRac-GAP in KNL-2 (Mis18BP1) affinity purification, which transiently localized to centromeres during late G1 phase. They showed that MgcRac-GAP together with the guanine nucleotide exchange factor

(GEF) Ect2, and the small GTPases Cdc42 and Rac, are essential for stability of newly deposited CENP-A at centromeres, suggesting that a GTPase molecular switch generated by MgcRac-GAP may facilitate centromere maintenance after CENP-A loading.

In summary, CENP-A assembly into centromeric nucleosome is a complex multistep process, starting with a priming event late in mitosis followed by HJURP mediated deposition of new CENP-A during late telophase/ early G1. The newly deposited CENP-A is then stabilized and maintained on centromeres by the concerted action of chaperones, chromatin remodelers and other factors. Finally, how the process of centromere priming, new CENP-A deposition, and maintenance are coordinated represent challenges for future investigation.

CHAPTER 4
CONCLUSION AND PERSPECTIVES

4.1 Concluding Remarks

The continuity of life depends on cell division. Proper cell division requires the faithful distribution of the replicated genome to daughter cells. Accurate chromosome segregation in mitosis and meiosis depends on the assembly of active kinetochore on a specialized chromosomal locus, called centromere. Chromosome segregation errors can lead to aneuploidy (the loss or gain of chromosomes), which has detrimental effects on both cell and organism. In human, aneuploidy is a major cause of congenital diseases (e.g. Down's syndrome, Edwards' syndrome and Patau's syndrome), spontaneous abortions, infertility [292], and it is also associated with tumor formation and cancer. Thus understanding how centromere location on chromosomes is stably maintained through generations is a key to understanding chromosome segregation mechanisms.

In higher eukaryotes, centromere specification is independent of the DNA sequence and is determined epigenetically by the presence of a unique nucleosome that contains a centromeric-specific histone H3 variant, CENP-A. The epigenetic mark generated by CENP-A nucleosome, is required for the assembly and maintenance of active centromere at a single locus on each chromosome over many generations. Understanding the mechanisms that govern the specific deposition of CENP-A exclusively at the preexisting centromeric region is a major goal in the field of epigenetics.

In our study, we have used powerful biochemical strategies combined with proteomics, to search for CENP-A deposition machinery in human cells. We show that HJURP (Holliday Junction Recognition Protein), a member of the CENP-A prenucleosomal complex, is essential for centromeric localization of CENP-A *in vivo*. HJURP recognizes and specifically binds to the CATD domain of CENP-A, *via* a highly conserved N-terminal domain, called CBD. Taken together, our data demonstrate that HJURP is a key chaperone responsible for the specific targeting and deposition of newly synthesized CENP-A at centromeres, and thus ensure proper propagation of epigenetic centromere identity.

4.2 Future Directions

In spite of recent advances in our knowledge on how centromere is specified and propagated from one generation to the next, in particular the identification of CENP-A deposition machinery; there are still many key questions that remain unanswered. As mentioned earlier, major challenges include the elucidation of the molecular functions of HJURP, the Mis18 complex, chromatin remodelers (Rsf1, SNF2h, & FACT), MgcRacGAP/small GTPases and other CENP-A assembly factors such as RbAp46/48 in CENP-A deposition and maintenance, and also understanding the role of these and other factors in the regulation of cell cycle dependent CENP-A assembly. Below, I highlight several outstanding questions and speculate on future research in the field of centromere biology.

CENP-A assembly is not coupled with DNA replication

In human, CENP-A deposition at centromeric DNA occurs during G1-phase and is not coupled with DNA replication. This uncoupling of CENP-A deposition from replication of centromeric DNA results in “dilution” of CENP-A at centromeres of daughter chromosomes. This raises the question how (equally or randomly) CENP-A gets distributed to the daughter centomeres, a question that is not yet solved. Whatever is the distribution of CENP-A, its “dilution” could result in at least three distinct scenarios for the changes in the centromeric chromatin structure after replication: (i) generation of nucleosome free gaps (ii) formation of heterotypic tetrasome (CENP-A-H4-H2A-H2B) [390, 391] and, (iii) incorporation of histones H3 which are later exchanged with CENP-A. To this end, a very recent study in HeLa cells shows that both H3.1 and H3.3 incorporates into centromeric chromatin during S phase and placeholder H3.3 is replaced by assembly of new CENP-A during G1 phase [397], but this issue has not been resolved till date.

Thus, it suggests that HJURP mediated deposition of CENP-A nucleosome in late telophase/early G1 occurs through a reaction in which H3.3 nucleosomes are exchanged with CENP-A nucleosomes. However, the mechanism responsible for H3.3 removal or exchange with CENP-A is not yet clear. We show that HJURP can deposit CENP-A/H4 onto naked DNA templates to assemble tetrasome, but whether

it can facilitate exchange of H3 nucleosomes for CENP-A nucleosomes is not known. It is possible that other molecular factors (histone chaperones or chromatin remodelers) are needed to destabilize or remove H3 nucleosomes prior to HJURP-mediated deposition of CENP-A. FACT complex could be an important candidate for this function, which is also required for CENP-A assembly [319, 396]. The subunits of FACT complex has been implicated in destabilizing and chaperoning H2A/H2B dimers in the course of elongation [398, 399], while the histone chaperone Spt6 associates with H3/H4 dimers [400]. Therefore, the FACT and Spt6 act in concert to promote the disassembly of H3 nucleosomes followed by replacement of new CENP-A nucleosomes, from soluble nuclear fraction. It will be interesting to determine how the activities of other factors and HJURP are coordinated to restrict CENP-A assembly at G1 phase. It is also possible that removal of H3.3 nucleosome may be linked to the priming events during late mitosis. Future studies are needed to address this and other relevant mechanisms.

What is the nature of CENP-A nucleosome?

For a long time, it was generally believed that the composition of CENP-A nucleosome is octameric containing two copies of each of CENP-A, H2A, H2B, and H4, wrapped by 146 bp of DNA in a left-handed manner similar to canonical H3 containing nucleosomes. However, several recent studies have provided evidence that support other contradictory models for the structure of CENP-A containing nucleosome. Following is the short overview of different models.

1. Classical octameric nucleosome: The most conventional structure of octamer containing two copies of each of CENP-A, H2A, H2B, and H4. This model is supported by various reports [4, 319, 349, 401-403].
2. Tetrasome: This tetrasome structure contains two copies of CENP-A and H4 but lack H2A and HA2B dimer [378].
3. Hemisome: The hemisome model containins one copy of each of CenH3CID/CENP-A, H2A, H2B, and H4, and reported to be found in *Drosophila melanogaster*. In addition, the DNA is wrapped in right-handed manner instead of left-handed twist as present in conventional nucleosome [378, 390, 404].
4. Octameric reversome: In this case the octamer of nucleosome shows the

same stoichiometry with right-handed wrapping of DNA [405].

5. Hexameric: Studies of the budding yeast *S. cerevisiae* led to the proposal of a hexasome model in which two copies of CENP-A^{Cse4} and H4 and two copies of Scm3 instead of H2A-H2B are contained in single nucleosome [374].
6. Trisome: A trisome of CENP-A^{Cse4}, H4 and Scm3 with right handed wrapping of DNA [404]. Whichever is true compositional model of CENP-A nucleosome, these recent studies suggested that centromeric nucleosome is highly variable structure.

Recently, it is also proposed that intermediate non-nucleosomal complexes of CENP-A with centromeric DNA may exist initially and which are then converted to the final octameric nucleosomes [406]. As both HJURP and Scm3 remains on centromeres for a considerable period of time during cell cycle, suggesting their association with intermediate CENP-A/DNA complexes. It will be interesting to determine the mechanism of HJURP removal from intermediate complexes, and assembly of octameric CENP-A nucleosome. In fact, the exact composition and nature of centromeric chromatin at different stages of cell cycle is not clear, and future studies are needed to address this issue in detail.

For example it would be crucial to study chromatin bound CENP-A complexes and identify the associated DNA, at different stages of cell cycle. This could be done by cell synchronization and subsequent immunoaffinity purification of CENP-A nucleosome in the presence of high salt. The size of associated DNA can be determine after extraction, which will provide a clue about the nature of CENP-A nucleosomal and non-nucleosomal complexes isolated during different cell cycle phases. Afterwards, it would be necessary to analyze the structure of purified CENP-A nucleosomal complexes to get in depth informations. The structural analysis could be done by electron microscopy (EM) or atomic force microscopy (AFM). Additionally, it would be interesting to identify intermediate structures for canonical H3 nucleosome, if exist.

What is the link between CENP-A deposition and proteolysis?

It would be interesting to understand the link between CENP-A stability and centromeric incorporation. In yeast the ubiquitin E3 ligase Psh1 mediates

degradation of mis-incorporated Cse4 [356, 357], but the existence of ubiquitination-mediated degradation of CENP-A in human is currently unknown. It is possible that distant Psh1 relatives or other type of E3 ubquitin ligases could be involved in CENP-A degradation in human. Our biochemical and proteomic strategies can be employed, to search for CENP-A specific ubiquitin ligases in human cells.

What is the role of transcription and non-coding RNAs in CENP-A loading and centromere function?

Several lines of evidence suggest that RNA and transcription have a role in CENP-A deposition and centromere formation. Transcription of genes within active centromeres, including neocentromeres, endogenous centromeres in plants, and human artificial chromosomes has been reported. In humans, it has been shown that artificially altering the chromatin to more open or closed states at centromere of human artificial chromosomes resulting in the loss of centromere proteins (CENP-A, CENP-C and CENP-B) and thereby affect kinetochore assembly [407]. Similarly, it has been shown that transcription across the centromere is important to deposit Cnp1 (a fission yeast CENP-A homolog) through an RNAi-mediated pathway [408]. The RNAi pathway processes the transcripts produced from the centromeric outer repeats to promote formation of flanking heterochromatin, which is required for the assembly of Cnp1 in fission yeast. Although, a number of studies suggested the role of RNAi in centromere assembly through formation of heterochromatin, but direct involvement of RNAi in CENP-A assembly requires further investigations.

It is interesting to understand whether transcription *per se* or product of transcription (RNA) is required for CENP-A assembly. In fact, several studies suggest that centromeric transcription might have a function in assembly of CENP-A chromatin. As discussed before, FACT complex, which is implicated in transcription, is found associated with CENP-A chromatin [319], and in cooperation with Chd1. It is shown to be required for centromeric localization of CENP-A [409]. In addition, an active mark of transcription *i.e.* H3K4me2 is found on interspersed H3 nucleosome, within centromeric regions [410]. A recent study, using synthetic human artificial chromosome, stresses the importance of alpha-satellite DNA transcription for HJURP recruitment and centromeric CENP-A assembly [392]. Taken together, these studies highlight the importance of transcription in CENP-A deposition. However, the exact

mechanism is not known and needs further studies. To understand the connection between CENP-A assembly and transcription, it would be good to compare the timing of CENP-A deposition with that of transcription and production of transcripts, during the cell cycle.

Different classes of transcripts generated from within the CENP-A chromatin region have been reported in plants, mice, and humans [411-414]. In addition to this, the non-coding transcripts have been found associated with CENP-C, INCENP (passenger protein), and the mitotic kinase Aurora B [415]. Recently, Dawe and colleagues have shown that the RNA transcript produced from the centromeric region alters the DNA-binding characteristics of CENP-C to target it to the inner kinetochore [416]. Although, the exact role of non-coding transcripts in CENP-A deposition is not clear, two possible mechanisms can be envisaged. First, these RNA transcripts may be required for the recruitment of CENP-A assembly factors. Second, it is also possible that non-coding transcripts will hybridize with their centromeric DNA templates to form R-loops, hybrids of RNA and DNA, which can elicit DNA damage response and lead to repair-coupled CENP-A deposition.

Future studies will be important to characterize RNA species produced from centromeric regions and define the role of transcription in centromere formation and function. Currently, we have identified RNA components in the preassembly CENP-A complex, in human cells (unpublished data). We speculate that these RNA transcripts may be responsible for guiding HJURP-CENP-A complex to centromeres. Further experiments need to be done to test this hypothesis. It would be important to isolate, clone and sequence these RNAs, in order to address the following questions. Do these transcripts show complete homology with majority of CENP-A-associated DNA sequence, or do they map only to a fraction of alpha satellite in centromere? Do these RNA components interact with HJURP, CENP-A or other partners of CENP-A complex? Afterwards, it would be necessary to block the expression of these RNAs, and monitor their outcome with respect to CENP-A deposition and centromere function. Are these RNA transcripts required for centromeric localization of CENP-A *in vivo*?

N-terminal tail and Post-translational modifications of CENP-A

Since the discovery of CENP-A, a lot of work has been dedicated to the C-terminal tail, particularly to histone-fold domain of CENP-A, but little is known about the function and post-translational modifications of the N-terminal tail of CENP-A.

Although the tail is dispensable for centromeric localization of CENP-A [4, 346], its deletion is lethal in yeast [417]. Tailless CENP-A^{CENH3} in *A. thaliana* localizes at the centromere but cannot rescue the embryonic lethality of mutant CENP-A^{CenH3} [348]. Replacement of the N-terminal tail of CENP-A with the corresponding one from H3 rescues mitosis, but the plants are sterile. Crossing the plants and expressing the GFP-tagged N-terminal swap CENP-A with plants expressing the GFP-tagged wild-type CENP-A triggers a fast elimination of the tail-swapped parental genome. The finding suggests that the CENP-A N-terminus may have an important function in meiosis. The underlying mechanism for the elimination of one parental genome is currently unknown.

Interestingly, the N-terminal tails of all known CENP-A proteins in different organisms are rich in lysine, arginine, serine and threonine residues thus providing potential sites for acetylation, methylation, ubiquitination and phosphorylation. However, little is known about the posttranslational modification of CENP-A and their possible important roles in centromere function. The only modification known for CENP-A is phosphorylation, but the relevance of this modification in CENP-A deposition and kinetochore assembly has not been tested. Therefore, it is important to determine whether CENP-A is subjected to other post-translational modifications, and how these modifications contribute to CENP-A deposition, centromere/kinetochore assembly and functions.

How CENP-A nucleosome builds a functional kinetochore?

An exciting and challenging future area of research is to understand the mechanisms, that how information in CENP-A chromatin is translated to assemble a fully functional kinetochore. CENP-C is an important candidate to link CENP-A chromatin to kinetochore formation. *In vitro* studies showed that CENP-C binds to the C-terminal tail of CENP-A nucleosome [418], suggesting the importance of CENP-A

C-terminal tail in kinetochore assembly [419]. However, the C-terminal tail of CENP-A is not conserved outside vertebrates. Therefore, it is crucial to understand how CENP-C recognizes CENP-A chromatin, *in vivo*. Kinetochore assembly is a complex process involving multiple protein-protein interactions. Biochemical strategies combined with imaging technologies will be helpful in future to uncover the composition and molecular organization of kinetochore.

Although, genetic and biochemical studies have identified proteins and protein complexes that play direct or indirect role in CENP-A deposition and maintenance at centromeres, the mechanisms responsible for centromere formation and perpetuation through infinite numbers of cell divisions, remain poorly understood. We have only begun to understand the mechanisms regulating centromere assembly and function. Fascinating and unexplored future lies ahead for centromere research.

CHAPTER 5
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Appendix A: PAPER 2

The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3

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The histone variant H3.3 marks active chromatin by replacing the conventional histone H3.1. In this study, we investigate the detailed mechanism of H3.3 replication-independent deposition. We found that the death domain-associated protein DAXX and the chromatin remodeling factor ATRX (α-thalassemia/mental retardation syndrome protein) are specifically associated with the H3.3 deposition machinery. Bacterially expressed DAXX has a marked binding preference for H3.3 and assists the deposition of $(H3.3–H4)_2$ tetramers on naked DNA, thus showing that DAXX is a H3.3 histone chaperone. In DAXX-depleted cells, a fraction of H3.3 was found associated with the replication-dependent machinery of deposition, suggesting that cells adapt to the depletion. The reintroduced DAXX in these cells colocalizes with H3.3 into the promyelocytic leukemia protein (PML) bodies. Moreover, DAXX associates with pericentric DNA repeats, and modulates the transcription from these repeats through assembly of H3.3 nucleosomes. These findings establish a new link between the PML bodies and the regulation of pericentric DNA repeat chromatin structure. Taken together, our data demonstrate that DAXX functions as a bona fide histone chaperone involved in the replication-independent deposition of H3.3.

[*Keywords*: Histone variant; H3.3; histone chaperone; PML-NBs]

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In addition to the conventional core histones, cells express low amounts of their nonallelic isoforms, the histone variants. The replacement of major histones by histone variants has emerged as an important way to control chromatin function by altering the biochemical makeup of the nucleosome (Kamakaka and Biggins 2005).

One of the best-studied histone variants is H3.3, which can replace the major species, H3.1. Although H3.1 and H3.3 are 96% identical, they exhibit important differences in behavior. H3.1 is synthesized in S phase and is deposited only during DNA replication. In contrast, H3.3 is expressed throughout the cell cycle and is incorporated at all phases of the cell cycle (Ahmad and Henikoff 2002a). Initial experiments performed in *Drosophila* showed that the deposition of H3.3 into chromatin appeared to be coupled to transcription (Ahmad and Henikoff 2002b; Schwartz and Ahmad 2005). Detailed analysis of H3.3 distribution patterns has revealed that both promoter remodeling and transcription elongation could be involved in the deposition of this variant (Chow et al. 2005; Mito et al. 2005; Wirbelauer et al. 2005). Consequently, H3.3 was proposed to be a marker of active chromatin and to be associated with the epigenetic maintenance of chromatin status (Henikoff et al. 2004; Ng and Gurdon 2008). This hypothesis is supported by the finding that H3.3 is enriched in post-translational modifications specific for active genes (McKittrick et al. 2004; Hake et al. 2006). Additionally, H3.3-containing nucleosomes are intrinsically less stable than those containing H3.1 (Jin and Felsenfeld 2007). This might facilitate the transcription by reducing the energy required to evict nucleosomes from active genes, and provide for the quick removal of existing epigenetic marks. Purification of the complexes responsible for the H3.1 and H3.3 deposition from epitope-tagged H3-expressing HeLa cell lines has revealed that these histones associate with distinct chromatin assembly complexes (Tagami et al. 2004). H3.1 was found mainly within a complex containing the replication-dependent Chromatin Assembly Factor 1 (CAF-1), whereas H3.3 copurified with a complex containing the HIRA protein (Tagami et al. 2004).

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The HIRA protein is believed to be a specific H3.3 chaperone able to deposit H3.3 independently of DNA synthesis (Tagami et al. 2004). The available data suggest that HIRA is involved in the deposition of H3.3 during decondensation of the *Drosophila* sperm pronucleus (Loppin et al. 2005). However, HIRA is not required for this deposition in embryos or in adult tissues (Loppin et al. 2005; Bonnefoy et al. 2007). In contrast, the chromatin remodeling factor CHD1 was found to deposit H3.3 not only in the *Drosophila* male pronucleus, but also during later stages of embryonic development (Konev et al. 2007). This supports the view that multiple and possibly redundant pathways are involved in the assembly of H3.3 nucleosomes.

In this study, we reinvestigated the mechanism that governs H3.3 deposition by purifying the H3.3-containing complexes from HeLa cells. Unexpectedly, we found that human HIRA did not form a stable complex with H3.3. Instead, our data identify HIRA as a member of a histone-less complex closely related to the previously described yeast HIR complex (Green et al. 2005). We show that the death domain-associated protein DAXX and the chromatin remodeling factor ATRX (α-thalassemia/mental retardation syndrome protein) are associated with the H3.3 preassembly complex. In addition, DAXX colocalizes with H3.3 into promyelocytic leukemia protein nuclear bodies (PML-NBs) and regulates the expression of mouse pericentric DNA repeats. We further present evidence that DAXX is a bona fide histone chaperone specific for H3.3.

Results

Isolation of H3.1 and H3.3 nucleosome preassembly complexes and identification of specific partners

We used the double-immunoaffinity purification method (Nakatani and Ogryzko 2003; Tagami et al. 2004; Ouararhni et al. 2006) to isolate the H3.1 and H3.3 nucleosome preassembly complexes. Histones H3.1 and H3.3 were expressed stably as fusion proteins with C-terminal Flag- and HA-epitope tags in HeLa cells (Fig. 1A). Epitope-tagged H3.1 and H3.3 (e-H3.1 and e-H3.3) nucleosome preassembly complexes were then purified from nuclear-soluble extracts by sequential immunoprecipitations with anti-Flag antibody, followed by anti-HA antibody (Ouararhni et al. 2006). Proteins associated with e-H3.1 and e-H3.3 nuclear complexes (NCs) were separated by SDS-containing 4%–12% polyacrylamide gradient gels and silver-stained (Fig. 1B). Numerous proteins were found to be associated physically with e-H3.1 and e-H3.3 (Fig. 1B). Mass spectrometry and immunoblotting analysis allowed the identification of the following partners as components common to the e-H3.1 and e-H3.3 complexes: core histones (H2A, H2B, H3, and H4), several well-characterized histone chaperones (anti-silencing factor 1a[ASF1a] and ASF1b, nuclear autoantigenic sperm protein [tNASP and the shorter form, sNASP] and p46/p48), histone acetyltransferase 1 (HAT1), Ku proteins (Ku70 and Ku80), Importin4, PARP-1, Topoisomerase II (TOP2), and DNA-dependent protein kinase (DNA-PK).

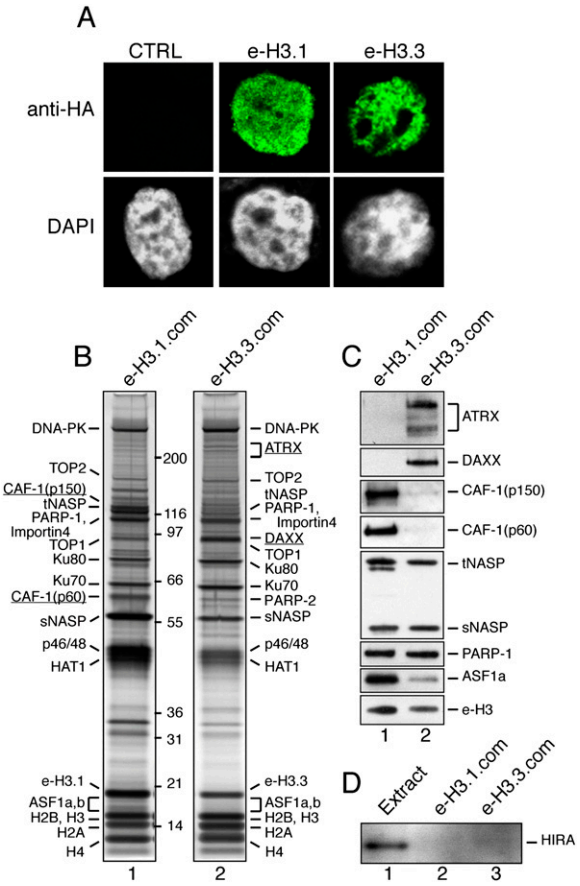

Figure 1. Immunopurification of e-H3.1 and e-H3.3 deposition complexes from soluble nuclear fractions. (*A*) Stable expression of e-H3.1 and e-H3.3 in HeLa cells. Cells expressing e-H3.1 or e-H3.3 and control cells (CTRL) were stained with anti-HA (*top*) and DAPI (*bottom*). (*B*) Silver staining of proteins associated with e-H3.1 (lane *1*) and e-H3.3 NCs (lane *2*). The complexes containing e-H3.1 (e-H3.1.com) and e-H3.3 (e-H3.3.com) were purified by double immunoaffinity from soluble nuclear extracts (NCs). Polypeptides identified by mass spectrometry analysis and the positions of molecular size markers are indicated. (*C*) DAXX and ATRX proteins are specific to the e-H3.3 NC. The e-H3.1 (lane *1*) and e-H3.3 (lane *2*) complexes were analyzed by immunoblotting with the indicated antibodies. (*D*) HIRA is not associated with H3.3. The e-H3.1 (lane *2*) and e-H3.3 (lane *3*) NCs were analyzed by immunoblotting with anti-HIRA antibody. (Lane *1*) HeLa whole-cell extract was used as a control.

Two of the three CAF-1 subunits (p150 and p60) were highly specific to the e-H3.1 complex, whereas the third CAF-1 subunit (p46/p48) was a component common to e-H3.1 and e-H3.3 complexes (Fig. 1B,C). This corroborated the reported data, showing that the CAF-1 subcomplex is part of the e-H3.1-containing complex (Tagami et al. 2004).

Concerning the specific partners of H3.3, HIRA was detected within the H3.3 complex after the first anti-Flag affinity (Supplemental Fig. 1), but was undetectable by mass spectrometry or by immunoblotting analysis after the second anti-HA affinity (Fig. 1B,D). We excluded the possibility that loss of HIRA after the anti-HA affinity step was due to an inefficient HA elution (see Supplemental

Fig. 2). This indicates that HIRA is not a stable component of the e-H3.3 complex. In contrast, after the double-immunoaffinity purification, we identified DAXX (originally found associated with CD95) (Yang et al. 1997) and ATRX (a member of the SNF2 family of chromatin remodeling factors) (Steensma et al. 2005) within the H3.3 complex, but not in the H3.1 complex (Fig. 1B). Immunoblotting of the purified complexes confirmed that both proteins were present only within the e-H3.3 complex (Fig. 1C).

DAXX is a stable component of e-H3.3–nucleosome preassembly complexes

DAXX is an acidic protein (pI 4.6) containing a stretch rich in glutamic and aspartic acid residues embedded within a yeast histone chaperone, Rtt106-like domain (Fig. 3A [below]; Supplemental Fig. 3). This raised the possibility that DAXX acts as a chaperone specific for H3.3. If this is the case, DAXX should interact strongly with H3.3 both in vitro and in vivo. With this in mind, we first checked that DAXX was stably associated with the H3.3 complex in vivo. To this end, isolated H3.1 and H3.3 complexes from nuclear-soluble extracts were fractionated on glycerol gradients. The different fractions were then run on a denaturing 4%–12% gradient gel, and proteins were silver-stained. Two distinct subcomplexes with different molecular masses were identified in both H3.1 and H3.3 NCs (Fig. 2A,B). These subcomplexes were termed LNC (for low-molecular-weight NC) and HNC (for high-molecular-weight NC). In parallel, e-H3.3 and e-H3.1 complexes were also purified from the cytosolic fractions (CC, for cytosolic complex) (Fig. 2C).

Immunoblotting analysis revealed that DAXX and ATRX were present within both the nuclear (LNC and HNC) and the cytosolic (CC) e-H3.3 subcomplexes (Fig. 2D), whereas CAF-1 p150 and p60 were detected only within the nuclear (LNC and HNC) e-H3.1 subcomplexes (Fig. 2D). NASP and ASF1 histone chaperones were common to both complexes (e-H3.3/e-H3.1), and were distributed within CC and LNC subcomplexes, but not in the corresponding HNC subcomplexes (Fig. 2C,D). Mass spectrometry and immunoblotting analysis revealed that the ASF1-containing complexes (CC and LNC) comprised e-H3.3–H4 or e-H3.1–H4, but not the other core histones (including the untagged endogenous histone H3.3/H3.1) (Fig. 2C; data not shown). This is consistent with the available data suggesting an interaction of ASF1 with one H3–H4 heterodimer by competing for the same surface of interaction that is normally occupied by the second H3–H4 dimer in the tetrameric complex (English et al. 2006; Agez et al. 2007; Natsume et al. 2007). In contrast, ASF1-free HNC complexes comprised the four core histones (including endogenous H3.3 and H3.1), suggesting that HNC complexes contained either histone octamers or heterotypic tetramers (H2A, H2B, H3, and H4) (Fig. 2C; Supplemental Fig. 4). To rule out the possibility that H2A–H2B dimers derive from contaminating chromatin, the e-H3.3 complex was treated with DNase I or ethidium bromide. The presence of the four core histones within the H3.3 complex was insensitive to both treatments, and consequently was not mediated by DNA (Supplemental Fig. 5). This observation raised interesting questions about the mechanism of histone deposition (see the Discussion).

In summary, these data demonstrate that (1) DAXX is a stable component of cytoplasmic and nuclear e-H3.3-containing complexes, but not of e-H3.1-containing complexes; and (2) a fraction of e-H3.3 or e-H3.1 contains the four core histones, and is present within an ASF1-free complex.

DAXX interacts directly and preferentially with H3.3 both in vitro and in vivo

We next investigated whether DAXX interacted directly with H3.3 in vitro using a GST pull-down assay. Purified GST-DAXX fusion protein was immobilized on glutathione–agarose beads and incubated with recombinant histone tetramers H3.1–H4 or H3.3–H4 (Fig. 3B). Bead-bound complexes were next washed with the indicated NaCl concentration, eluted, and fractionated on SDS-PAGE. Coomassie blue staining revealed that H3.3–H4 exhibited a more robust interaction (persisting at higher ionic strength) with GST-DAXX protein than H3.1–H4 (Fig. 3B, cf. lanes 3–5 and 7–9). This demonstrated that DAXX associates preferentially with H3.3. Similar results were obtained using native histones purified from chromatin of HeLa cells stably expressing e-H3.1 or e-H3.3 instead of recombinant histones (Supplemental Fig. 6).

To determine the region of DAXX involved in the recognition of H3.3, we generated deletion mutants containing either the N-terminal (1–302), central (302–495), or C-terminal (495–740) domain of DAXX. We expressed these mutants as GST fusion proteins and examined their binding to native e-H3.3–H4 histones. Immunoblotting using an anti-HA antibody revealed that the acidic central domain, containing the Rtt106-like motif, exhibited a strong interaction with e-H3.3–H4 (Fig. 3C). Note that the N-terminal domain of DAXX also interacted with histones, but to a lesser extent than did the central domain (Fig. 3C).

To further investigate the preferential binding of DAXX to H3.3 in a more physiological context, DAXX was stably expressed in HeLa cells as a fusion protein with C-terminal Flag- and HA-epitope tags. e-DAXX complex was purified using the same procedure described for e-H3.3. Mass spectrometry analysis identified ATRX as a partner of e-DAXX in both the CC and the NC fractions (Fig. 3D), as found previously by others using different approaches (Xue et al. 2003; Tang et al. 2004). In addition, both complexes contained p46/p48, TBA2 (Tubilin α2), UBP7, Agrin (Fig. 3D), and histones H3 and H4, but not ASF1 or NASP, two chaperones identified within e-H3.3 complex (Fig. 3D; Supplemental Fig. 7). We next performed a more detailed analysis to determine the precise nature of the H3 copurified with e-DAXX (i.e., H3.3 vs. other H3 isoforms). Mass spectrometry analysis identified several peptides corresponding exclusively to H3.3 (Supplemental Fig. 8). This result was then confirmed by immunoblotting using a specific antibody directed against H3.3 (Fig. 3E; Supplemental Fig. 9). Together, these data established that DAXX binds H3.3 preferentially in vivo (compared with H3.1) and could

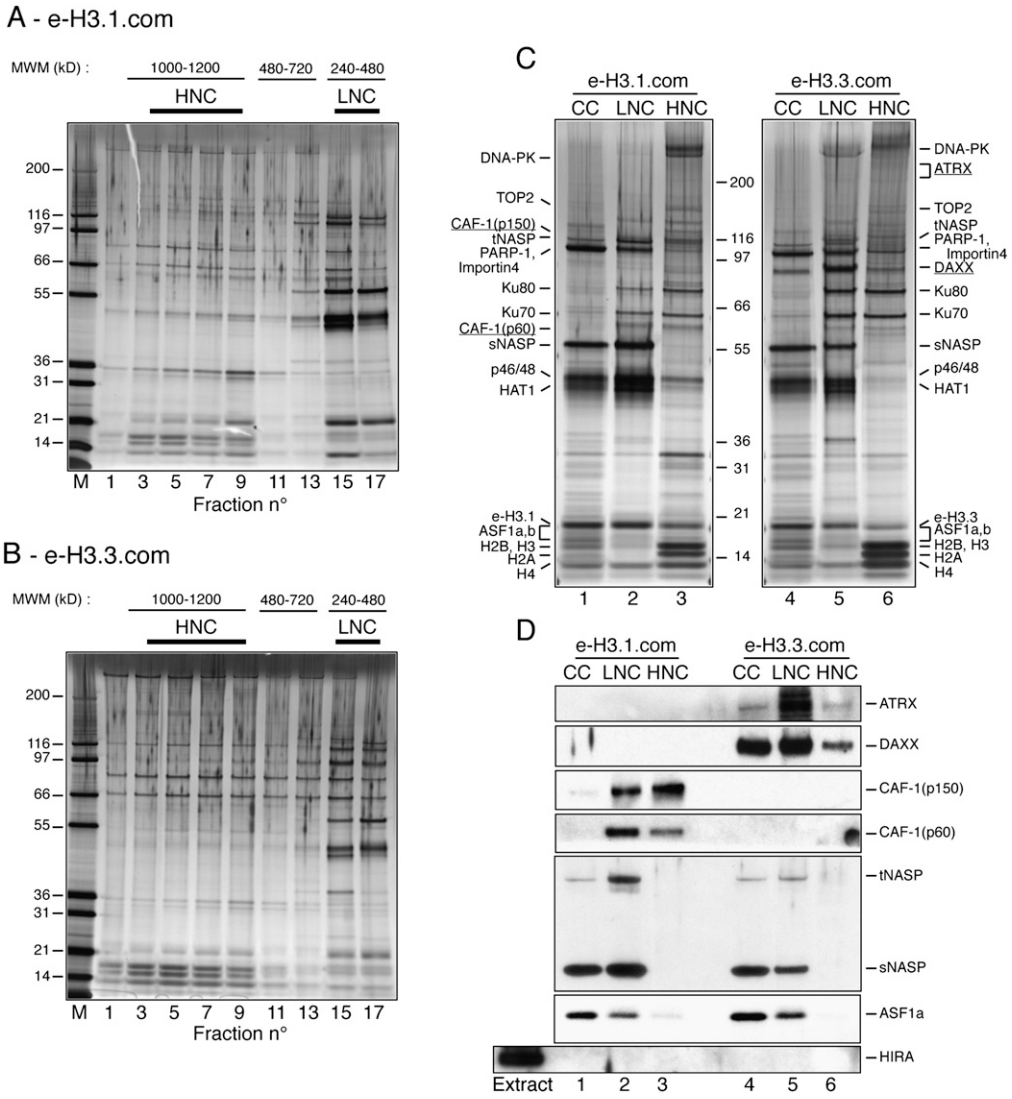

Figure 2. DAXX is stably associated with the H3.3, but not with the H3.1 complex. (*A*) Silver staining of nuclear e-H3.1 complex fractionated on a glycerol gradient. The e-H3.1 NC purified by double affinity was separated on a glycerol gradient. Fractions were pooled as indicated at the *top* of the gel. The approximate molecular weight of the different subcomplexes was estimated using the NativeMark molecular weight marker (MWM; Invitrogen). (*B*) Silver staining of nuclear e-H3.3 complex fractionated on a glycerol gradient. Experiments were performed as described in *A*. (*C*) Silver staining of pooled fractions containing e-H3.1 and e-H3.3 nuclear subcomplexes (LNC and HNC) and of e-H3.1 and e-H3.3 CCs. (*D*) Immunoblotting of pooled fractions containing e-H3.1 and e-H3.3 nuclear subcomplexes (LNC and HNC) and of e-H3.1 and e-H3.3 CCs with the indicated antibodies. Input fraction (extract) is shown for the blot with anti-HIRA.

explain why DAXX was found only in e-H3.3 complex (see Fig. 1A).

HIRA does not form a stable complex with H3.3 in HeLa cells

To gain insight into the relationship between HIRA and H3.3, a HeLa cell line stably expressing a Flag- and HA-tagged version of HIRA was generated (e-HIRA). Cabin1, Ubinuclein1, the hypothetical protein FLJ25778, ASF1a, and ASF1b were identified by mass spectrometry analysis as major components of the e-HIRA complex (Fig. 3D; Supplemental Fig. 7). Cabin1 has been characterized recently as a candidate human ortholog of Hir3, a component of the yeast HIR complex (Balaji et al. 2009). Ubinuclein1 and FLJ25778 (termed also Ubinuclein2) were also proposed to be candidate orthologs of another component of the yeast HIR complex, Hpc2 (Banumathy et al. 2009). Together, these data would imply that the HIRA/ASF1 complex is conserved through evolution. However, in contrast to the yeast HIR complex (Green et al. 2005), the e-HIRA complex did not contain histones (Fig. 3D,E). This is entirely consistent with our data showing that HIRA is not a stable component of the e-H3.3-containing complex (see the Discussion; Fig. 1B,D).

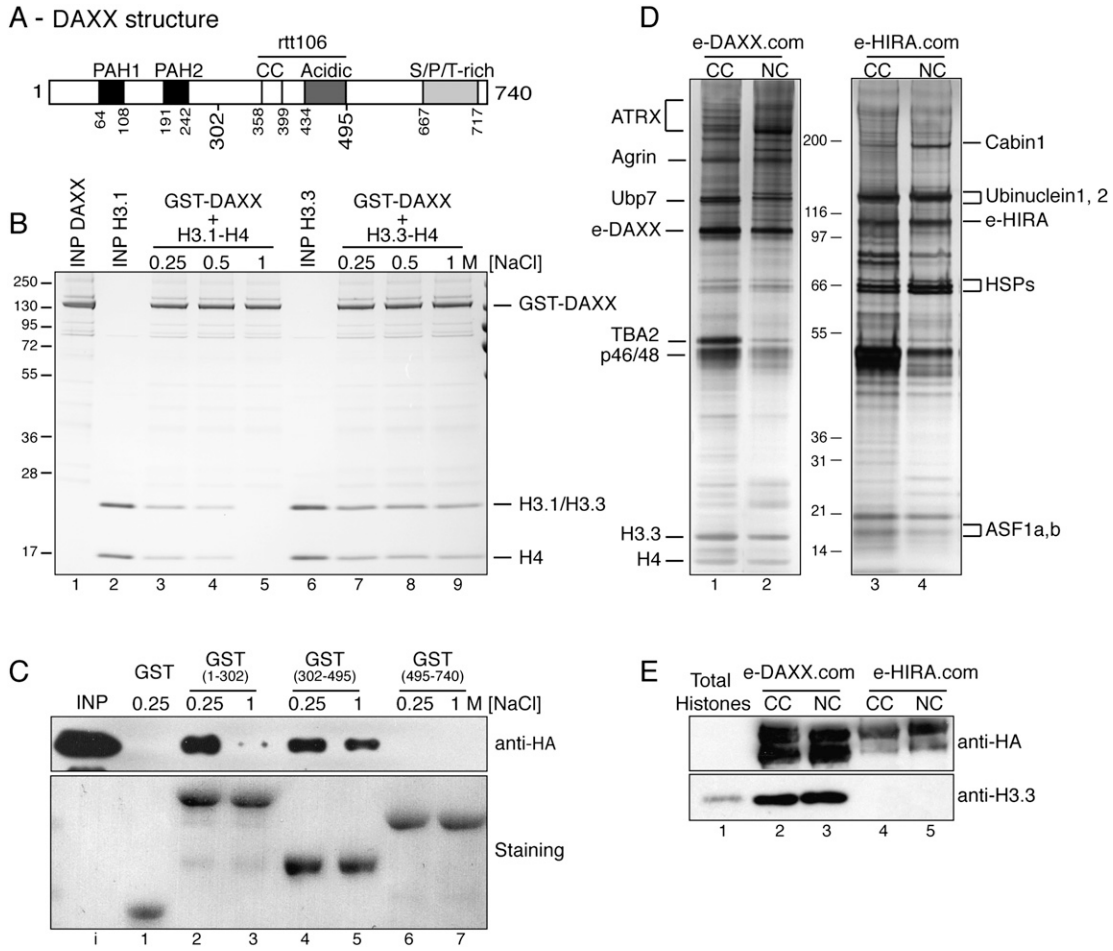

Figure 3. DAXX preferentially associates with H3.3 in vitro and in vivo. (*A*) Primary structure of DAXX. DAXX contains several putative domains: two paired amphipathic helices (PAH1 and PAH2), a coiled-coil (CC), an acidic domain (acidic), a Ser/Pro/Thr rich domain (S/P/T-rich), and an rtt106-like domain (rtt106). (*B*) DAXX preferentially associates with H3.3 in vitro. GST-DAXX, immobilized on glutathione-agarose, was incubated with recombinant histones H3.1–H4 (lanes *3–5*) or H3.3–H4 (lanes *7–9*). Bead-bound complexes were washed with the indicated concentration of NaCl. Eluted proteins were fractionated on SDS-PAGE and stained with colloidal blue. The input lanes (INP) represent the amount of proteins used for the pull-down. (*C*) The central part of DAXX contains a high affinity H3.3-interacting domain. N-terminal (1–302), central (302–495), and C-terminal (495–740) regions of DAXX were produced as GST fusion proteins. The fusion proteins (lanes *2–7*) and GST alone (lane *1*), immobilized on glutathione-agarose resin, were incubated with tetramers containing epitope-tagged H3.3. (*Top*) After washing with either 0.25 or 1 M NaCl, the resin-bound tetramers were analyzed by immunoblotting using anti-HA antibody. (*Bottom*) To compare the levels of the GST fusions used for the pull-down, the blot was first stained with Ponceau red. The input lane (i) represents 40% of the amount of tetramers used for the pull-down. (*D*) Silver staining of DAXX and HIRA complexes (e-DAXX.com and e-HIRA.com) purified by double immunoaffinity from either cytoplasmic extract (CC) or soluble nuclear extract (NC). The polypeptides identified by mass spectrometry analysis are indicated. (*E*) The DAXX complex, but not the HIRA complex, contains H3.3. The e-DAXX (lanes *2,3*) and e-HIRA (lanes *4,5*) complexes were analyzed by immunoblotting with anti-HA (*top*) and anti-H3.3 (*bottom*) antibodies. (Lane *1*) Total histones purified from HeLa cells were used as control.

DAXX facilitates the deposition of H3.3 in vitro

Acidic domains are known to bind basic proteins such as histones and to mediate nucleosome assembly (De Koning et al. 2007). We tested whether DAXX was able to assist the deposition of histones on DNA and to assemble a functional $(H3.3–H4)_2$ tetrameric particle (tetrasome). Labeled 359-base-pair (bp) DNA encompassing the *Drosophila Hp70A* promoter (Hamiche et al. 1999) was circularized under conditions that generate one negative supercoil corresponding to topoisomer −1. This negatively supercoiled DNA has been shown previously to be a very good substrate for histone $(H3–H4)_2$ deposition, and allows a clear in-gel visualization of tetrasome formation (Hamiche and Richard-Foy 1999). The negatively supercoiled DNA was then incubated with increasing amounts of H3.3–H4 histones (at the indicated histone/DNA ratio, rw), which were either preincubated or not with equimolar amounts of DAXX for 30 min (Fig. 4A); then the deposition of histones onto DNA was analyzed by EMSA (Fig. 4B). Under

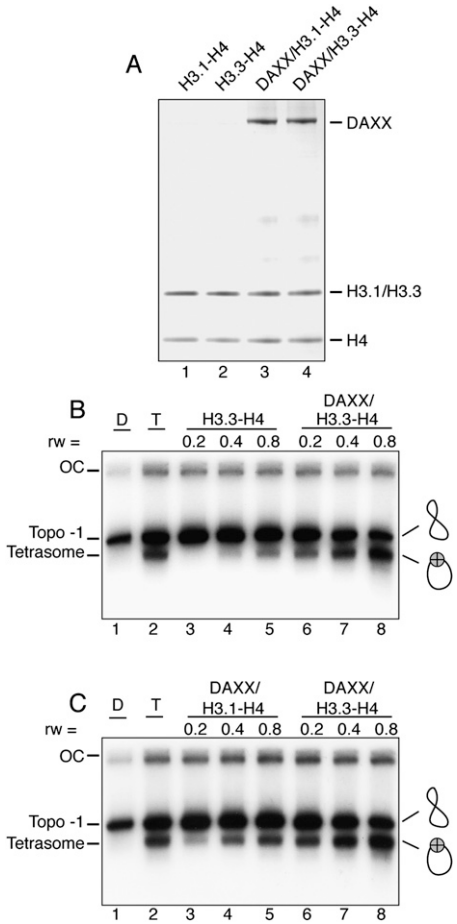

Figure 4. DAXX favors deposition of H3.3 in vitro. (*A*) Purification and reconstitution of recombinant DAXX/histone complex. Histones H3.1–H4 (lane *1*) or H3.3–H4 (lane *2*) and full-length DAXX were expressed in bacteria and mixed at equimolar ratio (lanes *3*,*4*). (*B*) DAXX facilitates the deposition of $(H3.3–H4)_2$ tetramers on DNA. Negatively supercoiled DNA corresponding to topoisomer −1 was incubated with increasing amounts of $(H3.3–H4)_2$ tetramers (at the indicated histone/DNA ratio, rw) either in the presence (lanes *6*–*8*) or the absence (lanes *3*–*5*) of equimolar (to the tetramers) amounts of GST-DAXX. The reaction products were then analyzed on native 4.5% polyacrylamide gel. (Lane *1*) Topoisomer −1 DNA. (Lane *2*) $(H3.3–H4)_2$ tetrasomes reconstituted on topoisomer −1 by salt dialysis. Positions of the open circular DNA (OC), the naked topoisomer −1 DNA, and the $(H3.3–H4)_2$ tetrasome are indicated. (*C*) DAXX deposits more efficiently $(H3.3–H4)_2$ than $(H3.1–H4)_2$ tetramers. Topoisomer −1 was incubated with increasing amounts (at the indicated histone/DNA ratio, rw) of $(H3.1–H4)_2$ (lanes *3*–*5*) or $(H3.3–H4)_2$ (lanes *6*–*8*) tetramers in the presence of equimolar (to the tetramers) amounts of GST-DAXX. The reaction products were then analyzed on native 4.5% polyacrylamide gel. (Lane *1*) Topoisomer −1 DNA. (Lane *2*) $(H3.3–H4)_2$ tetrasomes reconstituted on topoisomer −1 by salt dialysis. Positions of the open circular DNA (OC), the naked topoisomer −1 DNA, and the $(H3.3–H4)_2$ tetrasome are indicated.

these conditions (in the absence of DAXX), very low amounts of $(H3.3–H4)_2$ tetramer deposition were observed (Fig. 4B, lanes 3–5). However, in the presence of DAXX, a significant deposition of $(H3.3–H4)_2$ tetramers was visualized by EMSA (Fig. 4B, lanes 6–8). This shifted complex comigrated with $(H3.3–H4)_2$ particles reconstituted by dialysis (Fig. 4B, lane 2). The DAXX-mediated deposition of histones on DNA is more efficient compared with what could be obtained by the salt dialysis method.

As a control, we also assayed the ability of DAXX to favor the deposition of H3.1–H4. In contrast to what was observed with H3.3–H4 (Fig. 4B), DAXX poorly favored the deposition of H3.1–H4 (Supplemental Fig. 10). This finding suggested that H3.3 is the preferred substrate of DAXX. To further prove this, increasing amounts of $(H3.1–H4)_2$ or $(H3.3–H4)_2$ tetramers, preincubated with equimolar amounts of DAXX (Fig. 4A), were incubated with DNA topoisomer −1 for 30 min, and then the histone deposition was analyzed by EMSA (Fig. 4C). Under these conditions, very low amounts of $(H3.1–H4)_2$ tetramer deposition were observed (Fig. 4C, lanes 3–5), compared with amount of deposited $(H3.3–H4)_2$ (Fig. 4C, lanes 6–8). We conclude that DAXX is a histone chaperone specific for H3.3.

Distinct H3.3 deposition mechanism operates in the absence of DAXX

To further investigate the role of DAXX in the deposition of H3.3, we stably expressed epitope-tagged H3.3 in wild-type and $DAXX^{-/-}$ mouse embryonic fibroblasts (MEFs) (Ishov et al. 2004). Preliminary attempts revealed that e-H3.3 was still incorporated into chromatin in DAXX-deficient cells (Fig. 7 [below]; data not shown). This suggests that, in the absence of DAXX, another chaperone could be involved in the deposition of H3.3. In order to understand how H3.3 was deposited in the absence of DAXX, proteins associated with e-H3.3 were purified by the double-immunoaffinity method from soluble nuclear extracts of wild-type and $DAXX^{-/-}$ MEF cells (Fig. 5A). As expected for the wild-type MEF cells, DAXX and ATRX were identified among the proteins associated with e-H3.3 in mice (Fig. 5A,B), suggesting that the deposition machinery is conserved in mammals. Note that, again, HIRA was not detected within the partners of e-H3.3 (Fig. 5B).

The purification of e-H3.3-containing complex from $DAXX^{-/-}$ MEF cells revealed an altered protein composition when compared with the wild-type complex (Fig. 5A). This complex did not contain ATRX (Fig. 5A,B), suggesting that anchorage of this protein to the e-H3.3 complex is mediated by DAXX. We also noticed an increase in the amount of the histone chaperone tNASP (Fig. 5B). Initially, this chaperone was found associated with linker histone H1 (Richardson et al. 2000). By coexpression of tNASP with H3.3–H4 (or H3.1–H4) in bacteria, we found that tNASP binds also H3/H4 histones (Supplemental Fig. 11), as suggested by others (Wang et al. 2008). More surprisingly, we could clearly identify within this complex the replication-dependent deposition factor CAF-1, as evidenced by mass spectrometry and Western blotting using antibodies against CAF-1 p150 and p60 (Fig. 5A,B). This suggested that, in the absence of DAXX, the CAF-1 complex associates with a fraction of H3.3. Note that the amount of expressed CAF-1 p150 in both wild-type and $DAXX^{-/-}$ MEFs was identical (Supplemental Fig. 12), thus ruling out the possibility that a change in expression of this protein

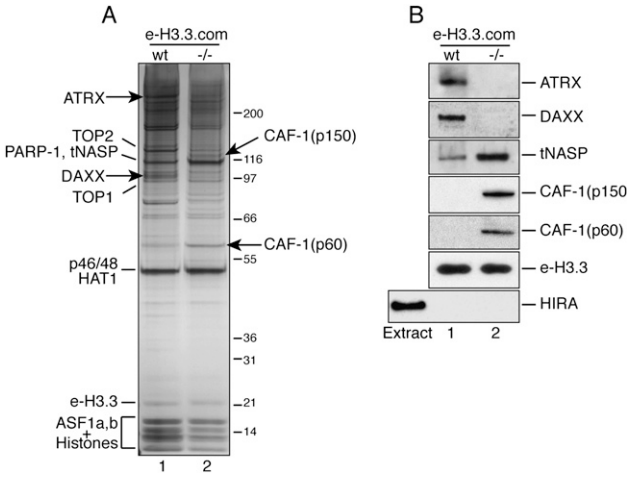

Figure 5. Purification of partners associated with e-H3.3 from extracts of stable wild-type and DAXX$^{-/-}$ MEFs. (*A*) Silver staining of proteins associated with e-H3.3 from soluble nuclear extracts of stable wild-type (lane *1*) and DAXX$^{-/-}$ (lane *2*) MEFs. H3.3 complexes were purified by double immunoaffinity. The polypeptides identified by mass spectrometry analysis are indicated. Arrows show the positions of partners specific to each complex. (*B*) Analysis by immunoblotting of proteins associated with e-H3.3 from extracts of stable wild-type (lane *1*) and DAXX$^{-/-}$ (lane *2*) MEFs using the indicated antibodies. Input fraction (extract) is shown for the blot with anti-HIRA.

might be responsible for its recruitment within the e-H3.3 complex in DAXX$^{-/-}$ cells. Together, these data imply that DAXX-deficient cells adapt to deposit H3.3 by using alternative mechanisms of deposition.

DAXX and ATRX-dependent deposition of H3.3 in pericentric heterochromatin

DAXX is a component of the PML-NBs and directly interacts with ATRX, which is highly enriched at pericentric heterochromatin in mammals (McDowell et al. 1999; Ishov et al. 2004). Consistent with this, our chromatin immunoprecipitation (ChIP) assays in MEF cells revealed that DAXX and ATRX were indeed strongly enriched on pericentric DNA repeats (major satellites) (Fig. 6A), and were only poorly detected on pseudo-*GAPDH*, another heterochromatin loci (Supplemental Fig. 13).

Recent studies have shown that pericentric DNA repeats are highly transcribed in mice (Lu and Gilbert 2007). The abundance of transcripts encoded by pericentric DNA repeats was therefore monitored in wild-type and DAXX$^{-/-}$ MEF cells. Real-time RT–PCR analysis revealed that these transcripts were more abundant in wild-type cells than in DAXX-deficient cells (Fig. 6B). Similarly, the knockdown of H3.3 (by a siRNA pool directed against the two H3.3 genes H3.3A and H3.3B) or ATRX strongly affected the transcription from pericentric DNA repeats (Fig. 6C; Supplemental Fig. 14). Note that the ablation of HIRA expression did not affect the expression from these repeats (Supplemental Fig. 14).

The above data suggested that H3.3, DAXX, and ATRX assist the transcription from pericentric chromatin. We hypothesized that this might be achieved through the DAXX-dependent assembly of H3.3 nucleosomes on pericentromeric chromatin in a replication-independent manner. To test this, DAXX$^{-/-}$ MEF cells were first

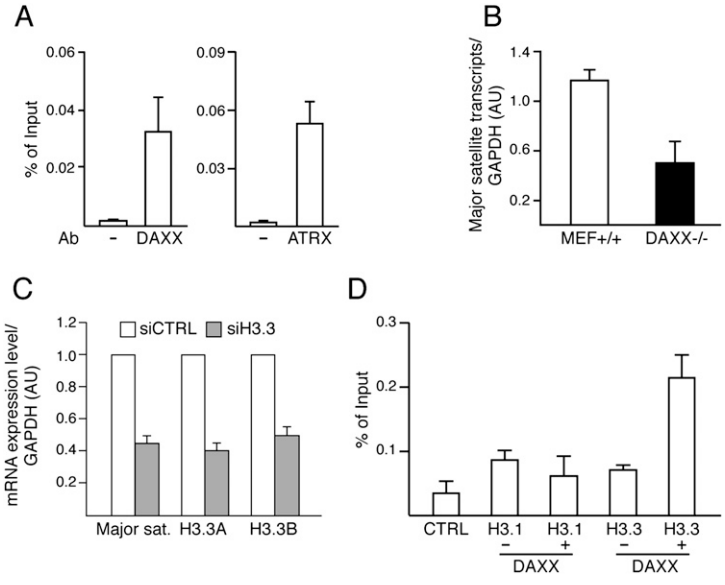

Figure 6. DAXX-dependent deposition of H3.3 on pericentric heterochromatin. (*A*) DAXX and ATRX are present on pericentric DNA repeats in wild-type MEFs. Presence of DAXX (*left* panel) and ATRX (*right* panel) on pericentric DNA repeats was investigated by ChIP assays using specific antibodies. (−Ab) Control sample in which primary antibody was omitted. Results are expressed as percentage of chromatin input used for immunoprecipitation. (*B*) The level of transcripts from pericentric DNA repeats is reduced in DAXX-deficient cells. Relative mRNA level for pericentric DNA repeats in wild-type and DAXX$^{-/-}$ MEFs was determined by quantitative RT–PCR. Results are represented as relative expression level of pericentric DNA repeats versus *GAPDH*. Mean ± standard deviation of four independent experiments. (*C*) Depletion of *H3.3A* and *H3.3B* resulted in a decrease in transcription from pericentric DNA repeats. MEFs were transfected with control siRNA (siCTRL) or a mixture of *H3.3A* and *H3.3B* siRNA (siH3.3). Relative mRNA levels for pericentric DNA repeats, *H3.3A*, and *H3.3B* were determined by quantitative RT–PCR. Results were normalized to *GAPDH* and were set at 1 in cells transfected with control siRNA. Mean ± standard deviation of three independent experiments. (*D*) DAXX is required for deposition of H3.3 onto pericentric DNA repeats outside of S phase. DAXX$^{-/-}$ MEFs were deprived of serum for 48 h before being cotransfected with empty vector (CTRL) or else epitope-tagged H3.1 or H3.3 expression vector in combination with DAXX expression vector where indicated. Forty hours later, cells were reinduced for 8 h with 20% FCS in the presence of aphidicolin and were subjected to ChIP assays. Results are expressed as percentage of chromatin input immunoprecipitated. Mean ± standard deviation of three independent experiments.

synchronized in G0 by serum starvation, and then they were transfected with either e-H3.3 or e-H3.1 constructs (or empty vector as control) in combination or not with a HA-DAXX construct. Forty hours later, cells were supplemented with serum and left to progress through the cell cycle, reaching S phase by 10 h after serum addition (data not shown). Before entry in S phase, the amounts of e-H3.1 and e-H3.3 integrated into pericentric repeats were evaluated by ChIP analysis 8 h after serum addition and in the presence of aphidicolin, an inhibitor of DNA polymerase. As expected, outside the S phase, e-H3.1 was poorly deposited on pericentric repeats even in the presence of DAXX (Fig. 6D). In the absence of DAXX, e-H3.3 was also poorly deposited. In contrast, addition of DAXX enhanced nearly threefold the e-H3.3 deposition in pericentric repeats (Fig. 6D). This stimulation was not due to an accumulation of e-H3.3 in DAXX-transfected cells, as verified by Western blot (data not shown). Moreover, we found that reduction of ATRX expression by siRNA led to a diminishment of DAXX-dependent deposition of H3.3 into pericentric repeats (Supplemental Fig. 14). In addition, the requirement of DAXX for H3.3 deposition seems to be restricted at least to pericentric DNA repeats, since H3.3 was deposited in the absence of DAXX at the active genomic site *DHFR*, although the presence of DAXX further increased H3.3 incorporation (Supplemental Fig. 13). Taken together, these results show that both DAXX and ATRX are required for H3.3 deposition onto pericentric DNA repeats outside the S phase, and suggest that the DAXX/ATRX complex uses H3.3 to modulate the transcription from these repeats.

DAXX is essential for tethering H3.3 to PML-NBs

A fraction of DAXX was found to accumulate in PML-NBs in the nucleus (Salomoni and Khelifi 2006). The PML-NBs form multiprotein structures where proteins accumulate and undergo post-translational modifications. These dot-like structures have been implicated in diverse biological functions, including the regulation of chromatin conformation (Bernardi and Pandolfi 2007). Interestingly, human pericentric DNA repeats can colocalize with DAXX within the PML-NBs (Luciani et al. 2006). Therefore, it is possible that DAXX, in addition to its role as a deposition factor, serves as a specific carrier that targets H3.3 to PML-NBs.

To study this possibility, resting DAXX$^{-/-}$ MEFs were transiently transfected with a green fluorescence-tagged H3.3 (GFP-H3.3) in combination with a HA-DAXX construct or an empty vector as control (Fig. 7). After 40 h, cells were reinduced for 8 h by serum addition in the presence of aphidicolin. Finally, cells were stained with DAPI and analyzed for the distribution of GFP-H3.3, DAXX, and PML in G1 phase. In the absence of DAXX (Fig. 7i–p), GFP-H3.3 showed a diffuse nuclear staining. The reintroduction of DAXX into the knocked-down cells resulted in a nice nuclear colocalization of H3.3 with DAXX and PML in a dot-like manner (Fig. 7a–h). We conclude that DAXX targets H3.3 to PML-NBs, suggesting a direct link between the accumulation of DAXX into PML-NBs and the deposition of H3.3 onto pericentric DNA repeats.

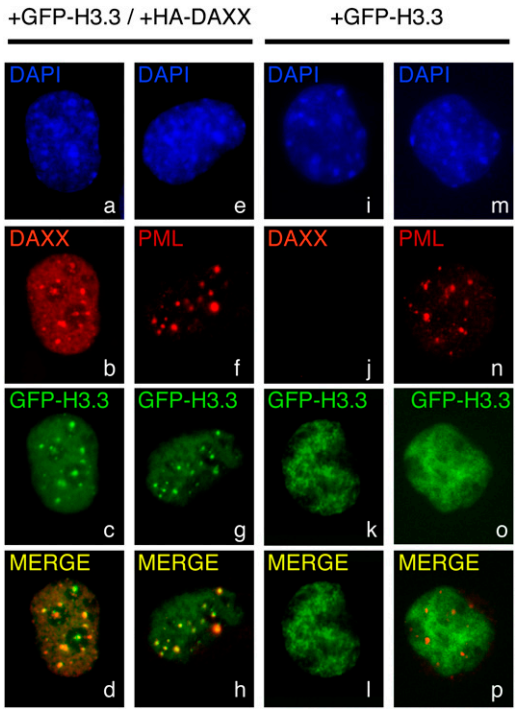

Figure 7. DAXX targets H3.3 to PML-NBs. Resting DAXX$^{-/-}$ MEF cells were transiently transfected with GFP-tagged H3.3 expression vector (+GFP-H3.3) in combination (*a–h*) or not (*i–p*) with HA-DAXX expression vector (+HA-DAXX). Forty hours later, cells were supplemented with serum and were paraformaldehyde-fixed after an additional 8 h. Distribution of HA-DAXX (*b,j*) or endogenous PML (*f,n*) in GFP-H3.3-positive cells was investigated by immunofluorescence staining using anti-HA or anti-PML antibody, respectively. (*a,e,i,m*) DNA was stained with DAPI. (*d,h,l,p*) Merged images correspond to the overlay of red (HA-DAXX or PML) and green fluorescence (GFP-H3.3).

Discussion

In this work, we studied in detail the mechanism of H3.3 deposition. We found that DAXX and the chromatin remodeling factor ATRX are crucial components of the H3.3 deposition machinery. Our data argue that DAXX functions as a chaperone involved in the replication-independent deposition of H3.3.

DAXX is a histone chaperone specific for H3.3

Although DAXX has been shown to be involved in apoptosis and transcriptional regulation, its physiological function remains poorly understood (Salomoni and Khelifi 2006). Our data unambiguously identify DAXX as a histone chaperone specific for H3.3, since (1) DAXX is associated stably with H3.3, but not with H3.1, in both the cytoplasm and the nucleus. This was demonstrated by immunopurifying both e-DAXX and e-H3.3 NCs and CCs. (2) DAXX contains a stretch rich in glutamic acid and aspartic acid residues embedded within an Rtt106-like domain, a feature that is shared by numerous histone chaperones, including the yeast Rtt106 chaperone (Huang et al. 2005), nucleoplasmin (Dutta et al. 2001), and FACT (Belotserkovskaya

et al. 2003). We demonstrate that, in vitro, this DAXX domain interacts both strongly and preferentially with H3.3. (3) DAXX assembles tetramers onto naked DNA minicircles. The assembly is more efficient using tetramers that contain H3.3 than H3.1, a requirement for a chaperone specific for H3.3.

DAXX is a H3.3 deposition factor

DAXX is an essential gene in mouse development: The loss of DAXX results in extensive apoptosis and embryonic lethality (Michaelson et al. 1999; Ishov et al. 2004). This embryonic lethality could be explained by the failure to deposit or target H3.3 correctly. In agreement with this, the absence of *H3.3A*, one of the two genes encoding H3.3, resulted also in mouse embryonic lethality (Couldrey et al. 1999).

Our results show that the majority of H3.3 is in a complex with DAXX, and the purification of H3.3 from the DAXX$^{-/-}$ cells supports the idea that, under some circumstances, other chaperones can substitute for DAXX and bind H3.3. Indeed, the H3.3 complex is enriched with NASP in the absence of DAXX. Since NASP was found to be a chaperone for H3/H4 (Supplemental Fig. 11; Wang et al. 2008), it suggests that this protein acts as a buffer for an excess of free H3.3. In addition, CAF-1 is also recruited within the H3.3 complex in DAXX$^{-/-}$ cells. This was quite surprising, since CAF-1 is considered to be a chaperone specific for H3.1 (Fig. 1B; Tagami et al. 2004). This indicates that a fraction of H3.3, in the absence of DAXX, could use the replication-dependent assembly pathway dedicated to H3.1. We hypothesize that DAXX prevents the association of H3.3 with CAF-1, thus not allowing the use of the replication-dependent assembly pathway by H3.3. Indeed, DAXX was found in both the cytoplasmic and the nuclear H3.3 complexes, whereas CAF-1 was present only in the nuclear H3.1 complex. Since DAXX associates with H3.3 in the cytoplasm, it is likely that the formation of a stable DAXX/H3.3 complex prevents further association of H3.3 with CAF-1.

H3.3, DAXX, ATRX, and transcriptional regulation of pericentric chromatin

Our results suggest that the deposition of H3.3 facilitates transcription from pericentric DNA repeats. In fact, although heterochromatin functions to silence transcription, transcripts spanning pericentric heterochromatin have been detected from fission yeast to mammals (Zaratiegui et al. 2007). While their role in mammals is still debated, these transcripts are required in fission yeast for the formation and maintenance of heterochromatin and for sister chromatid cohesion (Kato et al. 2005; Grewal and Jia 2007). Interestingly, depletion of mouse ATRX, the major partner of DAXX, leads to a defect in chromatid cohesion (Ritchie et al. 2008). Such a defect has not yet been reported for the DAXX-deficient cells, despite the description of shortened S-phase progression in these cells (Ishov et al. 2004). This could correspond to an alteration in heterochromatin formation, since replication of heterochromatin and S-phase progression are tightly interrelated (Quivy et al. 2008).

We still do not know the actual mechanism by which H3.3 facilitates transcription from pericentric DNA repeats. However, our results are in agreement with the recent report showing that down-regulation of H3.3 affects the induction of interferon-β-responsive genes (Tamura et al. 2009). This active role of H3.3 in transcription may be due, at least in part, to its ability to affect nucleosome stability, facilitating thereafter ejection of nucleosomes during the transcription process (Jin and Felsenfeld 2007).

One of the major DAXX-interacting partners is the ATP-dependent chromatin remodeling factor ATRX (Fig. 6; Xue et al. 2003; Tang et al. 2004). ATP-dependent chromatin remodeling factors use the energy derived from ATP hydrolysis to modulate histone–DNA contacts (Hamiche et al. 1999). They appear to function not only in remodeling of existing nucleosomes, but also in histone deposition during chromatin assembly. We found that ATRX is present on pericentric DNA repeats together with DAXX and that the reduction of ATRX expression by siRNA affects both the transcription and DAXX-dependent deposition of H3.3 onto these repeats. This indicates that ATRX assists DAXX in the deposition of H3.3 by using the energy provided by ATP hydrolysis. In agreement with this, the *Drosophila* ATRX homolog XNP has been shown recently to interact genetically and cytologically with H3.3 (Schneiderman et al. 2009).

DAXX and H3.3 in PML-NBs

Human pericentric DNA repeats have been shown to colocalize with DAXX and ATRX within the PML-NBs (Luciani et al. 2006). Our data suggest that a fraction of H3.3 is targeted to PML-NBs in a DAXX-dependent manner. Moreover, the knockdown of DAXX strongly affected transcription of pericentric DNA repeats and histone H3.3 deposition. Taken together, these data tend to demonstrate that PML-NBs could serve as specific structures that target H3.3 to pericentric DNA repeats. We cannot exclude, however, that these structures are the sites of H3.3 storage that could modulate the supply and demand of this histone. Indeed, PML-NBs appear to accumulate numerous proteins implicated in the regulation of chromatin conformation (Bernardi and Pandolfi 2007). Future studies will be necessary to understand in detail the connection between PML-NBs and H3.3.

HIRA and H3.3 deposition

Purification of the complexes responsible for the H3.1 and H3.3 deposition from epitope-tagged H3-expressing HeLa cell lines has revealed that H3.1 is found mainly within a complex containing CAF-1, whereas H3.3 copurified with a complex containing the HIRA protein (Tagami et al. 2004). Using a similar approach of purification by double affinity, we found that H3.1 is indeed associated with CAF-1. In contrast, our data suggest that HIRA is not a stable component of H3.3 complex, since it is retained after the first anti-Flag affinity, but is lost after the second anti-HA affinity (Supplemental Fig. 1). Rather, our results support the view that HIRA belongs to a histone-less complex containing ASF1a/b, Ubinuclein-1, Ubinuclein-2, and Cabin1.

Given the data showing a connection between HIRA and H3.3 in various remodeling processes, we cannot refute that HIRA can somehow be in contact with H3.3. As mentioned before, it was found, for instance, that HIRA assembles H3.3 nucleosomes during decondensation of the *Drosophila* sperm pronucleus (Loppin et al. 2005). However, according to the same investigators (Loppin et al. 2005; Bonnefoy et al. 2007), HIRA is not required for deposition of H3.3 in embryos or adult *Drosophila* tissues. The role of HIRA in DNA synthesis-independent nucleosome assembly in human cells was also challenged recently by work by Galvani et al. (2008). This supports the view that the HIRA/H3.3 interaction could be cell or tissue context-dependent, and that multiple pathways are involved in the deposition of H3.3.

We cannot exclude from our results the existence of distinct and possibly redundant pathways of H3.3 deposition that could involve HIRA. We found, indeed, that DAXX is required for assembly of H3.3 nucleosomes onto pericentric DNA repeats, while it is only partially involved in this assembly onto the active gene *DHFR* (although addition of DAXX further stimulates the efficiency of this process by a factor of nearly two). The purification of the H3.3 complex from DAXX-depleted cells tends to support the idea that cells adapt to deposit H3.3 at least on *DHFR*. Alternatively, one can propose the existence of distinct deposition machineries, including the DAXX complex and the HIRA complex, which can function in specialized genomic regions. However, it remains to be understood how HIRA contacts H3.3, since the HIRA/H3.3 interaction is probably not constitutive. Whether this interaction is regulated by post-translational modifications or by bridging proteins, such as ASF1, remains to be determined.

H3 is associated with distinct complexes into the cell

The biochemical purification of complexes containing unincorporated H3.1 or H3.3 offers new insights into the complexity of histone deposition pathways. H3 and H4 were found in complex with ASF1 in both the cytoplasm (the CC complex) (Fig. 2C) and the nucleus (the LNC complex) (Fig. 2C). Bearing in mind the previously reported data (English et al. 2006; Agez et al. 2007; Natsume et al. 2007), this complex should consist of a dimer of H3 and H4 and ASF1. In addition to the LNC, we identified in the nucleus another H3.1–H4 or H3.3–H4 complex, the HNC, which contains all four of the core histones in equimolar amounts (Fig. 2C). Our data do not us allow to discriminate whether this HNC comprises a full histone octamer $(H2A–H2B–H3–H4)_2$ or a heterotypic H2A–H2B–H3–H4 tetramer. We hypothesize that the HNC is the complex that is used to deposit histones onto DNA, and thus to assemble the nucleosome. If this is the case, the histones should be deposited as either a full histone octamer or a heterotypic tetramer, as was suggested recently for the *Drosophila* centromeric histone (Dalal et al. 2007). To our knowledge, there is as yet no compelling evidence against either possibility, since we still do not know the exact mechanism by which nucleosomes are assembled in vivo.

Materials and methods

Cell lines

H3.1, H3.3, DAXX, and HIRA proteins fused with C-terminal Flag- and HA-epitope tags (e-H3.1/e-H3.3/e-DAXX/e-HIRA) were stably expressed in cells by retroviral transduction (Ouararhni et al. 2006). The immortalized $DAXX^{-/-}$ MEF line was a kind gift of Dr. Gerd G. Maul (The Wistar Institute,) (Ishov et al. 2004).

Antibodies

Antibodies employed were as follows: monoclonal antibody anti-Flag M2 (Sigma); anti-HA 9E (Roche Diagnostics); anti-H3.3 (H00003021-M01, Abnova); anti-PARP-1 (Alexis); anti-H3 CT, pan (05-928, Upstate Biotechnologies); anti-H2B (07-371, Upstate Biotechnologies); polyclonal anti-NASP (ProteinTech Group); and polyclonal antibodies from Santa Cruz Biotechnology anti-DAXX (sc-7152), anti-ATRX (sc-15408), anti-CAF-1 p150 (sc-10772), anti-CAF-1 p60 (sc-10982), and anti-PML (sc-18425). Anti-polII was produced by the IGBMC facility. Anti-Asf1a is a kind gift of Dr. Carl Mann (Commissariat à l'énergie atomique de Saclay, France).

Double-immunoaffinity purification

Extracts were prepared using a modification of the Dignam protocol (Dignam 1990). Briefly, cells were lysed in hypotonic buffer (10 mm Tris-HCl at pH 7.65, 1.5 mm $MgCl_2$, 10 mm KCl) and disrupted by Dounce homogenizer. The cytosolic fraction was separated from the pellet by centrifugation at 4°C. The nuclear-soluble fraction was obtained by incubation of the pellet in high-salt buffer (to get a final NaCl concentration of 300 mM). Tagged proteins were immunoprecipitated with anti-Flag M2-agarose (Sigma), eluted with Flag peptide (0.5 mg/mL), further affinity-purified with anti-HA antibody-conjugated agarose, and eluted with HA peptide (1 mg/mL). The HA and Flag peptides were first buffered with 50 mM Tris-Cl (pH 8.5), then diluted to 4 mg/mL in TGEN 150 buffer (20 mM Tris at pH 7.65, 150 mM NaCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0 0.01% NP40), and stored at −20°C until use. Between each step, beads were washed in TGEN 150 buffer. Complexes were resolved by SDS-PAGE and stained using the Silver Quest kit (Invitrogen).

Identification of proteins was carried out using an ion-trap mass spectrometer (ThermoFinnigan LTQ-XL) or by Taplin Biological Mass Spectrometry Facility (Harvard Medical School, Boston, MA).

For glycerol density gradient, samples were loaded onto a 4.5-mL glycerol gradient (17%–42%) and spun at 300,000*g* in a Beckman SW50.1 rotor for 2 h. Fractions were collected from the bottom of the tube. The approximate molecular weight of the different subcomplexes was estimated using the NativeMark molecular weight standard (Invitrogen).

Immunofluorescence

Immunofluorescence was done using standard procedures on a Leica DMR microscope (Leica) using a 63 × 1.32 NA oil immersion objective.

Preparation of recombinant DAXX

The full-length and deletions mutants of DAXX were PCR-amplified from pcDNA3-HA-DAXX plasmid (a kind gift of Dr. Hsiu-Ming Shih, Academia Sinica, Taiwan) and subcloned into pGEX-5X.1 vector (GE Healthcare). GST fusion proteins were purified by standard methods.

Histones

Human histones H3.1, H3.3, and H4 cDNA sequences were PCR-amplified by using Vent-DNA polymerase (New England Biolabs). All of the histones were cloned in a homemade bicistronic pET28b vector (Clontech). H3.1 and H3.3 were cloned at the NdeI–BamHI sites of pET28b in frame with an N-terminal His tag, while RBS-containing Flag-tagged H4 was cloned at the EcoRI–NotI sites. PCR-generated cDNA were sequenced for verification. BL21-CodonPlus-RIL cells (Stratagene) were routinely grown at 37°C on LB medium plus 0.1% glucose and selective pressure (kanamycin and chloramphenicol). Expression was induced at an A600 of 0.6 by addition of isopropyl β-D-thiogalactoside to a final concentration of 1 mM, and the culture was incubated for 2 h at 30°C. Histones were purified using agarose anti-Flag M2 resin (Sigma). Purified histones were stored at −80°C until use.

Epitope-tagged (eH3.1/eH3.3–H4)$_2$ tetramers were prepared from HeLa cells expressing e-H3.1 and e-H3.3. Briefly, HeLa cell nuclei were digested with micrococcal nuclease to give predominantly mononucleosomes. Tagged mononucleosomes were next purified by the double-immunoaffinity method. Eluted material was bound to hydroxyapatite resin (Bio-Rad). Resin was washed successively with 0.65, 0.9 M NaCl, and bound (H3–H4)$_2$ tetramers were eluted with 2 M NaCl.

Protein–protein interactions

GST fusion proteins immobilized on glutathione Sepharose were incubated for 1 h at room temperature with tagged (H3–H4)$_2$ tetramers or recombinant histones in TGN buffer (20 mM Tris at pH 7.65, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 0.01% NP40) containing 250 mM NaCl. Beads were then washed extensively in TGN containing 250 mM, 500 mM, or 1 M NaCl. Bound proteins were eluted in SDS sample buffer and fractionated on SDS-PAGE. Native histones were probed with anti-HA antibody, while recombinant histones were stained by Coomassie blue.

To study the interaction between GST-tNASP and histones, GST-tNASP was coexpressed with the bicistronic H3.1–H4 or H3.3–H4 in *Escherichia coli* strain BL21-CodonPlus-RIL-pLysS (Stratagene), as described (Shuaib et al. 2010).

Transfection, synchronization, and RNAi

MEF cells were transiently transfected using a standard calcium phosphate method. For synchronization, cells were starved for 48 h in DMEM containing 0.5% FCS and were reinduced by the addition of 20% FCS, as described previously (Daury et al. 2006).

For siRNAs experiments, MEF cells were seeded onto six-well plates and transfected using Hiperfect (Qiagen) with a siRNA pool (Dharmacon) directed against *H3.3A* and *H3.3B* mRNA or an irrelevant siRNA (25 nM final). Cells were harvested 72 h post-transfection for the assessment of the expression level of endogenous *H3.3A* and *H3.3B* mRNA, and of transcripts from pericentric repeats by real-time quantitative PCR analysis.

The sequences of the siRNA were as follows: *mH3.3A*: ACGCGGAGAACGUGCUUAA, GCCAAACGUGUAACAAUUA, GUAAAGCACCCAGGAAACA, GUGAAGAAACCUCAUCGUU; *mH3.3B*: UGAGAGAGAUCCGUCGUUA, CCAGUUGGCUCGC CGGAUA, GAACCAAGCAGACCGCUAG, CACCAAGGCGGC UCGGAAA; *mATRX*: GGAAAGUGGAUCCGAAAUA, GUACAG AAAUCUCGCUCAA, AGAAAUGCAUCCUGCGCAA, AGAGA AGAAUGGCCGUAAA; *mHIRA*: ACGCAUGUUCUCCGGCU UA, CUCUCAAGCUGAUGAUCGA, CUUGGGAUCCCGUU GGUAA, CAGUCACGGGUGUGGUCAA; irrelevant siRNA: GCCGGUAUGCCGGUUAAGUTT.

Retrotranscription and real-time quantitative PCR

Total RNAs were purified using standard methods and cDNA was synthesized by random priming. Real-time quantitative PCR was done with the QuantiTect SYBR green PCR kit (Qiagen) and a LightCycler apparatus (Roche Diagnostics). Primer pairs used were ACGTGCTTAAGAGTCCACTA and TTCCACTCG CAATCATATAC for *H3.3A*, GGCTGGTAACACAACACTAA and AGATGATGCTGGTGTGAATA for *H3.3B*, GACGACTT GAAAAATGACGAAATC and CATATTCCAGGTCCTTCAG TGTGC for pericentric repeats (Lehnertz et al. 2003), and CATGGCCTTCCGTGTTCCTA and TGCCTGCTTCACCAC CTTCT for *GAPDH*. Results were normalized to *GAPDH*.

ChIP

ChIP assays were performed as described in Drane et al. (2004). Primer pairs used were GACGACTTGAAAAATGACGAAATC/ CATATTCCAGGTCCTTCAGTGTGC for pericentric DNA repeats (Lehnertz et al. 2003), GGTACCAGGAAGACATGAGA/ TGTAGTGCCTTCCAGTAACC for *DHFR*, and CCAATGTGT CCGTCGTGGATCT/GTTGAAGTCGCAGGAGACAACC for pseudo*GAPDH* (Daury et al. 2006). Results were normalized to input DNA.

Histone deposition assay

Assay of histone deposition in the presence of the histone chaperone DAXX was performed using a negatively supercoiled DNA topoisomer −1 prepared from the 359-bp hsp70 promoter. This fragment was purified from an EcoRI digest of the plasmid pBSK359x3 (Hamiche et al. 1999), ^{32}P end-labeled, and circularized in the presence of ethidium bromide (Hamiche and Richard-Foy. 1999). Recombinant (H3.1–H4)$_2$ and (H3.3–H4)$_2$ tetramers (100 ng/μL) were purified as described above. (H3.1–H4)$_2$ and (H3.3–H4)$_2$ were mixed or not with equimolar amounts of DAXX in 20 mM Tris-Cl (pH 7.5), 50 mM NaCl, and 3 mM $MgCl_2$, and were incubated for 30 min at room temperature. Fifty nanograms of labeled circular DNA corresponding to topoisomer −1 was added to each mixture (at the indicated histone to DNA ratio, rw), incubated for 30 min at 37°C, and analyzed on 4.5% native polyacrylamide gel 0.5× TG (0.025 M Tris, 0.192 M glycine), run at room temperature. Control tetrasomes were assembled on circular DNA according to the "salt jump" method as described in Hamiche and Richard-Foy (1999).

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Supplemental Material

"**The death-associated protein DAXX is a novel histone chaperone involved in the replication-independent deposition of H3.3.**"

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Supplemental Figure Legends

Supplemental Figure 1

(A) Schematics of the TAP-tag approach used for the purification of e-H3.3 complex. Histone H3.3 was stably expressed as fusion proteins with C-terminal FLAG- and HA-epitope tags in HeLa cells. Epitope-tagged H3.3 (e-H3.3) preassembly complex was then purified from nuclear soluble extracts by immunoprecipitation with anti-FLAG antibody followed or not by anti-HA antibody. Protein partners associated with e-H3.3 after the first (anti-Flag) or the second (anti-HA) purification step were fractionated on SDS-PAGE and silver stained (B) or probed with anti-HIRA (C).

(B) Silver staining of proteins associated with e-H3.3 after the first (lane 1) or the second affinity purification step (lane 2). The asterisks indicate proteins lost after the second purification step.

(C) Western blot analysis of HIRA in the e-H3.3 complex after the first anti-FLAG affinity purification (lane 1) or the second anti-HA affinity purification (lane 2) using an anti-HIRA antibody. As control, increasing amounts of e-HIRA complex were also blotted (lanes 3-5). M, molecular weight marker.

Supplemental Figure 2

(A) Schematics of the TAP-tag approach used for the purification of e-H3.3 complex from the cytosolic, nuclear or chromatin fractions of HeLa cells. e-H3.3-containing complex was purified by double immunoaffinity from cytosolic, nuclear or chromatin extract of HeLa cells stably expressing FLAG-HA tagged H3.3. After the second affinity purification and HA peptide elution, beads were collected and boiled in 2% SDS-containing buffer to elute residual bound proteins.

(B) Silver staining analysis of H3.3-associated proteins purified as described in A after the elution by HA-peptide (lanes 1-3) or by boiling in SDS after HA-elution (lanes 4-6). M, molecular weight marker.

Supplemental Figure 3

Partial alignment of *homo sapiens* DAXX protein (Acc number NP_001341.1) with *saccharomyces cerevisiae* rtt106 protein (Acc number NP_014193.1) using CLUSTAL 2.0.12.

Supplemental Figure 4

Coomassie blue staining of both H3.1.com (lane 2, top) and H3.3.com (lane 3, top) purified from nuclear extract of HeLa cells stably expressing H3-tagged proteins. Lane 1 (*top*), acid-extracted core histones from HeLa cells chromatin. Samples were also probed with anti-H3 pan (*middle panel*) or with anti-H2B (*bottom panel*).

Supplemental Figure 5

DNA does not mediate the presence of the four core histones within the H3.3 complex.

(A) Description of the experimental procedure. e-H3.3-containing complex was purified by a first anti-Flag affinity from nuclear extract of HeLa cells stably expressing H3.3-tagged protein. Equal amounts of purified material were then non-treated (lane 1) or treated with DNAse I (50U/ml) (lane 2) or with Ethidium bromide (1μg/ml) (lane 3) prior to a second anti-HA purification step.

(B) Silver staining of H3.3.com non-treated (lane 1) or treated with DNAse I (lane 2) or Ethidium Bromide (Lane 3). The position of histones is indicated.

Supplemental Figure 6

(A) DAXX preferentially associates with H3.3 *in vitro*. GST-DAXX, immobilized on glutathione-agarose, was incubated with purified tetramers containing either epitope-tagged H3.1 (lanes 1-3) or H3.3 (lanes 4-6). Bead-bound tetramers were washed with the indicated concentration of NaCl. Eluted proteins were analyzed by immunoblotting with anti-HA antibody (top). Blue staining of the top of the gel (bottom) is shown for comparing levels of GST-DAXX used. The input lanes (INP) represent 30% of the amount of tetramers used for the pulldown.

(B) Epitope-tagged H3.1 (lanes 1-3) or H3.3 (lanes 4-6) native tetramers immobilized on anti-FLAG agarose, were incubated with *in vitro* translated ^{35}S-DAXX. Bead-bound proteins were washed with the indicated concentrations of NaCl. Eluted proteins were analyzed by SDS-PAGE, and dried gels exposed for autoradiography. The input lane (INP) represents 10% of the amount of ^{35}S-DAXX used for the pulldown. Blue staining of the gel (bottom) is shown to compare level of e-H3.1- and e-H3.3-containing tetramers used. Note that the tetramers were isolated from stable cell lines expressing either e-H3.1 or e-H3.3, and thus they also contain endogenous (non-tagged) H3.1 and H3.3.

Supplemental Figure 7

Western blot analysis of NASP and ASF1a in HeLa nuclear extract (40μg) (lane 1 and 5) and in H3.3.com (lane 2), DAXX.com (lane 3) and HIRA.com (lane 4) purified by double immunoaffinity from nuclear extract of HeLa cells stably expressing e-H3.3-, e-DAXX- and e-HIRA-tagged proteins, respectively.

Supplemental Figure 8

Identification of H3.3 within the Daxx complex by "mass fingerprinting" of chymotryptic peptides. The band corresponding to H3.3 in the DAXX complex was excised from the gel, digested with chymotrypsin and analyzed by mass spectrometry using an LTQ-Orbitrap (Thermo-Finnigan).

Supplemental Figure 9

Specificity of the anti-H3.3 antibody used in this study. Purified recombinant histones (0.5, 0.2 and 0.1μg) H3.1-H4 (lanes 1-3) and H3.3-H4 (lanes 4-6) were Coomasie blue stained (top) or blotted with the anti-H3.3 antibody (Abnova, H00003021-M01) (bottom).

Supplemental Figure 10

DAXX poorly facilitates the deposition of $(H3.1\text{-}H4)_2$ tetramers onto DNA. Negatively supercoiled DNA corresponding to topoisomer -1 was incubated with increasing amount of $(H3.1\text{-}H4)_2$ tetramers (at the indicated histone/DNA ratio, rw) either in presence (lanes 7-9) or absence (lanes 4-6) of equimolar (to the tetramers) amounts of GST-DAXX. The reaction

products were then analyzed on native 4.5% polyacrylamide gel. The reconstitution by salt dialysis of tetrasomes (lane 1) and nucleosomes (lane 2) on topoisomer -1 is shown. Lane 3, topoisomer -1 DNA. Positions of the open circular DNA (OC), naked topoisomer-1 DNA, tetrasome and nucleosome are indicated.

Supplemental Figure 11

The histone chaperone tNASP strongly interacts with histones and do not discriminate between H3.1-H4 and H3.3-H4.

(A) Description of the experimental procedure. GST-tNASP fusion protein was coexpressed with the bicistronic H3.1-H4 (or H3.3-H4) in *E. Coli* strain BL21-CodonPlus-RIL after addition of IPTG. Complexes were purified from soluble extract using glutathione-Sepharose 4B.

(B) GST-tNASP coexpressed with histones H3.1-H4 (lanes 3-5) or with H3.3-H4 (lanes 7-9) was pulled-down from bacterial extract as described in A. Bead-bound complexes were washed with the indicated concentration of NaCl. Eluted proteins and total proteins from IPTG-induced bacterial extract (I) and of non-induced bacterial extract (NI) were separated on SDS-PAGE and stained with Coomassie blue. Asterisks indicate the position of GST-tNASP, H3.1 (or H3.3) and H4 within the extracts.

Supplemental Figure 12

(A) and (B) Cell cycle profile of WT (A) and DAXX-/- (B) MEF cells was determined using Propidium Iodide staining followed by flow cytometry analysis. Percentage of SubG1 cells was indicated. Mean ± standard deviation of 3 independent samples.

(C) Western blot analysis of CAF-1 p150, DAXX and β–actin in whole-cell extracts prepared from WT and DAXX-/- MEFs.

Supplemental Figure 13

(A) DAXX, ATRX and polymerase II (polII) are present on *DHFR* (+8274/+8419) in WT MEF cells. Presence of DAXX, ATRX and polII on *DHFR* was investigated by ChIP assays using specific antibodies. (-Ab) corresponds to a control sample in which primary antibody was omitted. Results are expressed as percentage of chromatin input used for immunoprecipitation.

(B) The recruitment of DAXX, ATRX and polymerase II (polII) on pseudo*GAPDH* was investigated as described in (A).

(C) DAXX favors the deposition of H3.3 onto *DHFR* during G1-progression. DAXX-/- MEF cells were deprived of serum for 48 h before being co-transfected with empty vector (CTRL) or else epitope-tagged H3.1 or H3.3 expression vector in combination with DAXX expression vector where indicated. Forty-hours later, cells were re-induced for 8 h with 20% FCS in presence of aphidicolin and were subjected to ChIP assays. Results are expressed as percentage of chromatin input immunoprecipitated. Mean ± standard deviation of 3 independent experiments.

(D) DAXX does not favor the deposition of H3.3 on pseudo*GAPDH* during G1-progression. Experiments were conducted as described in C. Results are expressed as percentage of chromatin input immunoprecipitated. Mean ± standard deviation of 3 independent experiments.

Supplemental Figure 14

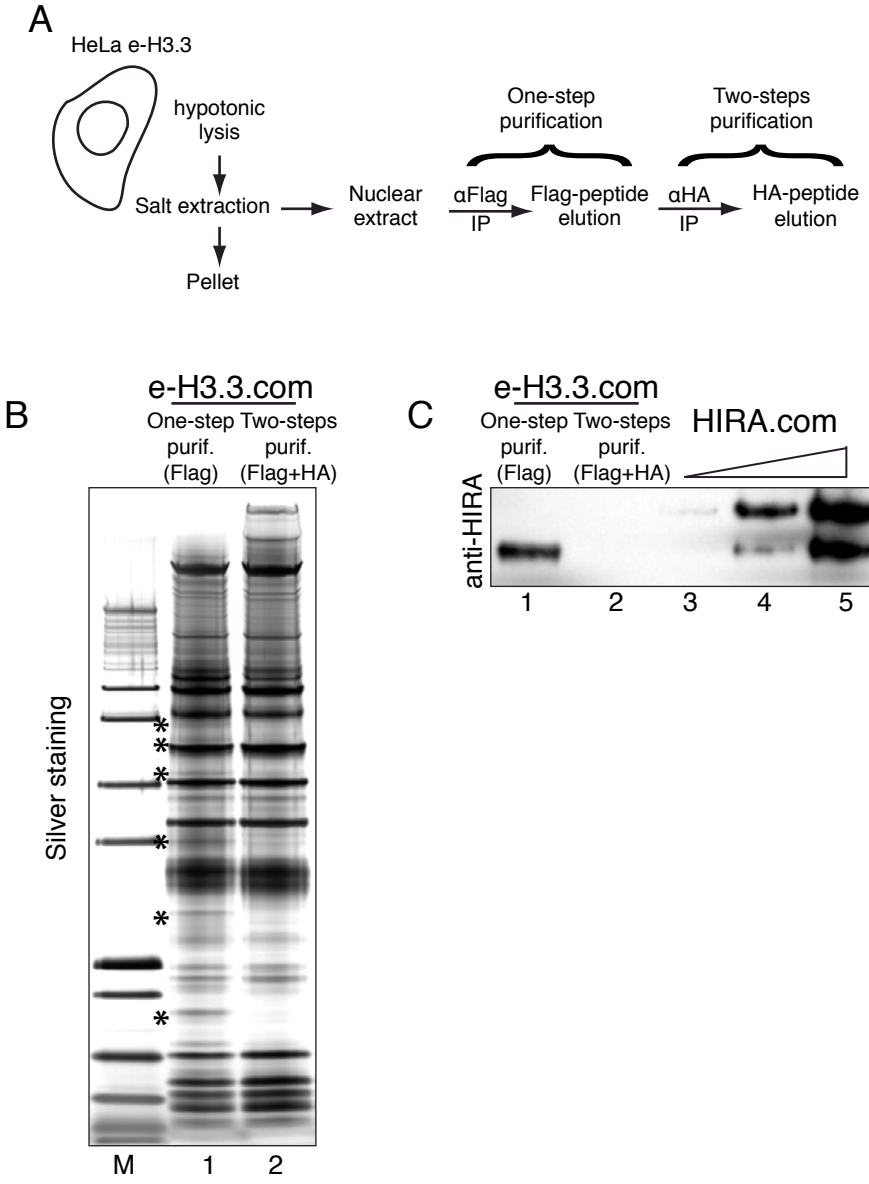
(A) Reduction of ATRX expression diminishes DAXX-dependent deposition of H3.3 onto pericentric DNA repeats. DAXX-/- MEFs were transfected with a siRNA directed against ATRX in complete medium. The day after, cells were transfected with the indicated plasmids in medium

containing 0.5% FCS. Forty-eight hours latter, cells were re-induced for 8 h with 20% FCS in presence of aphidicolin and were subjected to ChIP assays. Results are expressed as percentage of chromatin input immunoprecipitated.

(B) Western blot analysis of e-DAXX and e-H3.3 tagged proteins and of endogenous ATRX in extracts from the transfected cells described in A.

(C) siRNA ablation of ATRX but not HIRA impaired pericentric DNA repeats transcription. The level of transcripts from pericentric DNA repeats is reduced in MEF cells ablated for ATRX but not HIRA. Relative mRNA level for pericentric DNA repeats in WT and ATRX or HIRA knocked-down MEF cells were determined by quantitative RT-PCR. Results are represented as relative expression level of pericentric DNA repeats versus *GAPDH*. Mean ± standard deviation of 3 independent experiments.

(D) Western blot analysis of HIRA knock-down using specific siRNA pool.


A
HeLa e-H3.3
hypotonic lysis
Salt extraction
Pellet
Nuclear extract
One-step purification
αFlag IP
Flag-peptide elution
Two-steps purification
αHA IP
HA-peptide elution
B
e-H3.3.com
One-step purif. (Flag)
Two-steps purif. (Flag+HA)
Silver staining
M
1
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1
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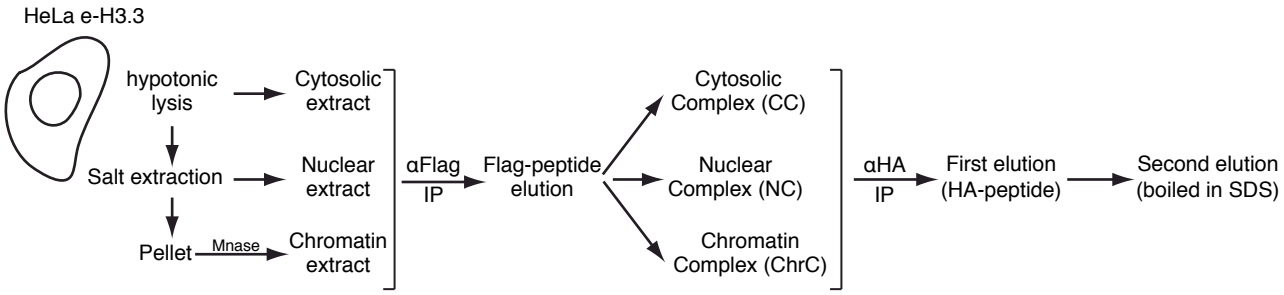
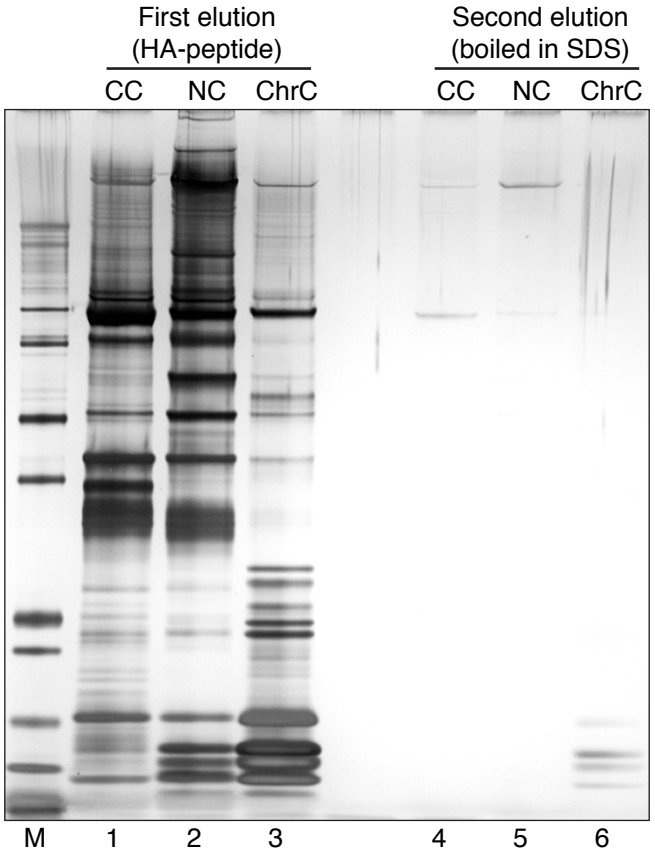
Drané_Sup-Fig. 2

A



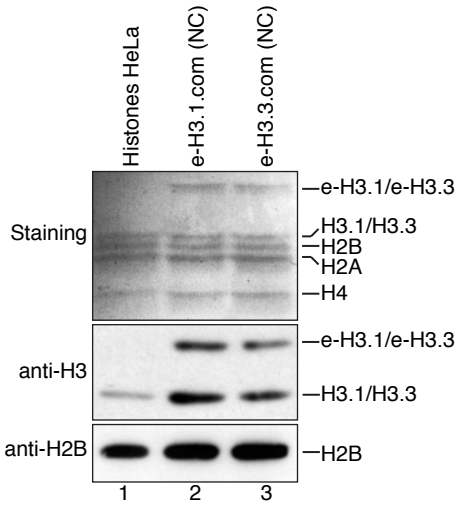
B

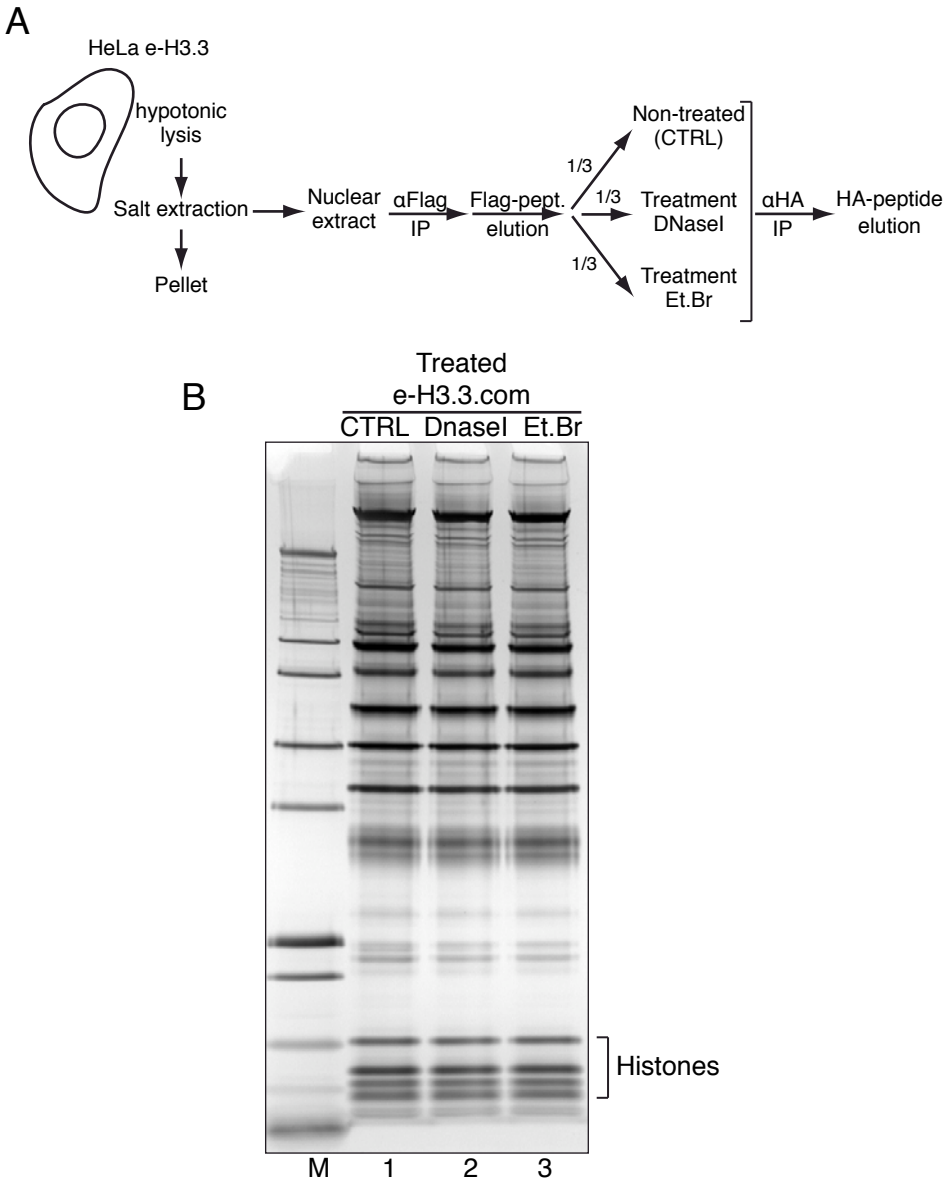


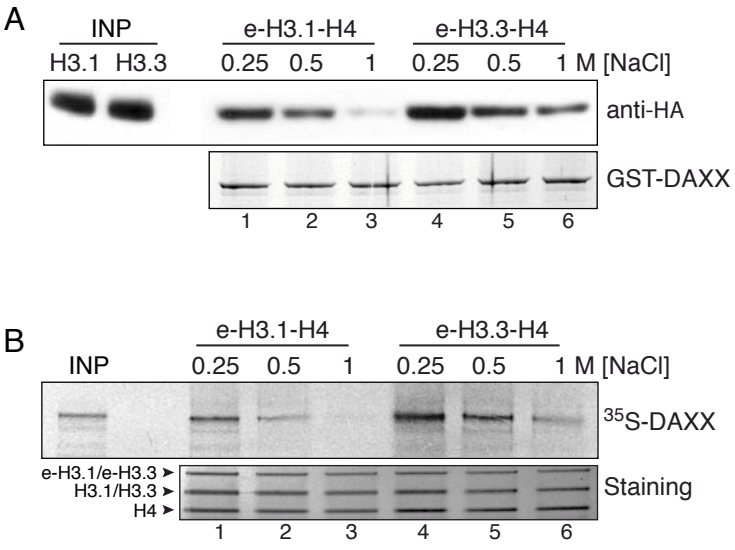
DAXX/Rtt106 sequence alignment (CLUSTAL 2.0.12)

```
DAXX       302 -LGLPRQQLQLMAQDAFRDVGIRLQ-------------ERRHLDLIYNFGCHLTDDYRPGV
Rtt106     242 -FGFKKPILLFDASDIESITYSSITRLTFNASLVTKDGEKYEFSMIDQTEYAKIDDYVK--
               :*: :  * : *.*    .   :              *: .:.:* :      ***

DAXX            DPALSDPVLARRLRENRSLAMSRLDEVISKYAMLQDKSEEGERKKRRARLQGTSSHSADT
Rtt106          RKQMKDKSMSEELKAKSKSKGQATDGTADQPSILQEATRQMQDEKKAGVFSDDDEENDQN
                   :.*  ::..*: : .   .  * . .: ::**: :.: : :*: . :.. .... :.

DAXX            PEASLDSGEGPSGMASQGCPSASRAETDDEDDEESDEEEEE-------------EEEEEE
Rtt106          FEAESDLSDGSGQESSDGAEDGEEAEEDDEEDDEEEDKKGQSALNRDNSFASINGQPEQE
                 **. * .:*..  :*:*. ....** ***:*:*.:::: :             : *:*

DAXX            EEATDSEEEEDLEQMQEGQEDDEEEDEEE- 484
Rtt106          LQYKEFKEPLELEDIPIEIDNDDDEDDED- 448
                 : .: :*  :**::    ::*::**:*:
```

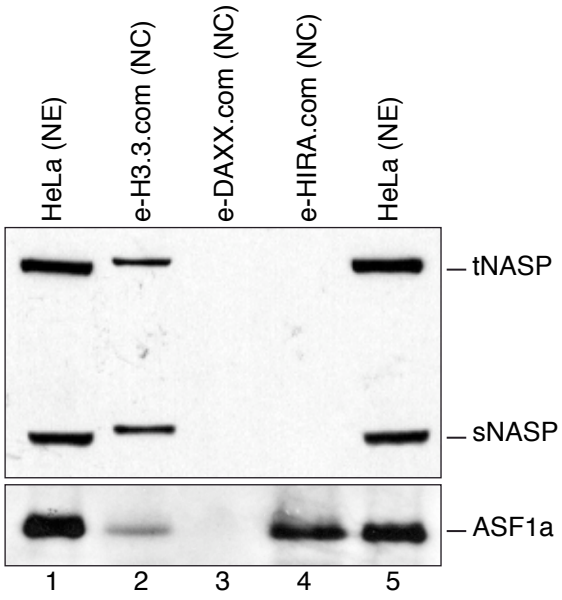
Drané_Sup-Fig. 4




A
HeLa e-H3.3
hypotonic lysis
Salt extraction
Pellet
Nuclear extract
αFlag IP
Flag-pept. elution
1/3
1/3
1/3
Non-treated (CTRL)
Treatment DNaseI
Treatment Et.Br
αHA IP
HA-peptide elution
B
Treated e-H3.3.com
CTRL DnaseI Et.Br
Histones
M 1 2 3


A
INP
e-H3.1-H4
e-H3.3-H4
H3.1 H3.3
0.25 0.5 1
0.25 0.5 1 M [NaCl]
anti-HA
GST-DAXX
1 2 3 4 5 6
B
e-H3.1-H4
e-H3.3-H4
INP
0.25 0.5 1
0.25 0.5 1 M [NaCl]
35S-DAXX
e-H3.1/e-H3.3
H3.1/H3.3
H4
Staining
1 2 3 4 5 6

Drané_Sup-Fig. 7



Identification of H3.3 within the DAXX complex by "mass fingerprinting" of chymotryptic peptides

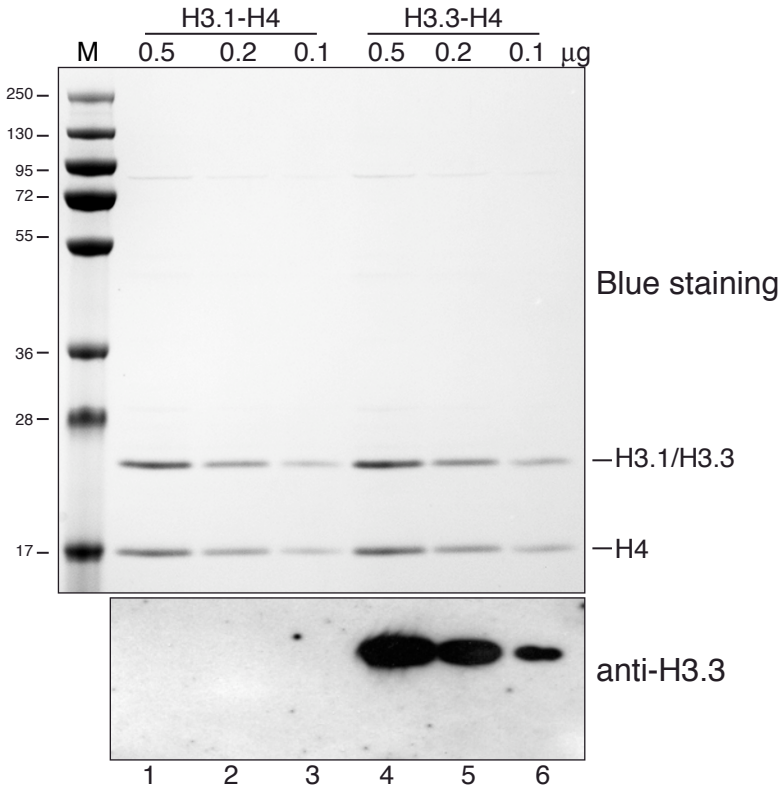
```
       1                                                  50
H3.3   MARTKQTARK STGGKAPRKQ LATKAARKSA PSTGGVKKPH RYRPGTVALR
H3.1   ---------- ---------- ---------- -A-------- ----------

       51                                                100
H3.3   EIRRYQKSTE LLIRKLPFQR LVREIAQDFK TDLRFQSAAI GALQEASEAY
H3.1   ---------- ---------- ---------- -------S-V M-----C---

       101                                 136
H3.3   LVGLFEDTNL CAIHAKRVTI MPKDIQLARR IRGERA
H3.1   ---------- ---------- ---------- ------
```

Position	MH+	Sequence	Histone
56-61	705.7862	QKSTEL	H3.1/H3.3
63-68	773.9969	IRKLPF	H3.1/H3.3
80-85	779.9145	KTDLRF	H3.1/H3.3
56-62	818.9456	QKSTELL	H3.1/H3.3
43-50	870.0427	RPGTVALR	H3.1/H3.3
62-68	887.1563	LIRKLPF	H3.1/H3.3
80-86	908.0452	KTDLRFQ	H3.1/H3.3
120-127	958.2041	IMPKDIQL	H3.1/H3.3
72-79	978.0929	VREIAQDF	H3.1/H3.3
41-49	1033.2187	RYRPGTVAL	H3.1/H3.3
128-136	1084.6446	ARRIRGERA	H3.1/H3.3
101-110	1121.2755	LVGLFEDTNL	H3.1/H3.3
69-79	1375.5705	QRLVREIAQDF	H3.1/H3.3
87-100	1381.4820	SAAIGALQEASEAY	H3.3
86-100	1509.6127	QSAAIGALQEASEAY	H3.3
101-114	1545.7936	LVGLFEDTNLCAIH	H3.1/H3.3
22-40	1893.1998	ATKAARKSAPSTGGVKKPH	H3.3
80-100	2270.5040	KTDLRFQSAAIGALQEASEAY	H3.3

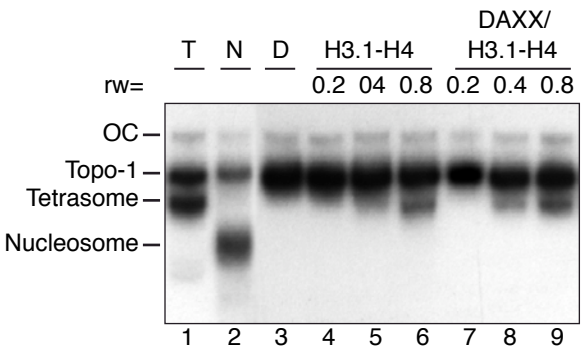
Protein coverage by amino acid count: 105/136 = 77.2%
Protein coverage by mass: 11822/15319 = 77.1%

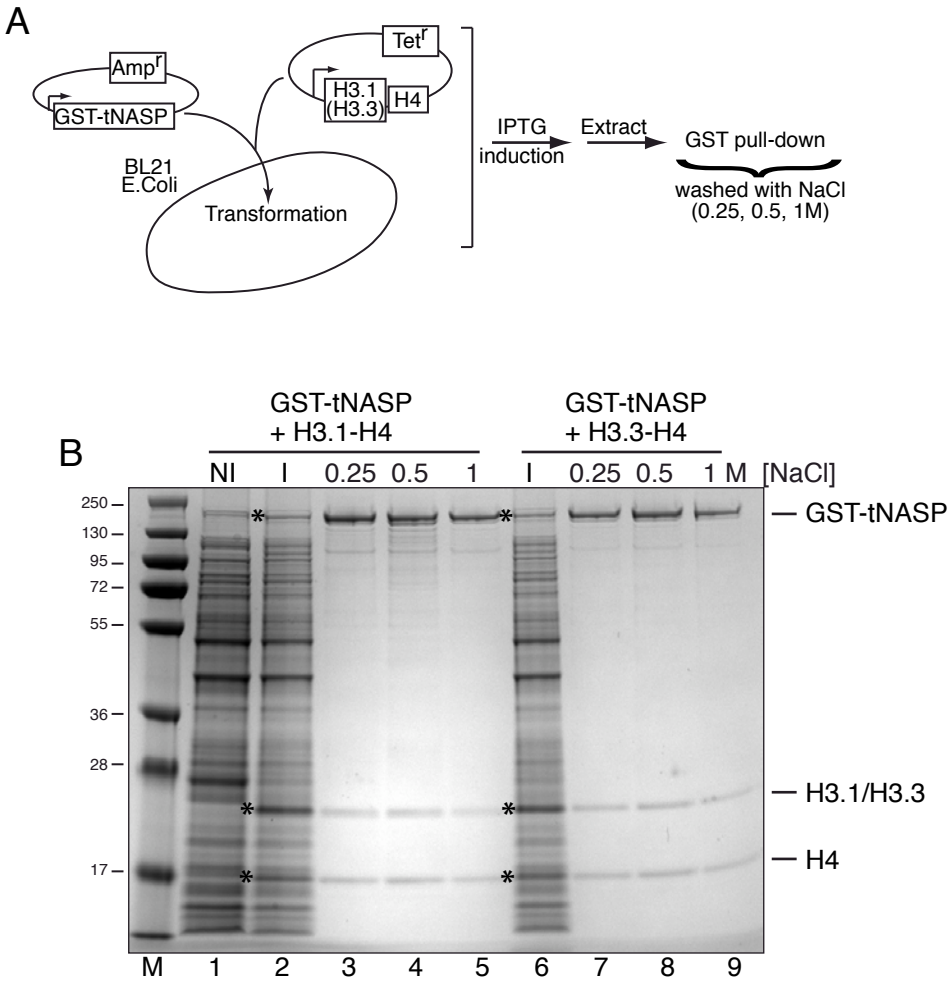
No specific-H3.1 peptide detected

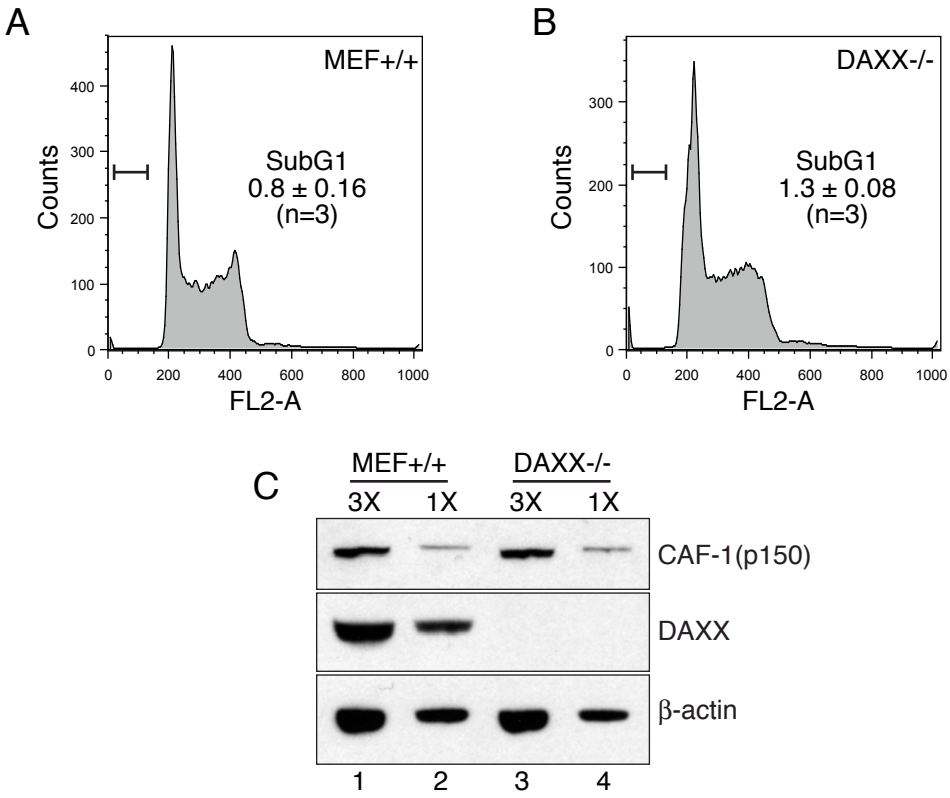
Drané_Sup-Fig. 9



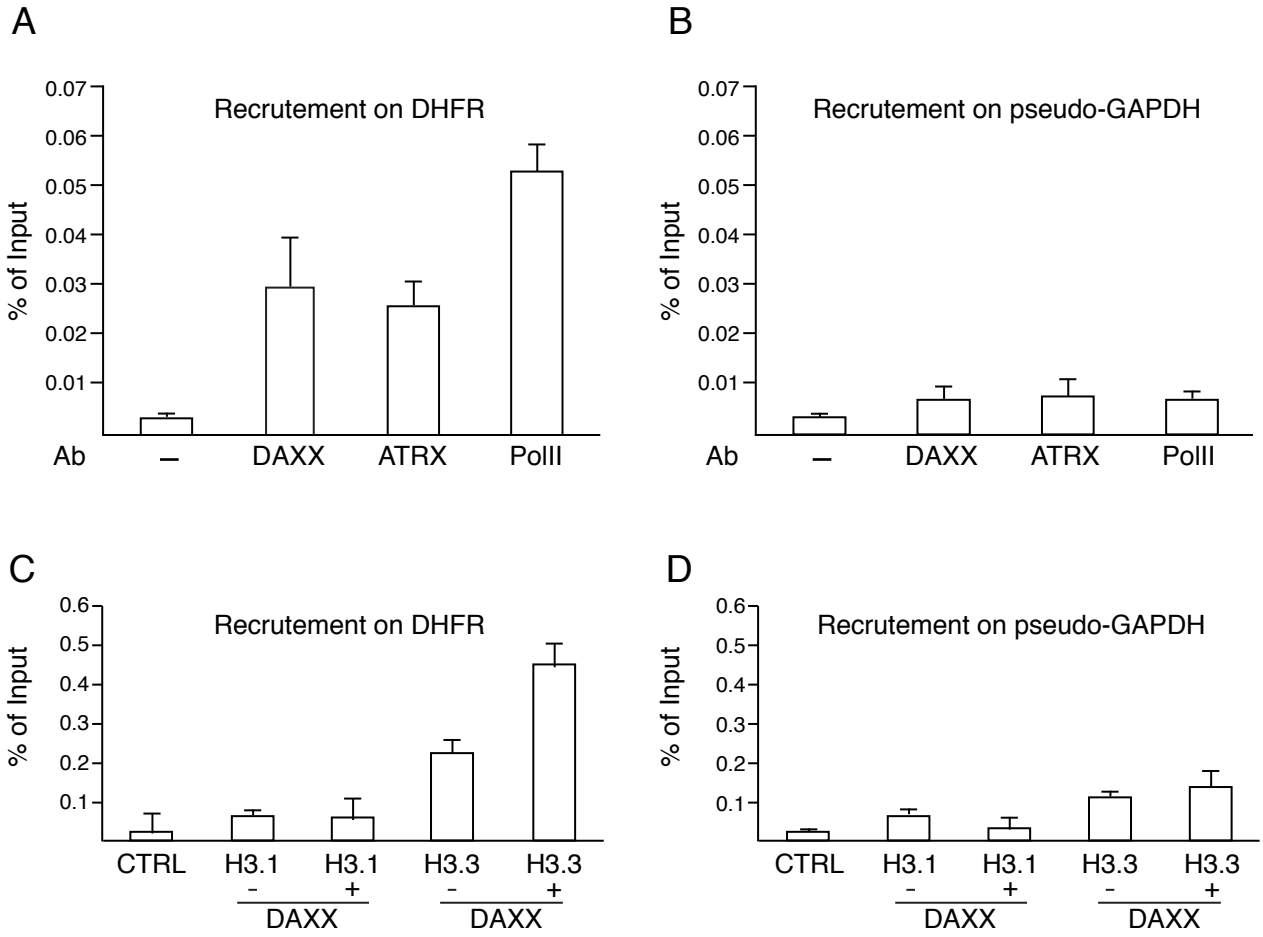
Drané_Sup-Fig. 10

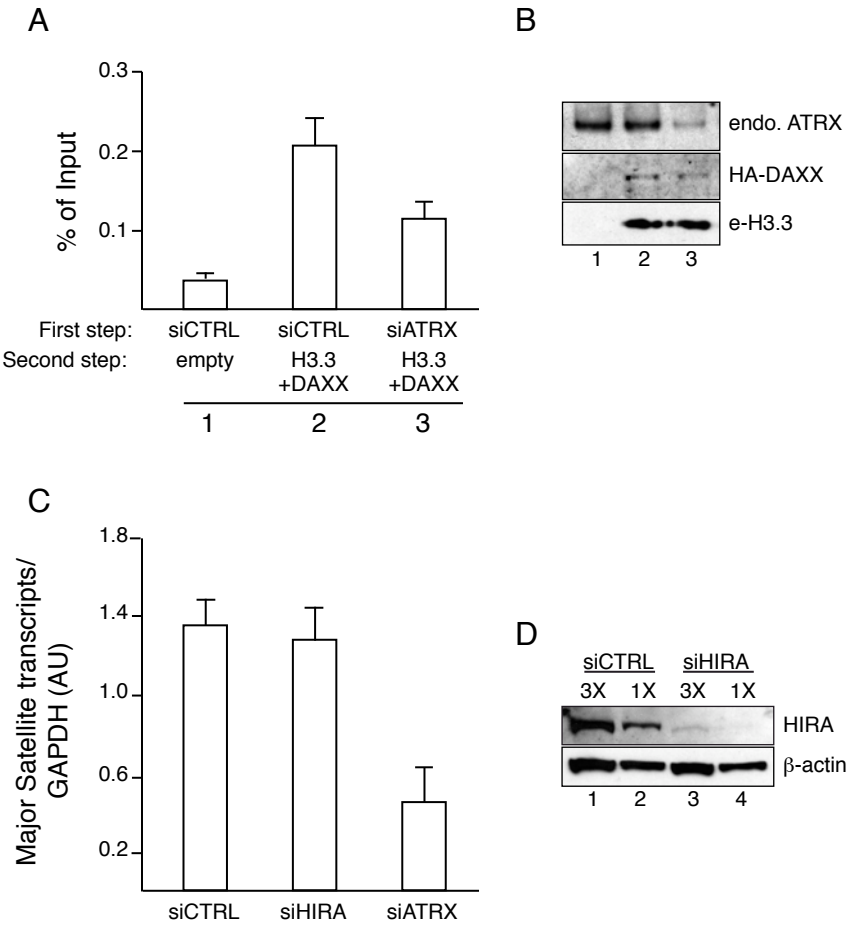



A
Amp^r
GST-tNASP
Tet^r
H3.1
(H3.3)
H4
BL21
E.Coli
Transformation
IPTG
induction
Extract
GST pull-down
washed with NaCl
(0.25, 0.5, 1M)
B
GST-tNASP
+ H3.1-H4
GST-tNASP
+ H3.3-H4
NI I 0.25 0.5 1 I 0.25 0.5 1 M [NaCl]
250
130
95
72
55
36
28
17
— GST-tNASP
— H3.1/H3.3
— H4
M 1 2 3 4 5 6 7 8 9


A
MEF+/+
SubG1
0.8 ± 0.16
(n=3)
Counts
FL2-A
B
DAXX-/-
SubG1
1.3 ± 0.08
(n=3)
Counts
FL2-A
C
MEF+/+
DAXX-/-
3X
1X
3X
1X
CAF-1(p150)
DAXX
β-actin
1
2
3
4

Drané_Sup-Fig. 13




A
B
0.3
0.2
0.1
% of Input
First step:
Second step:
siCTRL
empty
siCTRL
H3.3 +DAXX
siATRX
H3.3 +DAXX
1
2
3
endo. ATRX
HA-DAXX
e-H3.3
1 2 3
C
D
1.8
1.4
1.0
0.6
0.2
Major Satellite transcripts/ GAPDH (AU)
siCTRL
siHIRA
siATRX
siCTRL
siHIRA
3X 1X 3X 1X
HIRA
β-actin
1 2 3 4

Appendix B: PAPER 3

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Review

Chaperoning the histone H3 family ☆

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ABSTRACT

Chromatin is a highly dynamic nucleoprotein structure, which orchestrates all nuclear process from DNA replication to DNA repair, from transcription to recombination. The proper *in vivo* assembly of nucleosome, the basic repeating unit of chromatin, requires the deposition of two H3–H4 dimer pairs followed by the addition of two dimers of H2A and H2B. Histone chaperones are responsible for delivery of histones to the site of chromatin assembly and histone deposition onto DNA, histone exchange and removal. Distinct factors have been found associated with different histone H3 variants, which facilitate their deposition. Unraveling the mechanism of histone deposition by specific chaperones is of key importance to epigenetic regulation. In this review, we focus on histone H3 variants and their deposition mechanisms. This article is part of a Special Issue entitled:Histone chaperones and Chromatin assembly.

1. Introduction

The genome in eukaryotic cell is composed of DNA, histone and non-histone proteins, which are assembled into highly compact structure known as chromatin. The basic building block of chromatin, the nucleosome, contains 147 bp of DNA wrapped around an octamer of the four core histones (H2A, H2B, H3 and H4) in ~1.7 super helical turns. The nucleosomes are connected with linker DNA and the resulting structure is called the 10 nm chromatin filament. The 10 nm filament further compacts into the 30 nm fiber through interaction with linker histones. The higher order chromatin structures are formed upon folding of the 30 nm fibers [1]. Despite this high level of compaction, eukaryotic chromatin is highly dynamic and allows access to the DNA template during various vital cellular processes. This dynamic nature of chromatin structure is regulated by different protein factors, including histone chaperones, ATP-dependent chromatin remodeling factors, histone variants, histone post-translational modifications (acetylation, methylation and phosphorylation) [2] and still many other unknown factors.

Histone variants are non-allelic isoforms of conventional histones. All histones, except histone H4, possess histone variants. The family of H3 histones includes the conventional histones H3.1, H3.2 and the histone variants H3t, H3.3, CENP-A (Centromere Protein A), H3.X, H3.Y and H3.5 (Fig. 1).

The deposition of histones in chromatin is assisted by histone chaperones. In the context of chromatin assembly chaperones can be defined as histone binding proteins responsible for the safe delivery of histones to DNA without being part of the final reaction product. After the discovery in 1978 [3] by Laskey of nucleosoplasmin, the first chaperone, a variety of chaperones have been identified and characterized. Chaperones play a role in histone deposition on DNA in replication dependent and replication independent manner, but are also implicated in their storage, transfer, exchange and removal. Moreover, chaperones prevent the non-specific and deleterious interaction of histones with other factors and DNA. In this review, we focus on the role of histone chaperones in the supply and the deposition of histone H3 proteins on DNA.

2. Histone H3 family: conventional and variant histones

Human core histones are encoded by intronless multicopy genes, which are transcribed into non-polyadenylated mRNAs. In contrast, the variant histones are encoded by genes, which are located outside the canonical histone gene cluster. They are mostly present as single or few gene copies, contain introns and their mRNAs are polyadenylated. Histone variants are evolved from the corresponding canonical histones and differ from their canonical paralogs in primary sequence, expression timing and deposition mechanism. Canonical histones are expressed during S-phase of the cell cycle and the cell uses them for chromatin assembly during replication. In contrast, histone variants are expressed throughout the cell cycle and are used for deposition and exchange independent of DNA replication.

The eight human histone H3 proteins (H3.1, H3.2, H3t, H3.3, CENP-A, H3.X, H3.Y and H3.5 (Fig. 1) can be grouped, on the basis of their incorporation into chromatin, in two different categories: (i) canonical, replication dependent H3 histones (H3.1 and H3.2) and (ii) replication independent histone H3 variants (H3t, H3.3, CENP-A, H3.X, H3.Y and

☆ This article is part of a Special Issue entitled: Histone chaperones and Chromatin assembly.

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H3.5). It is noteworthy that H3.3, CENP-A, H3.X, and H3.Y are somatic histone variants, while H3t and H3.5 are testis specific variants. Interestingly, in yeast,there exists a single type of H3, which is equivalent to H3.3 of mammals. In fact, H3.3 gene is the common ancestor, which gave rise to the major H3 variants (H3.1, H3.2 and H3t), during the course of evolution in animals [4]. The single H3 isoform of yeast is deposited by both replication dependent and replication independent pathways.

The two canonical histones H3.1 and H3.2 differed by a single amino acid substitution (S96C). The H3.3 variant differs from the canonical H3.1 by five substitutions (A31S, S87A, V89I, M90G, and C96S), whereas the testis specific variant H3t has four amino acid substitutions compared with H3.1 (A24V, V71M, A98S, and A111V). Centromere specific H3 variant CENP-A is approximately 60% identical to H3.1 within its histone fold domain, but has a highly divergent N-terminal tail. H3.X and

A. Sequence alignment of human H3 variants

```
H3.1        -ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALREIRRYQKSTE 59
H3.2        -ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGTVALREIRRYQKSTE 59
H3t         -ARTKQTARKSTGGKAPRKQLATKVARKSAPATGGVKKPHRYRPGTVALREIRRYQKSTE 59
H3.3        -ARTKQTARKSTGGKAPRKQLATKAARKSAPSTGGVKKPHRYRPGTVALREIRRYQKSTE 59
H3.5        -ARTKQTARKSTGGKAPRKQLATKAARKSTPSTCGVK-PHRYRPGTVALREIRRYQKSTE 58
H3.X        -ARTKQTARKATAWQAPRKPLATKAARKRASPTGGIKKPHRYKPGTLALREIRKYQKSTQ 59
H3.Y        -ARTKQTARKATAWQAPRKPLATKAAGKRAPPTGGIKKPHRYKPGTLALREIRKYQKSTQ 59
CENP-A      GPRRRSRKPEAPRRRSP-SPTPTPGPSRRGPSLGASSHQHSRRR-QGWLKEIRKLQKSTH 58

H3.1        LLIRKLPFQRLVREIAQDFKT--DLRFQSSAVMALQEACEAYLVGLFEDTNLCAIHAKRV 117
H3.2        LLIRKLPFQRLVREIAQDFKT--DLRFQSSAVMALQEASEAYLVGLFEDTNLCAIHAKRV 117
H3t         LLIRKLPFQRLMREIAQDFKT--DLRFQSSAVMALQEACESYLVGLFEDTNLCVIHAKRV 117
H3.3        LLIRKLPFQRLVREIAQDFKT--DLRFQSAAIGALQEASEAYLVGLFEDTNLCAIHAKRV 117
H3.5        LLIRKLPFQRLVREIAQDFNT--DLRFQSAVVGALQEASEAYLVGLLEDTNLCAIHAKRV 116
H3.X        LLLRKLPFQRLVREIAQAISP--DLRFQSAAIGALQEASEAYLVQLFEDTNLCAIHARRV 117
H3.Y        LLLRKLPFQRLVREIAQAISP--DLRFQSAAIGALQEASEAYLVQLFEDTNLCAIHARRV 117
CENP-A      LLIRKLPFSRLAREICVKFTRGVDFNWQAQALLALQEAAEAFLVHLFEDAYLLTLHAGRV 118

H3.1        TIMPKDIQLARRIRGERA---------- 135
H3.2        TIMPKDIQLARRIRGERA---------- 135
H3t         TIMPKDIQLARRIRGERA---------- 135
H3.3        TIMPKDIQLARRIRGERA---------- 135
H3.5        TIMPKDIQLARRIRGERA---------- 134
H3.X        TIMPRDMQLARRLRGEGAGEPTLLGNLAL 146
H3.Y        TIMPRDMQLARRLRREGP---------- 135
CENP-A      TLFPKDVQLARRIRGLEEGLG------- 139
```

B. Different characteristics of H3 variants

		H3 variants	Amino acids	Identity with H3.1 (%)	Related Functions
Canonical		H3.1	136	_ _	DNA replication, repair, chromosome stability
		H3.2	136	99	_ _
Non-canonical	Somatic	H3.3	136	96	Transcription, Sperm pronucleus decondensation, Incorporation during mammalian meiotic sex inactivation (MSCI),Chromosome inactivation, Pericentric transcription,Telomere silencing
		CENP-A	140	42	Assembly of Kinetochore, Chromosome segregation
		H3.X	147	73	?
		H3.Y	136	77	Response to external stimuli
	Testis Specific	H3t	136	97	Expressed in testicular cells
		H3.5	135	85	Associated with actively transcribed genes Seminiferous tubules

Fig. 1. Sequence alignment and characteristics of H3 variants. (A) Amino acid sequence alignment of different human H3 variants (H3.1, H3.2, H3t, H3.3, H3.5, H3.X, H3.Y and CENP-A). Identical amino acids are represented in black letters and the amino acids differences among human H3 variants are shown in red letters. The residues of H3.3, H3.5, H3.X and H3.Y corresponding to replication independent deposition are highlighted in gray. The position 31 of H3.3 and H3.5 contain serine residue. Differences of CENP-A from other H3 variants are highlighted in green. (B) Different properties of human H3 variants include number of amino acids, % identity with H3.1 and major functions.

H3.Y, the recently identified histone H3 variants in primates [5], display interesting changes in amino acids that are known to be modified in H3.1, H3.2, and H3.3. Another newly identified histone H3 variant, H3.5 is specifically expressed in testis and shown to be associated with actively transcribed genes [6]. The amino acids differences between the different histone H3 provide the specificity for their differential chromatin assembly and regulation.

3. Chaperoning histones H3–H4 from the cytoplasm to the nucleus

The first step in the deposition of the newly synthesized histones is the transport from the cytoplasm to the nucleus, a process, which is assisted by distinct chaperones. The chaperone Asf1 (Anti-silencing Function 1) was the first chaperone identified to play a key role in supplying histones H3–H4 to the downstream chaperones, like CAF-1 (Chromatin Assembly Factor 1) and HIRA (Histone Regulatory homolog A) for nucleosome assembly [7–10]. Human Asf1 exists in two isoforms, Asf1a and Asf1b, coded by 2 different genes [11]. Structural and biochemical studies show that Asf1 binds only to one H3–H4 dimer [12–14], thus preventing the formation of H3–H4 tetramer. In addition, the cytosolic complex of ectopically expressed epitope-tagged H3.1 (e-H3.1) contains only the tagged H3.1 fusion, but not the endogenous H3 [15,16]. These data evidence that Asf1 is associated with a single H3–H4 dimer both *in vitro* and *in vivo* and that the H3–H4 dimer, and not the $(H3–H4)_2$ tetramer, is further delivered to the nucleus.

More recent reports demonstrate that the processing and the transport of newly synthesized H3–H4 are very intricate events requiring both the concerted action of numerous multi-chaperone complexes and the presence of specific post-translational modifications (PTMs) of histones [17]. Noteworthy, the specific histone PMTs are catalyzed by dedicated enzymes. For example, the acetylation of H4 on lys5 and lys12, a well-studied and highly conserved pre-deposition mark [17–19], is catalyzed by HAT1–RbAp46 holoenzyme [20]. Acetylation occurs before deposition [21] and is immediately removed after histone deposition onto DNA. The precise role of these pre-deposition PTMs in chromatin assembly is not well understood.

In two very recent studies, the biochemical purification of the cytoplasmic H3 complex has allowed both the identification of distinct H3 chaperones and the suggestion of a comprehensive mechanism(s) for the sequential assembly of H3–H4 dimers [22,23]. The first study, carried out by the group of D. Reinberg, identified six different H3 chaperones (HCS70, HSP90, tNASP, sNASP, RbAp46, and Asf1b) along with histone H4, importin4 and HAT1 [22]. This study [22] has not detected Asf1a as a part of the cytosolic H3 complex. Four distinct cytosolic complexes were found to be sequentially involved in the assembly of H3–H4 dimers [22]. After synthesis, histones H3 and H4 were sheltered from misfolding and aggregation by interaction with chaperones HSC70 and HSP90, respectively [22,23]. For transport and deposition onto DNA, histones H3–H4 first assembled to form the dimer, a process facilitated by HSP90 and tNASP [22]. Once the H3–H4 dimer was assembled, RbAp46 associates with the H4 carboxyl domain [22] and helps the recruitment of HAT1, which in turn acetylates H4 on lys5 and lys12. Then the acetylated histones are transferred to Asf1b and importin4 for nuclear transport [22].

The other study, carried out by the group of A. Loyola, reported some different results [23]. First, they described an interesting finding concerning the free soluble H3–H4 dimers. Loyola and colleagues showed that the free soluble endogenous histones H3 and H4 were transiently poly-ADP-ribosylated and that this mark was removed after dimer formation [23]. This has led to the hypothesis that the poly-ADP-ribosylation of H3–H4 was necessary for keeping the soluble histones H3 and H4 in folded state. In addition, and in contrast to ref. [22] the authors found that the H3–H4 dimers were associated with both Asf1a/b and importin4 in two different complexes [23]. Moreover, the two distinct translocation complexes contained H3–H4 dimers with distinct pre-deposition marks. The first translocation complex contained Asf1a, H3K9me1, and H3K14ac, while the second complex contained Asf1b and H3K9me1 [23]. This study also claimed that both H3 acetylation and methylation do not affect nuclear translocation, but may be instead linked to the nucleosomal histone H3 PTM patterns.

4. Chaperoning histone H3 proteins from nucleus to chromatin

Analysis of the preassembly complexes associated with the different human H3 variants has identified CAF1, Asf1a/b, HIRA, DAXX, and HJURP as the major histone chaperones controlling their targeting and deposition to specific chromatin loci (Table 1). CAF1 is the key chaperone in replication coupled chromatin assembly, while Asf1a/b plays a role in both replication coupled and replication independent deposition. The deposition of replication independent histone H3 variant H3.3 is assisted by HIRA and DAXX, while this of the centromeric variant CENP-A is assisted by HJURP. Chaperones involved in deposition of H3.5, H3t, H3.X and H3.Y histones are not known.

5. Replication coupled deposition of canonical H3 histones

The canonical histones H3.1 and H3.2 are synthesized and deposited during S-phase of the cell cycle in a replication-dependent manner. During replication the “old” nucleosomes are disassembled and the “new” ones are assembled. There are two sources of histones for the replication-coupled deposition: (i) “old” histones and, (ii) newly synthesized histones. According to the generally accepted view, replication-induced disruption of “old” nucleosomes produces two H2A–H2B dimers and H3–H4 tetramer [24]. H2A–H2B dimer dissociation and reassembly appeared to require both chaperones FACT (facilitate chromatin transcription) and Nap1 (Nucleosome assembly protein 1) [25]. An interesting question that remains elusive is how the old H3–H4 tetramer dissociates from “old” DNA and reassembled on new DNA strands. This process might involve either transfer of the whole H3–H4 tetramer or it's splitting into two half. Recently the group of Bing Zhu and She Chen [26] reported that during replication there is no splitting of the H3.1–H4 tetramer, while the H3.3–H4 tetramers did split during replication. Another recent study in budding yeast, using sequential chromatin immunoprecipitation of mononucleosome containing differentially tagged versions of H3, found that splitting of H3–H4 tetramers occurs only at highly active genes undergoing histone exchange [27]. Although, yeast contains a single H3.3 like isoform, which was shown to be permissive for splitting events in human cells [26]. The above described studies show that in many genomic loci the H3–H4 tetramer splitting is a rare event, which might occur in a chromatin region specific manner. Whether the splitting of H3–H4 tetramer is also histone variant specific still needs further investigations. The candidate chaperone for H3–H4 tetramer splitting during replication was suggested to be Asf1. Indeed, Asf1 binds the replicative helicase MCM2-7 via histone H3–H4. In this complex, the histones contain specific parental posttranslational modification marks [28].

6. Deposition of newly synthesized conventional H3.1 histones

As mentioned earlier CAF-1 is the bona fide chaperone for replication coupled chromatin assembly. CAF-1 was first identified in humans and was shown to promote chromatin assembly on replicating SV40 DNA *in vitro*[29]. In mammals, the CAF-1 complex is composed of three highly conserved subunits p150, p60 and p48 [30,31]. The p150 subunit of CAF-1 is recruited to the site of DNA synthesis through direct interaction with proliferating cell nuclear antigen (PCNA) and colocalizes with the replication foci and p60 during S-phase [32–35]. The interaction of CAF-1 with PCNA is enhanced by Cdc7–Dbf4 mediated phosphorylation of p150 subunit [36]. Down-regulation of CAF-1 decreases also chromatin assembly during DNA replication [37–39]. Importantly, CAF-1 was found associated *in vivo* with the replication dependent H3.1 complex and not with the replication independent H3.3 complex, a key finding

Table 1
Distinct histone chaperones for H3 variants deposition.

Histones	Name		Mass (kDa)	Functions (in context of chromatin assembly)
	Specific histone chaperones			
H3.1–H4	CAF-1	CHAF1A	105	Replication Coupled (RC)
		CHAF1B	61	deposition of H3.1–H4
		RbAp48	48	
H3.3–H4	HIRA		81	Replication Independent (RI) deposition of H3.3–H4 Genic regions
	DAXX		112	Telomeres, Pericentric heterochromatins
CENP-A–H4	HJURP		83	Deposition of CENP-A–H4 at centromeres
	Common histone chaperones			
H3–H4 family	ASF1A		23	Supply of H3.1/H3.3–H4 dimers
	ASF1B		22	for RC and RI
	NASP		85	Transport of histones to nucleus, Promotes H4 acetylation, Linker histone deposition
	RbAp46		48	Regulation of chromatin metabolism by assisting different enzymatic activities,
	RbAp48		48	Chromatin assembly
H3–H4	FACT	SSRP1	81	Transcription elongation,
H2A–H2B family		Spt16	120	Recombination
	hDEK		43	Possible chaperone in human Targeted deposition of H3.3 in Dorosophila
	JDP2		19	Transcription, Inhibition of HAT
	ANP32B		29	Deposition of core histones at promoter regions

further demonstrating the direct implication of CAF-1 in replication coupled deposition [15].

In the nucleus the newly synthesized H3–H4 dimers appeared to remain initially associated with Asf1. Next Asf1 supplies the newly synthesized H3–H4 dimers to CAF-1. This scenario is supported by several experiments (Fig. 2). For example, the replication-coupling assembly factor (RCAF), which comprises Asf1 and histones H3 and H4, was shown to promote CAF-1 mediated chromatin assembly *in vitro*[7]. Two different studies in chicken and human showed that Asf1 is critical for replication-coupled chromatin assembly *in vivo*[40,41]. Note also that it was described earlier that human Asf1 regulates the delivery of S-phase histones during replication *in vivo*[9]. The direct interaction of Asf1 and p60 subunit of CAF-1 both *in vitro* and *in vivo* facilitates the delivery of histones from Asf1 to CAF-1 for deposition [42]. However, the exact mechanism for the removal of Asf1 from H3–H4 dimer, transferring of the dimer to CAF-1 and formation of H3–H4 tetramer on CAF-1 still remains unclear.

7. Deposition of H3.1–H4 following DNA Repair

DNA repair process is coupled with disruption and restoration of chromatin structure. A well recognized model for DNA repair was suggested by Smerdon [43] called "Access-Repair-Restore". According to this model, in order the repair machinery to get access to DNA, chromatin has to be first disrupted and reassembled after completion of DNA repair. The role of chaperones (Asf1 and CAF-1) in chromatin restoration is relatively well understood compared to the chromatin disassembly during DNA repair. CAF-1 assists chromatin assembly *in vitro* on UV-damaged DNA [44] and along with PCNA it is recruited to the sites of UV repair *in vivo*[45]. The interaction of human Asf1 with CAF-1 synergistically facilitates chromatin assembly *in vitro* during nucleotide excision repair [8]. In addition, the incorporation of newly synthesized histones to the repair sites *in vivo* by CAF-1 is dependent on nucleotide excision repair [46]. CAF-1 is shown also to be recruited to the sites of DNA repair in quiescent human cells and its down regulation results in strong cell viability decrease and accumulation of DNA double-strand breaks (DSBs) [47]. Recent studies demonstrated that CAF-1 interacts with the repair machinery KU complex and 14-3-3 proteins [48]. CAF-1 mediated nucleosome assembly is required for regulating the degradation of the discontinuous mismatch-containing strands during mismatch repair [49]. All these data evidence for an important implication of CAF-1 and Asf1 in chromatin assembly during DNA repair.

8. Replication independent deposition of H3.3

Unlike canonical histones the expression and deposition of H3 variants occur throughout the cell cycle. It is well documented that the synthesis of histone H3.3 takes place outside S-phase [50]. The level of H3.3 transcript is constitutively maintained throughout differentiation [51]. This constitutive expression pattern makes H3.3 variants available for deposition and replacement independent of DNA replication. Noteworthy, H3.3 exhibits differences in the primary amino acid sequence and PTMs pattern compared to conventional H3, which conferred distinct properties of H3.3 histones. In fact, the substitution of any one amino acid in H3.1 toward H3.3 identities ($S_{87}/V_{89}/M_{90}$ to $A_{87}/I_{89}/G_{90}$) permits some replication independent deposition [52].

Several studies have analyzed in detail the localization of H3.3 both at specific regions of the nucleus and genome-wide. Deposition of H3.3 variant occurs at highly transcribed regions in flies and mammals [52–56]. Incorporation of H3.3 at regulatory sites of both active and silent genes has been reported [53,57,58]. The deposition of H3.3 in germline cells takes place in a replication independent manner. It has been also reported that nucleosome replacement involving the deposition of H3.3 occurs during mammalian meiotic sex chromosome inactivation (MSCI) [59]. H3.3 is deposited during decondensation of sperm nucleus in *Drosophila* and mouse [60,61]. Recently enrichment of H3.3 was found at telomeres and pericentric heterochromatin in mouse ES cells and MEF cells [62–65]. These last observations clearly show that H3.3 is not only accumulated at active chromatin but it is also deposited at silent genomic loci. The localization pattern of H3.3 variant in the genome playsan important role in the epigenetic marking of specific chromosome regions and regulation of gene expression by altering the local chromatin structure.

Importantly, the deposition of histone variants in different combinations, to different regions in the genome could result in quite different consequences for chromatin structure and gene regulation. A typical example is the study of the group of Felsenfeld [66]. This group reported that nucleosomes containing the two variants, H3.3 and H2A.Z, are less stable than nucleosomes with H3.3 and H2A. In addition, nucleosomes containing H3.1 and H2A.Z are as stable as H3.1/H2A nucleosomes [66].

Enrichment of H3.3 at different genomic regions suggests the existence of distinct deposition factors. Investigating the mechanism of H3.3 deposition and identifying dedicated chaperones will further increase our understanding on both the accumulation of H3.3 at specific regions and its function. Presently two specific chaperones HIRA and DAXX are known for replication independent deposition of H3.3 histone variant (Fig. 2).

9. HIRA mediated deposition of H3.3

HIRA was the first described chaperone responsible for H3.3 deposition. Initially, DNA replication independent chromatin assembly *in vitro* was found to be facilitated by HIRA in Xenopus egg extracts [67] and histones were identified as proteins able to specifically interact with HIRA [68]. The subsequent affinity purification study in human cells identified two distinct chaperones, CAF-1 and HIRA, for replication dependent and replication independent assembly of H3.1 and H3.3, respectively [15]. In addition to HIRA, H3.3 predisposition complex contains Cabin1 and Ubinuclein1/2 suggesting the role of these factors in HIRA mediated H3.3 deposition [15]. Moreover, a recent bioinformatics study predicted that the acidic HUN (for HPC2-Ubinuclein1) domain of ubinuclein could function as histone binding subunit of HIRA complex [69]. Asf1, a common

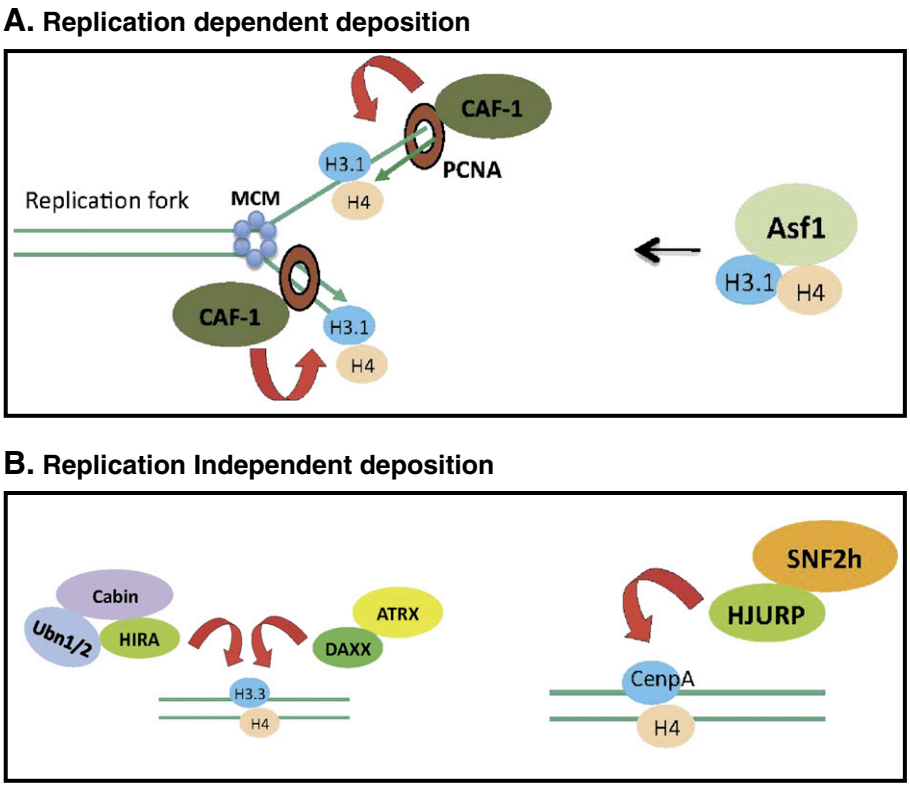

Fig. 2. Overview of histone H3 deposition pathways and corresponding histone chaperones. (A) Replication coupled deposition of newly synthesized histone H3.1-H4. Asf1 is the key histone chaperone for the delivery of histones H3.1-H4 to CAF-1. CAF-1 is recruited by PCNA to the site of DNA synthesis for deposition of H3.1-H4. (B) Replication independent deposition of H3.3 and CENP-A. (1) HJURP mediates the centromeric deposition of CENP-A-H4. Chromatin remodelling factor SNF2h play role in maintenance of centromeric CENP-A chromatin. (2) HIRA and DAXX mediate independent deposition of H3.3-H4 units.

partner of both H3.1 and H3.3 complexes, is believed to provide histones to CAF-1 and HIRA through chaperone–chaperone interactions [10,15,42]. The exact mechanism for this differential delivery of histones to CAF-1 and HIRA by Asf1 is not clear. Interestingly, HIRA is involved in the deposition of H3.3 during decondensation of the *Drosophila* sperm pronucleus [60] but is not required for H3.3 deposition in embryos or adult cells [60,70]. The chromatin-remodeling factor CHD1 was reported to be responsible for chromatin assembly of H3.3 in *Drosophila*[71]. This supports the idea that H3.3 deposition occurs through distinct pathways. HIRA is also required for H3.3 deposition in mouse zygote [72]. Recent study shows that enrichment of H3.3 at promoters and in the body of genes in ES cells is dependent on HIRA [62] in agreement with a role of HIRA in H3.3 deposition at these regions.

10. DAXX mediated deposition of H3.3

DAXX was initially linked to FAS-mediated apoptosis [73]. DAXX was found to colocalize with both the promyelocytic leukaemia (PML) nuclear body and the alpha-thalassemia/mental retardation X-linked syndrome protein (ATRX), which is highly enriched at pericentric heterochromatin [74,75]. Recently, our group [63] and the group of Allis [62,76] showed that DAXX, in complex with ATRX, facilitates H3.3 deposition. DAXX directly and specifically interacts with H3.3 both *in vivo* and *in vitro* and mediates deposition of bacterially purified recombinant H3.3–H4 tetramer on naked DNA *in vitro*[63]. A central acidic domain of DAXX strongly interacts with H3.3–H4 [63] and the motif "AAIG" of H3.3 was found sufficient for specific interaction with DAXX [76].

Interestingly, the DAXX–ATRX complex deposited H3.3 at regions different from the ones that contained H3.3 deposited by HIRA. Genome-wide enrichment study shows HIRA-independent localization of H3.3 at telomeres and transcription factors binding sites [62]. DAXX–ATRX dependent H3.3 deposition at pericentric heterochromatin in mouse embryonic fibroblasts (MEFs) was described, and the presence of H3.3 appeared to regulate transcription of pericentric DNA [63]. The exact mechanism of this transcription regulation is not known and needs further studies. In contrast, DAXX–ATRX dependent deposition of H3.3 at telomeres in ES cells is required for transcription repression from telomeric repeats [62]. This suggests that DAXX–ATRX mediated deposition of H3.3 at different genomic regions can play multiple roles. The DAXX capacity to assemble alone recombinant H3.3–H4 tetramers on DNA template [63,76] strongly indicates that ATRX is required for recruitment of DAXX containing H3.3 complex to specific regions. It is speculated that DAXX, in the absence of ATRX, can facilitate deposition of H3.3 at transcription factors binding sites [76]. Surprisingly CAF-1 was found associated with H3.3 predeposition complex in the absence of DAXX [63], suggesting that cells can use replication dependent assembly pathway to counterbalance the loss of DAXX. It seems that DAXX prevents the interaction of H3.3 with CAF-1 complex in order to promote replication independent chromatin assembly of H3.3. Structural study of DAXX–H3.3 complex will be helpful for better understanding this mechanism.

11. Mechanism of CENP-A deposition at centromeres

CENP-A synthesis and deposition at centromeres is cell cycle dependent. In human the peak of the synthesis of CENP-A occurs during G2-phase [77] and deposition of CENP-A at centromeric DNA starts late in mitosis and continues to early G1-phase [77,78]. Noteworthy, incorporation of CENP-A into centromeric chromatin is not coupled with DNA replication [79]. This uncoupling of CENP-A deposition from replication of centromeric DNA results in "dilution" of CENP-A at centromeres of daughter chromosomes. This raises the question how (equally or randomly) CENP-A gets distributed to the daughter centromeres, a question that is not yet solved. Whatever is the distribution of CENP-A, its "dilution" could result in at least three distinct

scenarios for the changes in the centromeric chromatin structure after replication: (i) incorporation of histones H3 which are later exchanged with CENP-A, (ii) generation of nucleosome free gaps and, (iii) formation of heterotypic tetrasome (CENP-A–H4–H2A–H2B) [80,81]. Which one of these or other scenarios is realized is currently unknown. The interesting point is that the "dilution" of CENP-A in daughter centromeres during S-phase and its subsequent restoration at the next G1-phase may be required for faithful cell division. In this context, how after synthesis CENP-A is transported into the nucleus, specifically delivered to centromeric chromatin and maintained on centromeres during cell cycle are the key issues. The centromeric localization of CENP-A depends on both intrinsic and extrinsic factors. A specific domain of CENP-A called CATD (CENP-A Targeting Domain) within the histone fold region is essential for the centromeric localization of CENP-A [82,83]. Different protein factors have been identified in different organisms, which play direct or indirect role in CENP-A assembly. Among these, the factors responsible for priming centromeres through regulation of centromeric nucleosome acetylating status include hMIS18α/β, MIS18BP1 and RbAp46/48 [84,85]. The ATP-dependent remodeling and spacing factor (RSF) complex is involved in maintenance of CENP-A chromatin [86].

Recent studies in humans identified a specific chaperone HJURP (Holliday Junction Recognition Protein) for CENP-A deposition at centromeres [87–89] (Fig. 2). HJURP directly interacts with CENP-A and its level rises during the time of CENP-A deposition in HeLa cells. Down regulation of HJURP results in decrease of the amount of CENP-A associated with centromeres. HJURP facilitates also the *in vitro* deposition of purified CENP-A–H4 at naked alpha-satellite DNA [89]. The N-terminal domain called CBD (CENP-A Binding Domain) of HJURP is necessary and sufficient for the interaction with CENP-A [89] and the CATD domain of CENP-A is required for HJURP binding [87]. HJURP is only conserved in fungi and vertebrates [89,90], but not in plants or invertebrates. Therefore, the function of HJURP could be replaced by other chaperones in other species. For example in flies, p55/RbAp48 [91] and CAL-1 [92] have been shown to be involved in CENP-A deposition.

Two recent structural analyses of CBD domain of HJURP [93] and Scm3 [94] with CENP-A–H4 and Cse4–H4 complexes (Cse4 is the analogs of CENP-A in yeast, while Scm3 is specific chaperone responsible for Cse4 deposition in budding and fission yeasts [95–99]) further confirmed the recognition of CENP-A by CBD. The crystal structure revealed that HJURP binds a CENP-A–H4 heterodimer and prevents tetramer formation [93]. This suggests either a stepwise assembly of two CENP-A–H4 dimers by HJURP at centromeres followed by the incorporation of two H2A–H2B dimers, or a one step assembly of a single CENP-A–H4–H2A–H2B heterotypic tetrasome. Indeed, heterotypic tetrasomes have been reported to exist in *Drosophila* and human interphase cells [80,81] and CENP-A was found, in HeLa cells, to reside in a soluble complex containing H4, HJURP and H2A–H2B [89]. The exact composition of centromeric nucleosomes is still under debate. To get more insight into the mechanism of CENP-A deposition it is important to understand how HJURP transfers CENP-A to DNA. HJURP and its yeast ortholog Scm3 compete with DNA for non-specific binding to the histone complex [93] and thereby, promote the nucleosome assembly. Surprisingly, Ser 68 residue, which is located outside the CATD domain of CENP-A, was found to provide specificity for HJURP interaction, while the corresponding residue Gln 68 in H3 prevents HJURP binding [93]. However, *in vivo* experiments are necessary to be performed for the clarification of this finding. Interestingly, the retention of Scm3 on centromeric DNA is mediated by distinct DNA binding domain of Scm3 and does not depend on Scm3 and Cse4–H4 interaction [94]. Whether this is also true for HJURP remains to be determined.

Presently it is not clear how the HJURP complex, carrying newly synthesized CENP-A, is specifically recruited to centromeres. A recent study, using synthetic human artificial chromosome, stresses the importance of alpha-satellite DNA transcription for HJURP recruitment and centromeric CENP-A assembly [100]. It can be suggested that the centromeric transcripts may guide HJURP-CENP-A complex to centromeres. Further studies are needed to test this hypothesis. The second important point is to understand the link between CENP-A stability and centromeric incorporation. In yeast the ubiquitin E3 ligase Psh1 mediates degradation of mis-incorporated Cse4 [101,102] but the existence of ubiquitination-mediated degradation of CENP-A in human is currently unknown.

12. Concluding remarks

Histone chaperones play essential roles in numerous nuclear processes. This review has highlighted the progress in our knowledge on the chaperones responsible for the deposition of the histones from the H3 family. Analysis of the reported data demonstrated the fascinating operation mechanism of H3 chaperones and how very little changes in the primary sequences of H3 histones resulted in changes in their structure, which are further recognized by specific chaperones. Despite the efforts invested, in particular during the last years, how histone H3 chaperones function is, however, not yet clearly understood. How are conventional H3 histones assembled at the replication fork? How the specific posttranslational modification pattern of histones preserve in the newly assembled. Why and how H3.3 histones deposit on different genome regions by distinct chaperones and how the presence of H3.3 confers specific properties to these regions? Is there some tissue specificity for the H3.3 chaperones DAXX and HIRA in animals? How the cell recognize specifically alpha-satellite DNA to deposit CENP-A? These and many other questions remain open for future studies.

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MÉCANISME EPIGENETIQUE IMPLIQUÉ DANS LA DÉPOSITION DE CENP-A AUX CENTROMERES

Résumé

La ségrégation fidèle des chromosomes est dirigée par le centromère, un locus chromosomique spécialisé qui est requis pour l'assemblage des kinetochores actifs. Les centromères sont marqués épigénétiquement par la présence d'un nucléosome unique qui contient un variant centromérique de l'histone H3 appelé Centromere protein A (CENP-A). Une question fondamentale est comment CENP-A est spécifiquement déposé aux centromères. L'objectif de ma thèse a été d'identifier les facteurs spécifiques de la déposition de CENP-A. Pour identifier les facteurs spécifiques impliqués dans la déposition de CENP-A aux centromères, j'ai utilisé la méthode de purification TAP-TAG à partir d'une fraction nucléaire soluble de cellules HeLa exprimant stablement une copie ectopique de CENP-A (e-CENP-A). J'ai ainsi pu identifié la protéine holliday Junction Recognition protein (HJURP). En utilisant un siRNA spécifique de HJURP, j'ai montré que la localisation et la déposition de CENP-A étaient fortement affectées. La protéine recombinante HJURP lie de manière stoechiométrique le tétramère CENP-A/H4 mais il ne lie pas le tétramère H3/H4. La liaison se fait grâce à un petit domaine conservé en position N-terminal de HJURP, dénommé CBD (CENP-A binding domain). De plus, j'ai pu mettre en évidence in vitro que HJURP facilitait la déposition du tétramère CENP-A/H4 sur de l'ADN satellite. L'ensemble de mes résultats démontre très clairement que HJURP est la principale chaperone responsable de la déposition de CENP-A aux centromères.

Mot clés: Histone variant, CENP-A, Centromères, Histone chaperone, HJURP

Summary

Centromere is a specialized chromosomal locus, where kinetochore assembles, which is required for correct chromosome segregation during cell division. In higher eukaryotes, centromere specification is independent of the DNA sequence and is determined epigenetically by the presence of a unique nucleosome that contains a histone H3 variant, called CENP-A. A fundamental question in centromere biology is that how CENP-A is specifically delivered to and maintained on centromeres. The aim of my thesis was to identify specific chaperone in human, responsible for CENP-A loading to centromeres, by using biochemical and proteomic strategies. To identify CENP-A deposition machinery, I purified the prenucleosomal CENP-A complex from HeLa cells stably expressing epitope tagged CENP-A. By mass spectrometry analysis of proteins present in CENP-A and H3.1 complex, I found HJURP uniquely in CENP-A prenucleosomal complex. Down regulation of HJURP by specific siRNA strongly diminished centromeric localization of CENP-A. Bacterially expressed HJURP specifically binds to the CATD domain of CENP-A, via a highly conserved N-terminal domain, called CBD. Finally, I showed that HJURP is able to facilitate the efficient deposition of CENP-A/H4 tetramer on naked DNA. Taken together, my data demonstrate that HJURP is a key chaperone responsible for the targeting and deposition of newly synthesized CENP-A at centromeres.

Key words: Histone variant, CENP-A, Centromeres, histone chaperone, HJURP