Control of histone variant H3.3 loading on chromatin

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List of publications

Paper I

Paper II

Paper III

* shared first authorship
## List of abbreviations

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<tbody>
<tr>
<td>A</td>
<td>alanine</td>
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<tr>
<td>ACF</td>
<td>ATP-utilizing chromatin assembly and remodeling factor</td>
</tr>
<tr>
<td>ADD</td>
<td>ATRX-DNMT3-DNMT3L</td>
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<tr>
<td>ALT</td>
<td>alternative lengthening of telomeres</td>
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<tr>
<td>ASF1A</td>
<td>anti-silencing function protein 1 homolog A</td>
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<tr>
<td>ASF1B</td>
<td>anti-silencing function protein 1 homolog B</td>
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<tr>
<td>ATRX</td>
<td>α-thalassemia/mental retardation X-linked syndrome protein</td>
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<tr>
<td>C</td>
<td>cysteine</td>
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<tr>
<td>CABIN1</td>
<td>calcineurin-binding protein 1</td>
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<td>CAF-1</td>
<td>Chromatin assembly factor-1</td>
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<td>centromere protein T</td>
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<td>CHD1</td>
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<td>chromodomain helicase DNA-binding domain 2</td>
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<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>ChIP-seq</td>
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<tr>
<td>DAXX</td>
<td>death domain-associated protein</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
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<tr>
<td>DSB</td>
<td>double strand break</td>
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<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
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<tr>
<td>EP400</td>
<td>E1A-binding protein p400</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem</td>
</tr>
<tr>
<td>FACT</td>
<td>facilitate chromatin transcription</td>
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<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
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<tr>
<td>G</td>
<td>glycine</td>
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<tr>
<td>H3.3[core]</td>
<td>H3.3 harboring a deletion from residue 3 to 35</td>
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<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
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<tr>
<td>HIRA</td>
<td>histone regulator A</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>TRIM</td>
<td>tripartite motif</td>
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<td>TSS</td>
<td>transcription start site</td>
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<tr>
<td>UBN1</td>
<td>ubinuclein 1</td>
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<tr>
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<td>W</td>
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1. Introduction

1.1. Basic principles of chromatin organization in the interphase nucleus

The hereditary identity of a living organism is defined by its genetic material encoded in DNA. Faithful transmission of genetic information from one generation to the next requires protection of DNA integrity. In eukaryotic cells, the nucleus segregates the nuclear DNA from the cytoplasm. The nucleus is bounded by the nuclear envelope, which consists of a double nuclear membrane perforated by nuclear pore complexes and underlined by the nuclear lamina, a filamentous network of intermediate filaments called lamins. In the nucleus, DNA is packaged into chromatin, a complex of DNA and DNA-bound proteins organized in several compaction levels (Fig. 1A). Compaction of DNA into chromatin not only facilitates the constraint of the genetic material in a small volume (the nucleus) but also, and maybe primarily, ensures regulated control of gene expression.

Figure 1. Basic organization of chromatin. (A) Chromatin compaction levels. Modified from (Horn and Peterson 2002). (B) Electron micrograph of heterochromatin and euchromatin in the nucleus; N, nucleus; H, heterochromatin; E, euchromatin. Modified from (Uranova et al. 2001). (C) FISH image of the gene-rich chromosome 19 (red) in the nuclear interior, and of the gene-poor chromosome 18 (green) at the nuclear periphery. Modified from (Bickmore 2013).
The basic repeating unit of chromatin is the nucleosome (Fig. 1A), which contains a core of proteins called histones. Nucleosomes appear by electron microscopy as “beads on a string” connected by thin bridges (the linker DNA) (Olins and Olins 1974). This first level of organization results in a five- to tenfold compaction of the DNA molecule (Kornberg 1974). The nucleosome string is folded into a more compact fiber of ~30 nm in diameter, which in turn folds into higher-order structures (Horn and Peterson 2002; Felsenfeld and Groudine 2003). The highest level of chromatin compaction is reached at mitosis, when each DNA molecule is packed into a mitotic chromosome; this extreme compaction level ensures correct partitioning of genetic information between the daughter cells.

Chromatin is non-uniformly distributed in the interphase nucleus. It is organized into compact electron-dense regions of heterochromatin, and looser electron-light areas of euchromatin (Fig. 1B). Whereas heterochromatin mainly consists of gene-poor and transcriptionally silent regions of the genome, euchromatin contains gene-rich and active regions. A fraction of heterochromatin consists of constitutive heterochromatin, which is compact, mostly transcriptionally silent and found in repetitive regions such as telomeres, centromeres and pericentromeres (Postepska-Igielska et al. 2013). Low levels of transcription occur in constitutive heterochromatin, which are necessary for heterochromatin homeostasis (Grewal and Elgin 2007). In contrast, facultative heterochromatin harbors a higher gene content and shows overall higher transcriptional activity or potential for gene activation. As described in sections 1.3 and 1.4, hetero- and euchromatic states are predominantly determined by their epigenetic signatures.

On a more global level, chromosomes occupy distinct positions in the nuclear space, which relate to their gene density (Fig. 1C). Whereas gene-rich chromosomes tend to localize in the nucleus center, gene-poor chromosomes tend to locate to the nuclear periphery (Boyle et al. 2001). Similarly, repressed regions of the genome tend to accumulate at the periphery (Fig. 1B), often in association with the nuclear lamina (Guelen et al. 2008). This spatial chromosome organization highlights the importance of specific chromosome allocations in the nucleus for proper control of gene activity.
1.2. The nucleosome

The structure of the nucleosome has been revealed by X-ray crystallography (Richmond et al. 1984; Luger et al. 1997). A nucleosome contains 146 base pairs of negatively charged DNA wrapped 1.65 times around a protein core containing two copies of each H2A, H2B, H3 and H4 histones – a histone octamer. Tight association between DNA and core histones protects DNA from nuclease digestion. Histones harbor a globular domain and an unstructured N-terminal tail. The globular domain is indispensable for histone-histone and histone-DNA interactions. Nucleosome formation begins with binding of H3 and H4 to form a heterodimer, and self-association of two (H3-H4) dimers via interactions between histones H3 to form a tetramer. Independently, histones H2A and H2B form heterodimers which associate with both sides of an H4-H3:H3-H4 tetramer through an H4:H2B interaction (Fig. 2). The histone octamer is wrapped by DNA via 14 contact points (Luger et al. 1997). All steps of nucleosome assembly are mediated by histone chaperones specific for each histone type.

Figure 2. Assembly and disassembly of the nucleosomes. The nucleosome assembly is a stepwise process where histones H3 and H4 form heterodimers which subsequently dimerizes to tetramer. H2A and H2B form dimers independently from H3 and H4 and bind to both sides of tetramer. Each step is mediated by histone chaperones. Histone H2A is in yellow, H2B in red, H3 in blue and H4 in green. Taken from (Das et al. 2010).
The N-terminal tails of core histones protrude from the nucleosome and are essential for mediation of inter-nucleosomal interactions and organization of higher-order chromatin structure (Allan et al. 1982). Histone tails can be post-translationally modified in a manner that affects chromatin organization and gene expression. Histone post-translational modifications (hPTMs) are part of a so-called epigenetic “code” essential for the regulation of gene activity.

### 1.3. Histone post-translational modifications

All histones can be modified on a total of approximately 130 amino acids by over 70 PTMs including methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, glycosylation, carbonylation, biotinylation, ADP-ribosylation, citrullination, proline and aspartic acid isomerization, N-formylation, crotonylation, propionylation and butyrylation (Tan et al. 2011; Sadakierska-Chudy and Filip 2015). These hPTMs are combinatorial and have been proposed to constitute an epigenetic “code” (Jenuwein and Allis 2001). This code is established “on top of” DNA sequence information and constitutes the premise of epigenetics (epi- meaning “on top of” in Greek). Many definitions have been ascribed to epigenetics; however we favor a view of epigenetics referring to heritable modifications of chromatin which modify gene expression without altering the DNA sequence. These modifications include DNA methylation and derivatives thereof (e.g. 5-hydroxymethylation (Chen et al. 2016)), post-translational modifications of histones, and the inclusion of histone variants. The histone code hypothesis proposes that hPTM combinations (both on canonical histones and histone variants) form unique recognition sites for proteins (“readers”) with the ability to interpret the code by interacting with histones through modification-specific binding domains (Jenuwein and Allis 2001). Chromatin readers such as transcription factors, chromatin modifiers and effector proteins can activate downstream signaling or block access of remodeling complexes (Lawrence et al. 2016). As such, they are key players in an intricate epigenetic signaling network involving cross-talks between different hPTMs.

The best characterized hPTMs are acetylation, methylation, phosphorylation and ubiquitylation (Fig. 3). Acetylation is mediated by histone acetyltransferases (HATs) on lysines and is associated with sites of transcriptional activity (Koch et al. 2007). Acetylation weakens interactions between DNA and histones nucleosomes by
neutralizing the positive charge of lysines, causing chromatin relaxation and facilitating binding of transcription factors (Shahbazian and Grunstein 2007). Methylation is catalyzed on lysine or arginine residues of H3 and H4 by histone methyltransferases (HMTs) (Bhaumik et al. 2007; Sadakierska-Chudy and Filip 2015). Methylation generates docking sites for chromatin remodeler (Strahl and Allis 2000) harboring specific recognition domains such as chromodomains (Bannister et al. 2001; Lachner et al. 2001), Tudor domains (Huyen et al. 2004) or PHD fingers (Wysocka et al. 2006). Histone methylation can be associated with active or repressed chromatin domains depending on the methylated lysine; for instance, di- and trimethylation of H3 lysine 4 (H3K4me2, H3K4me3) are associated with transcriptionally active regions. In contrast, H3K9me2 and H3K9me3 are mostly found in constitutive heterochromatin while H3K27me3 marks silenced facultative heterochromatin (Koch et al. 2007). Both histone acetylation and methylation are reversible modifications; this reversibility enables versatility of gene expression.

![Figure 3. Post-translational histone modifications on N-terminal tails.](image)

Figure 3. Post-translational histone modifications on N-terminal tails. S, serine; K, lysine; T, threonine; R, arginine. Taken from (Lawrence et al. 2016).

Phosphorylation of histones is related to cell cycle progression and regulates many cellular functions including mitosis (H3S10ph), meiosis (H4S1ph), DNA damage response (H2A.XS139ph), gene expression (H3S10ph, H3T11ph) and apoptosis.
Histone ubiquitination is catalyzed by ubiquitin ligases (Wilkinson 2000) and plays a role in transcription, gene silencing, DNA repair and proteolysis (Wilkinson 2000; Zhang 2003). Phosphorylated and ubiquitinated residues both serve as targets for regulatory complexes.

There is increasing evidence for cross-talk between hPTMs, in that by being in the same neighborhood they can affect each other, resulting in distinct outcomes. Cross-talk can occur within the same histone tail. For example, H3K9 methylation can inhibit H3K4 methylation and H3 acetylation; H3K4 methylation can facilitate acetylation and inhibit H3K9 methylation, promoting a transcriptionally favorable state of chromatin (Wang et al. 2001). Cross-talk can also arise between modifications on different histones. For example, H3K9me3 is required for H4K20me3 (Kourmouli et al. 2004), and H2B ubiquitylation facilitates methylation on H3K4 (Zhang 2003). hPTM cross-talk enables spatially and timely controlled binding of chromatin modifiers and transcription regulators.

1.4. Histone variants

Histones are necessary for the formation of nucleosomes and organization of distinct chromatin compaction levels. The five major histone families can be classified into two categories: core histones build the nucleosome core particle (H2A, H2B, H3 and H4), while linker histones (H1) stabilize nucleosomes. Nucleosome core histones are of 11-15 kDa molecular weight and conserved among eukaryotes while linker histones are larger (~22 kDa predicted molecular weight) and less conserved (Baxe vanis and Landsman 1996). In addition, histone H1, H2 and H3 (but not mammalian H4) contain both canonical forms and variants (Fig. 4A, B). Histone variants differ from canonical histones either by a few amino acids or by the larger domains which confer them distinct mechanisms of deposition into chromatin. Canonical histones are synthesized and incorporated into chromatin in a replication-dependent manner, i.e. during S phase, while synthesis and incorporation of variants is independent of DNA synthesis and can occur during and outside S phase (Szenker et al. 2011). Histone variants, also called replacement histones, often replace canonical histones under specific conditions such as transcription or repair of damaged chromatin, and in non-dividing cells, and thus are essential for maintenance of chromatin integrity.
Figure 4. Human histone variants. (A) Linker H1 histone variants; globular domains are shown in brown, tail domains are shown in white. (B) Core histone variants; H2A are shown in yellow, H2B in red, H3 in blue and H4 in green. Modified from (Maze et al. 2014).
1.4.1. Histone H1 variants

Linker histone H1 is essential for formation of higher-order chromatin organization. It binds DNA more weakly and is more mobile than core histones (Misteli et al. 2000). By binding to DNA at the entry site of nucleosomes, H1 stabilizes the nucleosome, controls the length of linker DNA between two adjacent nucleosomes (Blank and Becker 1995) and contributes to regulating gene expression (Shen and Gorovsky 1996; Fan et al. 2005). Interestingly, histone H1 has been shown to be excluded from sites of active transcription where chromatin is kept in an accessible configuration, by histone variant H3.3 (discussed below) (Braunschweig et al. 2009). Histone H1 thus plays a critical role in chromatin organization by modulating nucleosome density. Mammals have eleven H1 variants: five replication-dependent somatic variants (H1.1, H1.2, H1.3, H1.4 and H1.5), two replication-independent somatic-type H1 (H1.0 and H1X), three testis-specific variants (H1t, H1T2m and HILS1) and one oocyte-specific variant (H1oo; Fig. 4A) (Happel and Doenecke 2009; Cheema and Ausio 2015). Shortly after fertilization, somatic H1 variants replace testis- and oocyte-specific variants, indicating extensive chromatin remodeling of the maternal and paternal genomes at this stage (Godde and Ura 2009).

1.4.2. H2A and H2B variants

The histone H2A family contains a large number of variants in eukaryotes (Fig. 4B) (Malik and Henikoff 2003). MacroH2A isoforms are only found in vertebrates and are distinguishable from all other H2A’s by their large globular (macro) domain (Buschbeck and Di Croce 2010; Zink and Hake 2016). It is enriched on the inactive X chromosome and heterochromatic domains (Costanzi and Pehrson 1998; Zhang et al. 2005; Gamble et al. 2010). H2A.Bbd (Barr body deficient) is also found only in vertebrates. It is 48% identical to H2A and as its name indicates is excluded from the inactive X (Chadwick and Willard 2001). H2AX has a unique extension in the C-terminal part (Fig. 4B). Perhaps the best known function of the H2A.X variant is in the recognition of DNA double strand breaks (DSBs). Upon DSB detection, serine 139 of H2A.X is rapidly phosphorylated (H2A.XS139ph) (Rogakou et al. 1998); H2A.XS139ph, also called γH2A.X, accumulates around DSBs where it recruits DNA repair factors (Li et al. 2005; Lou et al. 2006). H2A.Z shares ~60% similarity with canonical H2A (Henikoff and Smith 2015). Two genes encode H2A.Z.1 and H2A.Z.2 sub-variants (Eirin-Lopez et al. 2009) (Fig. 4B). H2A.Z prevents nucleosome-H1 interactions (Thakar et al. 2009), contributing to
loosening chromatin structure, and plays a role in DNA repair (Xu et al. 2012). H2A.Z predominantly associates with H3 variant H3.3 to form H2A.Z-H3.3 nucleosomes; these appear to characterize regions of chromatin instability, suggesting a high turnover rate of these nucleosomes (see also section 1.5) (Yukawa et al. 2014). H2A.Z plays a role in transcription regulation in multiple ways (Soboleva et al. 2014); in mammals, H2A.Z correlates with transcriptional activity but negatively correlates with transcription in yeast (Soboleva et al. 2014); this discrepancy has been proposed to be related to H2A.Z positioning relative to the TSS.

The histone H2B family contains three variants in addition to canonical H2B (Fig. 4B), each with specialized functions. Two play a role in chromatin compaction during gametogenesis: TSH2B is a sperm-specific variant that replaces most canonical H2B in elongating spermatids and facilitates replacement of somatic histones by protamines during spermiogenesis (Montellier et al. 2013). H2BFWT is expressed exclusively in testis but its role remains unclear (Churikov et al. 2004). The most recently identified replication-independent H2B variant, H2BE, is expressed exclusively in mouse olfactory neurons where it modulate a transcription and life span of these neurons (Santoro and Dulac 2012).

1.4.3. Variants of histone H3

The histone H3 family consists of a large number of variants (Fig. 4B) that differ between species (Hake and Allis 2006). CENP-A (also known as cenH3) is a centromere-specific H3 found in all eukaryotes and is necessary to form a functional centromere. CENP-A shows only 50% similarity in its globular domain with the other H3s (Malik and Henikoff 2003). In mammals, CENP-A is deposited in centromeric regions in telophase and early G1 in a replication-independent manner (Jansen et al. 2007), where it is essential for kinetochore formation and chromosome segregation (Howman et al. 2000). In human cells CENP-A can form functional neo-centromeres at ectopic sites independently of centromeric α-satellite sequences (Amor et al. 2004), suggesting that CENP-A may function as a key factor organizing centromeres. Supporting this view, CENP-A remains associated with centromeres throughout spermatogenesis while all other histones are replaced by protamines (Palmer et al. 1990). CENP-A is therefore a key component of centromere identity.
Three additional H3 variants are constitutively expressed in mammalian cells, namely H3.1, H3.2 and H3.3. Other higher eukaryotes harbor only two non-centromeric H3 variants (H3, which is identical to mammalian H3.2, and H3.3) and yeasts express only one (H3.3-like in *Saccharomyces cerevisiae* and a hybrid H3 containing amino acids of both H3.3 and H3.2 in *Saccharomyces pombe*) (Hake and Allis 2006) (Fig. 5). H3.3 is the focus of this thesis and is discussed in more detail below. H3.1 and H3.2 are canonical core histones expressed and loaded on chromatin during S phase (Ahmad and Henikoff 2002; Tagami et al. 2004). H3.1 is deposited into chromatin after DNA damage as well (Polo et al. 2006). Human H3.1 and H3.2 differ by one amino acid at position 96, where H3.1 contains a cysteine and H3.2 a serine. Despite their high similarity, H3.1 and H3.2 are enriched in distinct hPTMs. At the promoter level, H3.2 has been found on a large proportion of sites marked by H3K9me3 and/or H3K27me3 (Delbarre et al. 2010), in agreement with mass spectrometry data showing H3.2 enrichment in K27me2 or K27me3 (Hake et al. 2006). H3.1 can be marked by K9me2 and accordingly it is found in heterochromatin (Hake et al. 2006; Tamura et al. 2009; Stroud et al. 2012). The differences in PTMs of H3.1 and H3.2 suggest that these H3 variants may be associated with distinct biological functions. This is in agreement with observation that in human cells H3.1, but not H3.2, is in a very close proximity to C-terminus of kinetochore protein CENP-T (Abendroth et al. 2015). Moreover, it is interesting to note that serines and cysteines have distinct biochemical properties: while a serine can undergo PTMs including phosphorylation, cysteines are involved in the formation of disulfide bonds. Whether these residues contribute to the distinct functions associated with H3.1 and H3.2 is a possibility that remains to be investigated.

Much less is known about the remaining H3 variants. H3.4 (also known as H3t or H3.1t) is thought to be testis-specific (Witt et al. 1996), although it has also been found in low amount in somatic cells (Govin et al. 2005). H3.4 varies from H3.1 by four residues, forms less stable nucleosomes than H3.1 or H3.2 containing nucleosomes, and has been proposed to play an important role in chromatin reorganization during spermatogenesis (Witt et al. 1996; Tachiwana et al. 2010). H3.X and H3.Y are two primate-specific variants (Wiedemann et al. 2010). They differ by four amino acids in their overlapping regions, while H3.X has a long unique C-terminal tail. H3.X and H3.Y have a higher sequence homology with H3.3 than with H3.1 or H3.2. Interestingly, starvation combined with high cell density can increase levels of H3.Y and knock down of H3.Y affects cell
growth and cell cycle control in U2OS cells (Wiedemann et al. 2010). Moreover, H3.Y depletion leads to an increase of H3.X mRNA level suggesting functional connections between these variants (Wiedemann et al. 2010). Recently, genomic localization analysis of the H3.Y revealed its enrichment around the TSSs of actively transcribed genes, suggesting that H3.Y may regulate the transcription status of certain genes (Kujirai et al. 2016). The latest discovered H3 variant is the hominid-specific H3.5 (also known as H3.3C) (Schenk et al. 2011). H3.5 is expressed in seminiferous tubules in human testis, is enriched in transcribed genes, and has been suggested to be able to substitute for H3.3 in maintaining cell growth (Schenk et al. 2011).

The emergence of H3 variants during evolution attests of the essential roles of H3 in genome organization. These processes require machinery that properly targets and incorporates H3 and its variants into chromatin. These aspects are key to this thesis and are addressed below.

![Figure 5. Major non-centromeric H3 amino-acid differences in fungi and metazoans.](Key amino acid differences are indicated with letters and additional differences are shown as dots. Taken from (Elsaesser et al. 2010).)
1.4.4. Histone variant H3.3

Histone H3 variant H3.3 is deposited into chromatin in replication-independent manner (Ahmad and Henikoff 2002; Tagami et al. 2004). H3.3 is encoded by two genes, \(H3F3A\) and \(H3F3B\) (Frank et al. 2003). Expression level of both H3.3 genes is not always concordant. In mouse embryonic stem (ES) cells, expression of \(H3f3b\) accounts for the majority of H3.3 protein levels (Udugama et al. 2015). However, tissue-specific differences have been reported in \(H3f3a\)- or \(H3f3b\)-null mouse fetuses, with more H3.3 encoded by \(H3f3a\) in the brain and similar amounts of H3.3 encoded by both \(H3f3a\) and \(H3f3b\) in liver and lung (Tang et al. 2015). H3.3 transcript levels also change during differentiation of C2C12 myoblasts (Song et al. 2012), raising the hypothesis that H3.3 encoded by either \(H3F3A/H3f3a\) or \(H3F3B/H3f3b\) may play different roles in a cell type- or tissue-specific manner. Indeed, inactivation of \(H3f3a\) in mice by gene-trap causes perinatal lethality (Couldrey et al. 1999), whereas knockout of \(H3f3b\) results in reduced viability and infertility in almost all survivors (Bush et al. 2013). \(H3f3a\)-null mice have also been shown to be viable to adulthood, with females being fertile and males subfertile, whereas \(H3f3b\)-null mutants are growth-deficient and die at birth (Tang et al. 2015). In \(Drosophila\), the lack of functional copies of both H3.3 genes leads to reduced viability and sterility in males and females (Sakai et al. 2009).

Histone H3.3 differs from H3.1 and H3.2 by five and four amino acids, respectively (Fig. 4B). Differences are at positions 31, 87, 89 and 90 where H3.1 and H3.2 have residues A, S, V and M (single-letter amino acid code), and H3.3 contains S, A, I, and G. In addition, amino acid 96 is a cysteine (C) in H3.1 while both H3.2 and H3.3 harbor a serine (S). Mutational analysis shows that amino acids A87, I89 and G90 in H3.3 confer independence of H3.3 deposition on DNA replication (Ahmad and Henikoff 2002). In addition, in mouse ES cells, mutations of H3.3 into H3.2 or into H3.1 (yet with the S31 of H3.3; “H3.1S31”) are able to alter the genome-wide distribution of H3.3 (Goldberg et al. 2010). These studies indicate that a handful of amino acids in the H3.3 globular domain are sufficient to confer a unique genomic enrichment pattern; this may be linked to differential association with distinct histone chaperones (see below).

H3.3 has been found to be enriched primarily in transcriptionally active chromatin (Ahmad and Henikoff 2002; Chow et al. 2005). Moreover, ChIP and immuno-FISH analyses show that H3.3 is incorporated in the promoter region of actively transcribed
genes, persists at these sites during mitosis, and is associated with H3 acetylation and H3K4 methylation, which mark active genes (Chow et al. 2005). Accordingly, mass spectrometry confirms that H3.3 harbors marks of active chromatin (Hake et al. 2006), and a ChIP-promoter array hybridization (ChIP-chip) study from our laboratory shows that H3.3-enriched promoters have stronger H3K4me3 enrichment at the TSS than all promoters in the RefSeq database, and H3.2-enriched promoters (Delbarre et al. 2010). In line with these data, ChIP-seq analyses of endogenously tagged H3.3 indicate that H3.3 is enriched in the body of active genes (Goldberg et al. 2010). Importantly though, H3.3 can also be found on promoters of non-expressed genes (Delbarre et al. 2010; Goldberg et al. 2010). The genomic localization of H3.3 is also altered upon cell differentiation, particularly on cell type-specific genes, e.g. after differentiation of ES cells into neuronal precursors (Goldberg et al. 2010). Moreover, in bivalent genes activated upon differentiation, H3.3 is maintained around the TSS and is incorporated into the gene body, whereas on bivalent genes that remain repressed, H3.3 enrichment is reduced at the TSS, with no gene body enrichment (Goldberg et al. 2010). These findings suggest that H3.3-containing nucleosomes may play a role in the establishment or maintenance of this bivalent promoter state in stem cells.

Contrasting with the initial view that H3.3 was a marker of active chromatin, an increasing number of studies have highlighted the association of H3.3 with regions of heterochromatin. Using antibodies against its phosphorylated serine (S)31 (H3.3S31ph), H3.3 was shown to accumulate at pericentric heterochromatin in HeLa (Hake et al. 2005) and mouse cells (Wong et al. 2009; Drane et al. 2010; Santenard et al. 2010), and epitope-tagged H3.3 targets telomeres in mouse ES cells (Wong et al. 2009; Goldberg et al. 2010). Interestingly, H3.3 loading at telomeres was recently shown to be essential for maintenance of the repressed state of these heterochromatic regions by providing a substrate for K9 trimethylation (H3.3K9me3) (Udugama et al. 2015). H3.3 is also distributed in heterochromatic regions in the mouse genome, including endogenous retroviral repeats (Elsasser et al. 2015) and silenced imprinted differentially methylated regions (Voon et al. 2015). These data support an emerging view that H3.3 may play a role in the repression of specific heterochromatic regions through trimethylation of H3.3K9.

The replacement histone function of H3.3 has been put forward not only in a transcription context, but also situations of DNA damage (Adam et al. 2013) and alterations in
chromatin causing nucleosome-depleted regions (Ray-Gallet et al. 2011; Schneiderman et al. 2012). These studies lead to the view of H3.3 playing a role in a nucleosome “gap-filling” process (Ray-Gallet et al. 2011; Schneiderman et al. 2012). Notably, H3.3 deposition at sites of UV-induced DNA damage sites is necessary for reactivation of transcription after DNA damage repair (Adam et al. 2013), and for replication fork progression (Frey et al. 2014). These studies, along with work presented in this thesis (Paper II and Paper III), suggest that H3.3 is deposited at any accessible regions left by the loss of H3.1-H4 nucleosomes. This may be necessary to avoid leaving nucleosome-free regions which would compromise genome integrity.

1.5. **Turnover of histone H3**

Chromatin is a dynamic structure regulating the DNA accessibility for transcription, replication, recombination and DNA repair (Venkatesh and Workman 2015). One of the mechanisms that modulate chromatin structure is histone exchange, or histone turnover. Histone turnover is a process by which entire nucleosomes or subsets of nucleosomal histones are replaced by “fresh ones”. Both newly synthesized and parental histones can be used in the assembly of a new nucleosome (Hamiche and Shuaib 2013). During nucleosome disassembly, the H2A-H2B dimer is released first, prior to eviction of the H3-H4 dimer (Fig. 2) (Henikoff 2008). It remains unclear whether the H3-H4 tetramer is split into two halves or is evicted as a whole before nucleosome reassembly (Katan-Khaykovich and Struhl 2011).

Several factors have been shown to facilitate histone exchange, including chromatin remodelers, histone chaperones and hPTMs. Chromatin remodelers use energy from ATP hydrolysis for nucleosome sliding, eviction and histone exchange (Becker and Workman 2013). They can generate an open DNA region amenable for deposition of new histones (see also section 1.6) or cooperate with histone chaperones to wrap DNA around histones (Becker and Workman 2013). Histone chaperone ASF1 (anti-silencing function protein 1) has been found to participate not only in histone deposition but also in H3-H4 eviction at yeast promoters (Schwabish and Struhl 2006). This is line with the ability of ASF1 to disrupt H3-H4 tetramers into dimers *in vitro* (Natsume et al. 2007). PTMs, such as histone acetylation, also affect nucleosome stability and histone eviction by opening or
Histone variants are also important elements in histone turnover, to ensure maintenance of proper chromatin structure by replacing canonical histones. Turnover of H3.3, likely together with turnover of H2A.Z, in regions containing H2A.Z-H3.3 nucleosomes (which tend to be unstable; see section 1.4.2), is more frequent than that of canonical H3 because H3.3 is the dominant histone H3 available outside S phase, and H3.1 or H3.2 cannot be deposited into chromatin in the absence of DNA replication (Ahmad and Henikoff 2002; Ray-Gallet et al. 2011). Therefore, H3.3 turnover is especially important in non-dividing cells, such as neurons. Indeed, accumulation of H3.3 has been detected in neuronal and glial chromatin with age and has been shown to be critical for neuronal activity-dependent gene expression (Maze et al. 2015). The rate of H3.3 turnover is different in distinct genomic regions (Fig. 6). In mammalian cells, H3.3 turnover is highest at active promoters and enhancers (which also contain H2A.Z) and lowest in heterochromatin regions including telomeres and pericentromeres (Huang et al. 2013; Kraushaar et al. 2013) (Fig. 6). There, histone turnover may be controlled by a replication-dependent deposition pathway (Kraushaar et al. 2013; Huang and Zhu 2014). Thus, distinct mechanisms linked to transcription and DNA replication regulate H3.3 turnover at distinct sites in the genome.
1.6. Deposition of histone H3 into chromatin by specific histone chaperones

H3 variants are incorporated in chromatin at distinct sites by different histone chaperones (Wong et al. 2009; Drane et al. 2010; Goldberg et al. 2010; Hamiche and Shuaib 2013). These are histone-associated factors responsible for histone storage, folding, exchange, removal and deposition into chromatin (De Koning et al. 2007; Hamiche and Shuaib 2013). We address here the chaperones specific for canonical H3 and H3.3.

1.6.1. Nuclear import of newly synthesized histone H3

Histones are incorporated into chromatin through multiple steps. First, newly synthesized histones are imported into the nucleus. In the cytoplasm, soluble non-nucleosomal human H3 and H4 are found in at least four protein complexes. During synthesis, H3 and H4 are transiently poly-ADP-ribosylated until they form a dimer; this has been proposed to help keeping individual histones in a properly folded state until dimerization occurs (Alvarez et al. 2011). Immediately after synthesis H3 and H4 interact with the chaperones HSC70 (Heat shock cognate 71 kDa protein) and HSP90/70 (Heat shock protein 90/70), respectively, to prevent mis-folding and aggregation. H3-H4 dimerization is facilitated by HSP90 and tNASP (testicular nuclear autoantigenic sperm protein) (Campos et al. 2010; Alvarez et al. 2011). H3-H4 dimers associate with sNASP (somatic nuclear autoantigenic sperm protein) and RBAP46 (retinoblastoma-associated protein 46), and the N-terminal tail of H4 is presented to the HAT1 acetyl transferase for acetylation on K5 and K12 (Campos et al. 2010). Finally, acetylated histone dimers interact with anti-silencing function protein 1 homolog (ASF1) A and/or B and are transferred into the nucleus by Importin 4 (Campos et al. 2010). Note that other importins, including Importin 5, Importin 7, Importin β and Transportin may mediate nuclear import of H3 (Baake et al. 2001; Muhlhausser et al. 2001; Mosammaparast et al. 2002).

1.6.2. Replication-coupled deposition of canonical H3

Canonical histone H3.1 is incorporated into chromatin during DNA replication and UV-damage by the chromatin assembly factor-1 (CAF-1) (Gaillard et al. 1996; Shibahara and Stillman 1999; Tagami et al. 2004; Polo et al. 2006). In the nucleus, ASF1A/B (see above)
in a complex with H3-H4 acts as a histone donor for CAF-1 which then loads H3-H4 on chromatin (Mello et al. 2002; English et al. 2006; De Koning et al. 2007). In yeast, ASF1 promotes acetylation of H3K56, which weakens the ASF1-(H3-H4) interaction and allows CAF-1 and Rtt106 (regulator of Ty1 transcription protein 106) to bind the H3-H4 dimer (Li et al. 2008; Burgess and Zhang 2013; Zhang et al. 2013). The chaperone FACT (facilitates chromatin transactions) promotes chromatin deposition of newly synthesized H3-H4 dimers during S phase through its association with Rtt106 and the (H3K56Ac-H4) dimer (Yang et al. 2016). In human cells, CAF-1 can dimerize, likely promoting the formation of the H3-H4 tetramer (Quivy et al. 2001). CAF-1 also interacts with PCNA (proliferating cell nuclear antigen) and colocalizes with the replication fork (Fig. 7A), coupling H3-H4 loading onto newly replicated DNA (Shibahara and Stillman 1999; Moggs et al. 2000).

To my knowledge, a chaperone specific only for H3.1 or H3.2 has not yet been identified. Taking into account that H3.1 and H3.2 only differ by one amino acid, CAF-1 may recognize both histones. Indeed, CAF-1 association with H3.2 has been demonstrated in HeLa cells using double affinity purification followed by tandem mass spectrometry analysis (Latreille et al. 2014). This is also supported by observations that CAF-1 depletion leads to defects in both H3.1 and H3.2 incorporation in mouse embryos (Akiyama et al. 2011). MCM proteins together with ASF1 have been also identified as H3.2 interacting factors (Latreille et al. 2014). However, this interaction is not specific for H3.2, and is observed for H3.1 and H3.3 (Latreille et al. 2014).

1.6.3. Replication-independent deposition of H3.3

Histone variant H3.3 is incorporated into different chromatin sites in a replication-independent manner by two major distinct histone chaperone complexes: histone regulator A (HIRA) in a complex with UBN1, CABIN1 and ASF1A (HUCA complex), and the death domain-associated protein (DAXX) in a complex with α-thalassemia/mental retardation X-linked syndrome protein (ATRX) (Fig. 7B) (Drane et al. 2010; Goldberg et al. 2010; Rai et al. 2011). HIRA was initially found to play a role in chromatin assembly in a replication-independent manner in Xenopus leavis egg extracts (Ray-Gallet et al. 2002). It was later identified in a complex with H3.3 (Tagami et al. 2004) and shown to be necessary for H3.3 deposition in the male pronucleus in
*Drosophila* and mouse zygotes (Loppin et al. 2005; van der Heijden et al. 2005). HIRA-dependent deposition of H3.3 also occurs on active and bivalent promoters and in transcribed genes (Goldberg et al. 2010; Pchelintsev et al. 2013). In addition, HIRA can deposit H3.3 at replication sites after inhibition of H3.1 loading by CAF-1, and incorporates H3.3 in nucleosome-free regions (Ray-Gallet et al. 2011) and at sites of DNA damage (Adam et al. 2013). Therefore HIRA is generally considered as a chaperone loading H3.3 in active genomic sites.

![Figure 7. The pathways of histone H3 deposition.](image)

(A) Replication-coupled deposition of canonical H3. CAF-1 and FACT, in cooperation with ASF1 and Rtt106, deposit H3-H4 into chromatin. Modified from (Yang et al. 2016) (B) Replication-independent deposition of H3.3 into specific domains by HIRA and DAXX/ATRX. Taken from (Xiong et al. 2016).
Prior to deposition, the H3.3-H4 dimer is presented to HIRA by ASF1A (Tang et al. 2006). Since ASF1A binds both CAF-1 and HIRA through the same region, HIRA competes with CAF-1 for having the opportunity to bind ASF1A (Tang et al. 2006). This competition is regulated by phosphorylation of H4 on S47, which promotes assembly of H3.3-H4 nucleosomes by increasing the binding affinity of HIRA to H3.3; it notably also inhibits assembly of H3.1-H4 dimers by reducing the association of CAF-1 with H3.1-H4 (Kang et al. 2011). Recently, the HIRA-binding protein UBN1 was identified as a novel H3.3-specific binding protein interacting with the residue G90 of H3.3 (Daniel Ricketts et al. 2015). The UBN1-binding region of H3.3-H4 is distinct from ASF1A-binding site (English et al. 2006). HIRA interacts with initiating and elongating forms of RNA polymerase II (Pol II) (Ray-Gallet et al. 2011), and this may promote incorporation of H3.3 in transcribed genes. HIRA can also associate with histone methyltransferase Wolf-Hirschhorn syndrome candidate 1 (WHSC1), which methylates H3K27, H3K36 and H4K20, and with Polycomb repressive complex 2 (PRC2) which trimethylates H3K27 (Banaszynski et al. 2013; Sarai et al. 2013). This suggests that H3.3 can be post-translationally modified at sites of chromatin deposition by HIRA, ensuring the maintenance of epigenetic states.

The H3.3 chaperone DAXX (Drane et al. 2010; Lewis et al. 2010) was originally identified as a protein associated with FAS-mediated apoptosis (Yang et al. 1997). Biochemical studies indicate that DAXX directly binds to H3.3 via the unique AAIG motif of H3.3 (Lewis et al. 2010) (Fig. 4B). This was confirmed by crystallography demonstrating that DAXX binds the H3.3-H4 heterodimer (Elsasser et al. 2012; Liu et al. 2012). The primary determinant for DAXX binding is the H3.3 residue G90, which also needed for UBN1 binding (Elsasser et al. 2012; Daniel Ricketts et al. 2015). Moreover, DAXX covers H3.3-H4 dimer at the same place as ASF1 (Elsasser et al. 2012). This suggests that the DAXX and HIRA complexes compete for the H3.3-H4 dimer.

During neuronal activation, DAXX is able to mediate H3.3 loading at regulatory elements of activity-regulated genes through a mechanism involving a calcium-dependent phosphorylation switch (Michod et al. 2012). DAXX also associates with ATRX, which belongs to the SNF2 chromatin remodeling protein family of helicase/ATPases, and promyelocytic leukemia (PML) nuclear bodies (Tang et al. 2004). The DAXX/ATRX complex has primarily been found to be critical for H3.3 deposition at pericentric heterochromatin and at telomeres, at least in pluripotent cells (Drane et al. 2010;
Goldberg et al. 2010; Lewis et al. 2010; Wong et al. 2010). DAXX/ATRX also deposits H3.3 in other heterochromatic regions in the mouse genome, including DNA-methylated alleles of imprinted and non-imprinted genes (Voon et al. 2015), endogenous retroviral repeats (ERVs) (Elsasser et al. 2015), retrotransposons and telomeres (He et al. 2015). Strikingly, ATRX has been shown to inhibit excessive H3.3 loading at endogenous intracisternal A particles (IAPs) and to secure efficient heterochromatin formation (Sadic et al. 2015). Most sites enriched in DAXX/ATRX and H3.3 are also enriched in H3K9me3, which is lost after DAXX, ATRX or H3.3 depletion; this has been linked to reactivation of these silenced regions (Elsasser et al. 2015; He et al. 2015; Jang et al. 2015; Udugama et al. 2015; Voon et al. 2015). Interestingly, the ATRX N-terminal region contains an ATRX-DNMT3-DNMT3L (ADD) domain that recognizes both unmodified H3K4 and H3K9me3 (Dhayalan et al. 2011; Eustermann et al. 2011; Iwase et al. 2011). This suggests that ATRX can recruit DAXX and H3.3 in H3K9me3-enriched regions and facilitate H3.3 deposition. Furthermore, ATRX interacts with heterochromatin protein 1 (HP1) (Lechner et al. 2005). HP1 notably binds H3K9me3 and recruits the SUV39H HMT (Felsenfeld and Groudine 2003) that trimethylates H3.3K9 at least on telomeres (Udugama et al. 2015). In addition, DAXX interacts with SUV39H and KAP1, which catalyzes H3.3K9me3 at ERVs (Elsasser et al. 2015; He et al. 2015). These data indicate that the DAXX/ATRX complex incorporates H3.3 at sites of heterochromatin and may be important for heterochromatin homeostasis.

In addition to HIRA and DAXX/ATRX, four proteins have been involved in H3.3 incorporation; these include chromatin helicase DNA-binding protein 1 (CHD1), chromodomain helicase DNA-binding domain 2 (CHD2), E1A-binding protein p400 (EP400) and DEK (Konev et al. 2007; Sawatsubashi et al. 2010; Harada et al. 2012; Siggens et al. 2015; Pradhan et al. 2016). EP400 contributes to gene regulation via deposition of H3.3 into promoters and enhancers (Pradhan et al. 2016). CHD1 was found to interact with HIRA and to be necessary for H3.3 incorporation into the male pronucleus in Drosophila embryos (Konev et al. 2007). CHD2 has been identified as a MyoD-interacting protein incorporating H3.3 at myogenic promoters to facilitate differentiation (Harada et al. 2012). CHD2 is also recruited to DSBs by polyADP-ribose polymerase 1 (PARP1), where it elicits H3.3 deposition (Luijsterburg et al. 2016). Lastly, the chromatin-bound factor DEK, which also potentially acts as an H3.3 chaperone, is discussed in section 1.8.
In light of the studies presented above, it is becoming clear that H3.3 incorporation into chromatin by distinct chaperones is not limited to active regions but also occurs in heterochromatin. However, how H3.3 is distributed between the different complexes remains poorly understood. In Paper I-III, we show that PML nuclear bodies are sites of co-localization of H3.3 chaperones and are key players in the routing of newly synthesized H3.3 to chromatin.

1.7. PML nuclear bodies

The promyelocytic leukemia (PML) protein is a tumor suppressor protein which has been identified as a fusion protein in acute promyelocytic leukemia caused by the chromosomal (15; 17) translocation resulting to a fusion of the PML and the retinoic acid receptor alpha (RARA) genes (Kakizuka et al. 1991). Due to alternative splicing of a single PML gene, up to 7 PML protein isoforms (designated PML1-7) are produced in humans (Fig. 8A) (Bernardi and Pandolfi 2007), six being nuclear and one being cytoplasmic (Nisole et al. 2013). All isoforms harbor a conserved N-terminus containing the RBCC (Really Interesting New Gene – (RING) finger domain, two cysteine/histidine-rich B-Box domains and an α-helical coiled-coil domain)/TRIM motif involved in PML dimerization and binding to other proteins (Jensen et al. 2001). PML isoforms however differ in their C-terminal end, suggesting that PML function may be isoform-dependent (Nisole et al. 2013). According to the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/), mice only harbor three PML isoforms designated PML1, PML2 (full length PML) and PML3.

In the nucleus, PML proteins are enriched as spherical structures of 0.1 to 1 μm in diameter called PML nuclear bodies - also known as nuclear domain (ND) 10 or PML oncogenic domains (PODs) (Fig. 8B) (Lallemand-Breitenbach and de The 2010). PML bodies are heterogeneous and dynamic, and contain up to over 100 different proteins (Van Damme et al. 2010); these notably include DAXX and ATRX (Li et al. 2000; Tang et al. 2004), enzymes controlling PTMs including kinases, HATs and methyltransferases (Sahin et al. 2014a), and SUMO (small ubiquitin-like modifier) proteins necessary for integrity of PML bodies (Lang et al. 2010; Sahin et al. 2014a). The formation of PML bodies is hierarchical. First, PML proteins are oxidized and form multimers that are organized into structures (bodies) associated with the nuclear matrix (Sahin et al. 2014a;
Guan and Kao 2015). Second, PML proteins are sumoylated by the SUMO-conjugating enzyme UBC9 (Sahin et al. 2014a). Interestingly, arsenic trioxide (As$_2$O$_3$)-induced attachment of higher weight poly-SUMO chains to PML proteins leads to PML ubiquitination and proteasome-dependent degradation (Nisole et al. 2013). The third step of PML body formation is protein partner recruitment. A common feature of most PML body components and of PML itself is their ability to be sumoylated and to harbor one or more SUMO interacting motifs (SIMs) (Sahin et al. 2014b). It is thus thought that PML partners may initiate association with PML bodies through sumoylation and/or the SIM, and sumoylated proteins recruited to PML bodies may gain additional PTMs through other enzymes also targeted to PML bodies (Sahin et al. 2014a). Thus, combinatorial association of PML with many proteins form multiple PML bodies which can participate in many processes including protein modification, degradation and sequestration, apoptosis, senescence, response to DNA damage, or resistance to micro-organisms and viral infections (Fig. 8C) (Lallemand-Breitenbach and de The 2010).

**Figure 8. PML isoforms and PML bodies.** (A) PML isoforms generated by alternative splicing of the PML gene containing nine exons; abbreviations: R – RING motif, B – B-boxes, CC – coiled-coil domain, NLS – nuclear localization signal, asterisk – frameshift. Modified from (Bernardi and Pandolfi 2007). (B) Immunofluorescence and electron micrographs of PML bodies; the red arrow indicates a single PML body. Modified from (de The et al. 2012). (C) PML body-containing proteins and associated functions. Taken from (de The et al. 2012).
PML bodies are also implicated in transcription regulation (Bernardi and Pandolfi 2007). Using ChIP, immuno-TRAP labeling and FISH approaches, PML has been localized in the vicinity of transcribed regions (Kumar et al. 2007; Gialitakis et al. 2010; Ulbricht et al. 2012; Ching et al. 2013) and shown to co-localize or interact with transcription factors and HATs, linking PML to transcription (Pearson et al. 2000; Zhong et al. 2000). In contrast, PML can also associate with repression-linked proteins such as HP1 (Seeler et al. 1998), histone deacetylases (Khan et al. 2001) and the histone methyltransferase SETDB1 which methylates H3K9 (Cho et al. 2011). Moreover, in cancer cells exhibiting ALT (alternative lengthening of telomeres), a specific kind of PML bodies called ALT-associated PML bodies (APBs) has been found to be associated with telomeric DNA and telomere-binding proteins (TRF1 and TRF2) (Wu et al. 2003). PML bodies also co-localize with telomeres and are necessary for H3.3 loading at telomeres by ATRX in mouse ES cells (Chang et al. 2013). Depletion of PML causes loss of ATRX and loss of H3.3 binding at telomeres, leading to telomeric dysfunction phenotype (Wong et al. 2009; Wong et al. 2010; Chang et al. 2013). These studies suggest that PML bodies play a role in chromatin organization through non-random association with genomic regions and might serve as platforms for H3.3 chaperones and H3.3 deposition, at least in mouse cells. We rationalized in this thesis work that PML bodies might be functionally linked to H3.3 chaperones and H3.3 incorporation into chromatin (Papers I-III).

1.8. The oncoprotein DEK

The work presented in this thesis (Paper II) also suggests that H3.3 deposition into chromatin may also be controlled by chromatin-associated protein complexes. Of such protein is DEK, a non-histone chromatin-associated protein highly conserved in higher eukaryotes. DEK is a 43 kDa protein with no identified enzymatic activity (Kappes et al. 2001; Privette Vinnedge et al. 2013; Mattrka et al. 2015). It has been discovered as a fusion protein in acute myeloid leukemia, caused by the (6;9) translocation which fuses two genes, DEK and CAN, the latter encoding a nuclear pore complex protein (von Lindern et al. 1992). DEK contains three DNA-binding domains, namely a central SAF-box, a pseudo-SAF/SAP box N-terminal to the SAF box, and a C-terminal DNA binding domain (Privette Vinnedge et al. 2013; Pease et al. 2015). By binding to DNA, DEK can bend it and introduce positive supercoils (Waldmann et al. 2002).
Whether association of DEK with DNA is sequence-specific has remained controversial. On one hand, using competition electrophoretic mobility shift assay (EMSA), DEK has been shown to have DNA sequence binding specificity towards the human immunodeficiency virus type 2 (HIV-2) peri-ets (pets) site, which is a TG-rich element in the HIV-2 enhancer (Fu et al. 1997). Mutational analysis further shows that DEK displays binding specificity to distinct sequence variants of the class II major histocompatibility complex (MHC) promoter (Adams et al. 2003). However, additional EMSA analyses, where purified DEK was incubated with wild-type or mutated pets sequences, supercoiled, relaxed, linear, duplex or cruciform DNA, have revealed that DEK binding to DNA is likely not sequence-specific, but rather shows preference for supercoiled and cruciform DNA structures (Waldmann et al. 2003). Nevertheless, more recently, using a proteomic analysis of isolated chromatin segments (PICh), wherein specific DNA sequences are pulled-down and associated proteins identified, Drosophila DEK has been also found to bind telomere-associated repeats (Antao et al. 2012). Thus, DEK may show some sequence specificity, at least in repeat regions.

Association of DEK with chromatin influences chromatin organization. DEK overexpression induces ectopic DEK association with mitotic chromosomes, leading to mitotic defects such as lagging chromosomes, anaphase bridges, and micronuclei (Matrka et al. 2015). This suggests that elevated expression of DEK causes chromosome instability, which is known to favor tumorigenicity. It is also consistent with observations that high levels of DEK are detected in cancer cells (Carro et al. 2006).

DEK is a multi-functional protein highly expressed in fast proliferating and cancer cells, with however, a decreasing expression level during differentiation (Carro et al. 2006; Wise-Draper et al. 2009; Privette Vinnedge et al. 2013). High expression of DEK in proliferating cells may be explained by upregulation of the transcription factor E2F which controls the transition from G1 to S phases of the cell cycle (Privette Vinnedge et al. 2013; Sanden and Gullberg 2015). Indeed, ChIP experiments reveal that E2F binds the DEK promoter and induces DEK expression (Carro et al. 2006). These results suggest that DEK is important in maintaining cell proliferation. In agreement with this, DEK level decreases as cells reach their proliferation capacity, whereas DEK overexpression bypasses senescence (Wise-Draper et al. 2005). Conversely, DEK knockdown reduces proliferation potential, slows down replication fork velocity, increases DNA damage at mitosis after induction of a replication stress (Deutzmann et al. 2015) and elicits apoptosis.
(Wise-Draper et al. 2006). These findings are consistent with previous results showing that DEK depletion in human cancer cells induces a DNA damage response (Kavanaugh et al. 2011). Collectively, these observations show that DEK promotes cell growth and survival by suppressing cellular senescence and apoptosis, and by contributing to DNA repair.

DEK has also been shown to be involved in gene regulation, with, however, apparently contradictory roles. On one hand, imaging and immunoprecipitation studies indicate that DEK is associated with euchromatic regions (Hu et al. 2007; Sawatsubashi et al. 2010) and preferentially binds promoters and genes that are highly active (Sanden et al. 2014). Accordingly, DEK is enriched at DNase-I hypersensitive sites, albeit in a transcription-dependent manner, as binding is reduced after inhibition of RNA Pol II (Hu et al. 2007). These studies therefore link DEK to transcriptional activity of genomic regions it associates with. On the other hand, DEK may also be implicated in conferring a repressive chromatin conformation. DEK has been identified in a complex with histone deacetylase II (Hollenbach et al. 2002) and to be important for heterochromatin integrity (Kappes et al. 2011; Saha et al. 2013). In human cells, loss of DEK correlates with global and locus-specific reduced levels of H3K9me3 and results in an increasing proportion of MNase-sensitive chromatin (Kappes et al. 2011). Conversely, DEK overexpression correlates with increased level of H3K9me3 (Kappes et al. 2011), and similarly, a rescue of DEK-depleted cells with recombinant DEK not only restores H3K9me3 levels but also leads to a more compact, MNase-resistant, chromatin conformation (Saha et al. 2013). Lastly, DEK directly binds to the HP1α and enhances HP1α interaction with H3K9me3 (Kappes et al. 2011); this notably leads to recruitment of the HMT SUV39H1/2 that further maintains the heterochromatic state by methylating H3K9 (Fig. 9A) (Felsenfeld and Groudine 2003). Altogether, these studies indicate that DEK can influence both gene activation and repression.

To date, little is known on how DEK might be implicated in the functions outlined above. One possible mechanism may involve PTMs of DEK itself. DEK contains over 70 lysine residues which can be potentially polyADP-ribosylated or acetylated (Mattrka et al. 2015). DEK polyADP-ribosylation leads to DEK dissociation from chromatin (Gamble and Fisher 2007; Kappes et al. 2008); as DEK polyADP-ribosylation occurs during apoptosis (Gamble and Fisher 2007; Kappes et al. 2008), this may provide a mechanism for the role of DEK in cell survival. Similarly, DEK acetylation reduces its binding to DNA in
glioblastoma cells (Cleary et al. 2005) and results in its re-localization to interchromatin granule clusters, sub-nuclear domains containing RNA-processing and transcription factors (Cleary et al. 2005). This may couple DEK acetylation to its potential role in gene expression. Finally, DEK contains 57 potential phosphorylation sites (Matrka et al. 2015), some of which are substrates for casein kinase 2 (CK2) (Kappes et al. 2004a). In vitro, DEK phosphorylation leads to its dissociation from DNA and to its multimerization (Kappes et al. 2004a; Kappes et al. 2004b); however, phosphorylated DEK remains bound to native chromatin through dimerization with unphosphorylated DEK (Kappes et al. 2004a), so to my knowledge the role of DEK phosphorylation on its association with chromatin in cells remain uncertain.

Figure 9. The oncoprotein DEK chromatin-related functions. (A) DEK importance for heterochromatin integrity; by interacting with HP1, DEK facilitates its binding to H3K9me3 and HP1 recruits SUV39H1/2 that methylates H3K9; DEK also binds DNA and thus helps maintain a heterochromatic state. Taken from (Broxmeyer et al. 2013). (B) Nucleosome reconstitution assay showing that DEK is a histone chaperone dependent on CK2. Taken from (Sawatsubashi et al. 2010). (C) Immunofluorescence co-localization of DEK (green) with H3.3 (red). Taken from (Sawatsubashi et al. 2010).
DEK phosphorylation by CK2 appears to be important for its claimed histone chaperone activity in vitro (Sawatsubashi et al. 2010) (Fig. 9B). In addition, imaging studies show that DEK co-localizes with H3.3 in Drosophila salivary gland cells (Fig. 9C), and facilitates H3.3 assembly during puff formation (Sawatsubashi et al. 2010). This suggests that DEK is involved in the deposition of H3.3 into chromatin (Sawatsubashi et al. 2010). Paper II in this thesis evaluates a role of DEK in the loading of H3.3 on chromatin, not solely from a chaperone point of view, but also, and primarily, from a chromatin standpoint.

1.9. H3.3 mutations and cancer

Somatic heterozygous mutations in the H3F3A have been identified in brain tumors such as DIPGs (diffuse intrinsic pontine gliomas) and glioblastoma multiform (Schwartzentruber et al. 2012; Wu et al. 2012). These mutations occur on K27, with K mutated to a methionine (K27M) or an isoleucine (K27I), and on G34, with G substituted to an arginine (G34R) or a valine (G34V) (Schwartzentruber et al. 2012; Wu et al. 2012; Castel et al. 2015). These mutations are mutually exclusive and show distinct localizations in the brain and distinct ages of diagnosis (Fig. 10). The K27M/I mutation is mostly located in the brain stem of young children whereas G34R/V is restricted to cerebral hemispheric tumors in adolescents and adults (Jones and Baker 2014; Castel et al. 2015). Moreover, K27M/I and G34R/V mutations negatively affect global methylation level of H3K27 and H3K36 methylation levels on the same tail of mutated H3.3, respectively (Bender et al. 2013; Lewis et al. 2013; Kallappagoudar et al. 2015). These large-scale epigenetic alterations are likely to have profound implications on transcription, and may directly or indirectly affect tumorigenicity of the mutated cells, although the mechanisms behind these effects remain largely unknown. Mutations in H3F3B resulting in H3.3K36M and G34W or G34L substitutions have respectively been identified in chondroblastoma and giant cell tumors of bone (Behjati et al. 2013).

The H3.3 incorporation machinery has also been linked with cancer. Strikingly, ATRX/DAXX mutations have been found to co-exist with all G34/V mutations in brain tumors (Schwartzentruber et al. 2012). ATRX/DAXX mutations have also been identified in pancreatic neuroendocrine tumors characterized as ALT cancer cells (Heaphy et al.
Mutations in H3.3 and its chaperones causing cancer point to the importance of H3.3 in maintaining proper chromatin state and genome stability.

Figure 10. Age and neuroanatomical localization of tumors harboring H3.3 mutations. K27M mutation (red star) mainly occur in brainstem and thalamic location of younger children; K27I (green star) has been found only in pontine tumors of younger children; G34V/R occurs in cerebral hemispheres of adolescents and adults. The size of the stars illustrating mutations is approximately proportional to % of identified tumors in (Khuong-Quang et al. 2012; Schwartzentruber et al. 2012; Sturm et al. 2012; Castel et al. 2015).
2. Aims of the study

Histones are fundamental proteins necessary for DNA packaging, establishment of chromatin states, and regulation of gene expression. Histone variant H3.3 is deposited into distinct genomic areas by specific chaperones. How is H3.3 dispatched to its different chaperones and what regulates the interplay between these chaperones has long remained poorly understood. This thesis seeks to elucidate mechanisms of H3.3 loading on chromatin. More specifically, our work focuses on cross-talks between H3.3 chaperones and on functional relationships between H3.3, H3.3 chaperones and chromatin-associated proteins.

The aims of the study were to:

- Investigate processes by which newly synthesized non-nucleosomal H3.3 is targeted to chromatin (Paper I).
- Determine the role of DEK, a chromatin-bound protein, in the deposition of H3.3 into chromatin (Paper II).
- Map the distribution of promyelocytic (PML) protein in the genome and assess its impact in the incorporation pattern and dynamics of H3.3 into chromatin (Paper III).
3. Summary of publications

Paper I

DAXX-dependent supply of soluble (H3.3-H4) dimers to PML bodies pending deposition into chromatin

Erwan Delbarre, Kristina Ivanauskiene, Thomas Küntziger, and Philippe Collas

Genome Research (2013) 23, 1580-1589. doi: 10.1101/gr.159400.113

The replication-independent chromatin deposition of histone variant H3.3 is mediated by several chaperones. We report here a multi-step targeting of newly synthesized epitope-tagged H3.3 to chromatin via promyelocytic leukemia (PML) nuclear bodies. We find that H3.3 is recruited to PML bodies in a DAXX-dependent manner, a process facilitated by ASF1A. DAXX is required for enrichment of ATRX, but not ASF1A or HIRA, with PML. Nevertheless, these chaperones co-localize with H3.3 at PML bodies and are found in one or more complexes with PML. Both DAXX and PML are necessary to prevent accumulation of a soluble, non-incorporated pool of H3.3. H3.3 targeting to PML bodies is enhanced with an (H3.3-H4)_2 tetramerization mutant of H3.3, suggesting H3.3 recruitment to PML as an (H3.3-H4) dimer rather than as a tetramer. Our data altogether support a model of DAXX-mediated recruitment of (H3.3-H4) dimers to PML bodies. We propose that PML bodies may function as sorting (triage) centers for H3.3 deposition into chromatin by distinct chaperones.
Paper II

The PML-associated protein DEK regulates the balance of H3.3 loading on chromatin and is important for telomere integrity

Kristina Ivanauskiene, Erwan Delbarre, James D. McGhie, Thomas Küntziger, Lee H. Wong and Philippe Collas


Histone variant H3.3 is deposited in chromatin at active sites, telomeres and pericentric heterochromatin by distinct chaperones, but the mechanisms of regulation and coordination of chaperone-mediated H3.3 loading remain largely unknown. We show in this paper that the chromatin-associated oncoprotein DEK regulates differential HIRA- and DAXX/ATRX-dependent distribution of H3.3 on chromosomes in somatic cells and in embryonic stem cells. Live cell imaging studies show that non-nucleosomal H3.3 normally destined to PML nuclear bodies is re-routed to chromatin after depletion of DEK. This results in HIRA-dependent wide-spread chromatin deposition of H3.3, and H3.3 incorporation in foci of heterochromatin, in process requiring the DAXX/ATRX complex. In embryonic stem cells, loss of DEK leads to displacement of PML bodies and ATRX from telomeres, redistribution of H3.3 from telomeres to chromosome arms and pericentric heterochromatin, induction of a fragile telomere phenotype and telomere dysfunction. Our results indicate that DEK is required for proper loading of ATRX and H3.3 on telomeres and for telomeric chromatin architecture. We propose that DEK acts as a ‘gate-keeper’ of chromatin, controlling chromatin integrity by restricting broad access to H3.3 by dedicated chaperones. Our results also suggest that telomere stability relies on mechanisms ensuring proper histone supply and routing.
PML protein organizes heterochromatin domains and regulates histone H3.3 loading by ATRX

Erwan Delbarre, Jane Spirkoski, Kristina Ivanauskienė, Akshay Shah, Kristin Vekterud, Thomas Küntziger and Philippe Collas

Manuscript under revision at the time of this writing.

Maintaining chromosome integrity in the cell nucleus entails proper delivery of histones, post-translational histone modifications and histone variants to chromatin. The interplay between different histone chaperones regulating the supply of histone variants to distinct chromatin domains remains largely unknown. Using biochemical, live cell imaging and genomics approaches, we show here a role of the promyelocytic leukemia (PML) protein in routing histone variant H3.3 to chromatin and in the organization of broad, intergenic and heterochromatic PML-associated domains which we refer to as PADs. The absence of PML alters the heterochromatic states of PADs by shifting the histone H3 methylation balance from K9 towards K27 trimethylation in these domains. Loss of PML also impairs the H3.3 loading function of ATRX in PADs and elicits H3.3 deposition and H3K27 trimethylation in these regions by HIRA. Our findings demonstrate a PML-dependent role of ATRX in H3.3 deposition in well-defined heterochromatic areas, and a compensatory H3.3 loading activity by HIRA in these regions when ATRX function is compromised. They also unveil a hitherto unappreciated role of PML in the large-scale organization of chromatin. We suggest that H3.3 loading by HIRA and H3K27 trimethylation constitute a mechanism ensuring maintenance of a heterochromatic state in PADs when integrity of these domains is compromised.
4. Discussion

The work presented in this thesis aims to better understand the mechanisms controlling histone variant H3.3 loading into chromatin using human and mouse cells. We show that a fraction of H3.3 transits via PML nuclear bodies in a DAXX-dependent manner before being incorporated into chromatin. Our data further support a model where PML is necessary for the function of H3.3 chaperone ATRX and for the heterochromatic organization of PML-associated domains. In parallel, we also demonstrate that the chromatin-bound protein DEK coordinates differential deposition of H3.3 through DAXX/ATRX and HIRA chaperone complexes.

4.1. PML bodies as a triage center for H3.3 before deposition into chromatin

H3.3 is deposited into chromatin by at least two distinct complexes: the HIRA/UBN1/CABIN1 complex that interacts with ASF1A, and the DAXX/ATRX complex (Drane et al. 2010; Goldberg et al. 2010; Rai et al. 2011). In paper I we report the accumulation of H3.3 together with HIRA, ASF1A, DAXX and ATRX at PML bodies in human primary cells. We suggest that PML bodies might act as a sorting center where a fraction of H3.3 can speculatively be sorted and presented to different chaperone complexes prior deposition into chromatin. We show that H3.3-H4 dimers are recruited to PML bodies by DAXX. Interestingly, previous studies show that ASF1 can bind free H3.3-H4 dimers, as well as dimers from a preformed DAXX-H3.3-H4 complex (Elsasser et al. 2012). Therefore, ASF1A may interact with H3.3 at PML bodies and present H3.3 to HIRA. Nevertheless, the relatively low proportion of cells containing detectable ASF1A at PML bodies (paper I) argues that a putative delivery of H3.3 to HIRA by ASF1A might only occur in a sub-population of cells. Moreover in paper II, we show that DEK, another H3.3 chaperone, also accumulates at PML bodies, supporting the view of PML bodies being a site of co-localization of H3.3 chaperones and H3.3. DEK has been found to associate with DAXX (Hollenbach et al. 2002), and co-localizes with DAXX at PML bodies (our unpublished data). However, it is not clear whether DEK acts as a chaperone in a complex with DAXX.
Reasons why H3.3 is recruited to PML bodies and how it might be distributed to different complexes remain unclear and will require further investigations. It is possible that before deposition into chromatin, H3.3 is post-translationally modified. Evidence supporting this idea emanates from a work showing that H3.3 is modified by K9 mono- and dimethylation (H3.3K9me1 and H3.3K9me2), and K9 and K14 acetylation (H3.3K9ac and H3.3K14ac) before deposition into chromatin (Loyola et al. 2006). Since PML bodies harbor HMTs, kinases and HATs (Sahin et al. 2014a) which may modify H3.3, it is formally plausible that modifications of H3.3 could occur at PML bodies. Moreover, if H3.3 is modified at PML bodies, these modifications may play a role in the distribution of H3.3 to distinct complexes. This hypothesis would be in agreement with the deposition of H3.3 in differently marked chromatin regions by distinct chaperone complexes: extrapolating on the Loyola et al. (2006) findings, one may speculate that the HIRA complex would load acetylated H3.3 on sites containing marks of active chromatin, whereas the ATRX/DAXX complex would deposit K9-methylated H3.3 in transcriptionally repressive domains (Goldberg et al. 2010; Lewis et al. 2010; Wong et al. 2010).

PML bodies contain the SETDB1 HMT which trimethylates H3K9 (Cho et al. 2011), and ATRX (paper I-III) that could potentially recognize H3.3K9me3 at PML bodies via the ADD domain (Dhayalan et al. 2011; Eustermann et al. 2011; Iwase et al. 2011). Subsequently, ATRX may load H3.3K9me3 at specific chromatin sites. Supporting this idea, we show in paper III that ATRX is responsible for H3.3 deposition in PML-associated domains (PADs) and at telomeres, and that this loading function of ATRX is reduced or lost in the absence of PML. It would be informative to perform a mass-spectrometry analysis of H3.3 co-isolated with PML to assess the nature of H3.3 PTMs in these structures.

In Drosophila, the N-terminal tail of H3 is required for its deposition during replication but not for the replication-independent deposition of H3.3 (Ahmad and Henikoff 2002). However, we found that H3.3 deleted for residues 3 to 35 (‘H3.3[core]’) accumulates at PML bodies and shows long-term (up to 7 days) residence at these sites (Fig. 11A; our unpublished data). Moreover, FRAP experiments revealed that H3.3[core] is more dynamic than full-length H3.3 (Fig. 11B) and remains dynamic even after 7 days. This suggests that a fraction of H3.3[core] is not incorporated into chromatin and that the H3.3 tail is essential for proper H3.3 deposition. One critical factor positively influencing H3.3
loading in this context would be PTMs on the H3.3 N-terminal tail (Loyola et al. 2006). Altogether, these studies suggest that PTMs on H3.3 may play an important role in its deposition into chromatin, possibly by the recognition of distinct modifications by different chaperone complexes.

Figure 11. Recruitment of H3.3[core] to PML bodies. (A) H3.3[core] co-localization with PML. Human primary cells expressing H3.3[core]-mCherry were fixed 24 h after transfection. Scale bar, 7 μm. (B) Dynamics of H3.3[core]-EGFP at 24 and 168 h (7 days) after transfection (green and red lines, respectively). Full-length H3.3-EGFP was used as a control, and shows slower recruitment to PML bodies (blue line). Ivanauskiene, Delbarre, Collas, unpublished data.

4.2. PML nuclear bodies retain a non-nucleosomal pool of H3.3 and regulate its loading at specific sites

In contrast to replication-dependent incorporation of canonical histones, H3.3 is deposited throughout the cell cycle (Ahmad and Henikoff 2002; Tagami et al. 2004) and is implicated in processes such as transcription and de novo deposition of nucleosomes in DNA damage sites prior to repair (Goldberg et al. 2010; Ray-Gallet et al. 2011; Adam et al. 2013). In replicating cells, H3.3 can compensate at least in part for chromatin assembly defects occurring after impaired incorporation of H3.1 (Ray-Gallet et al. 2011).
Furthermore, in embryonic and adult neuronal cells, where H3.3 accumulates at near-saturating levels throughout the genome, a reduced available H3.3 pool results in stalled nucleosome turnover and in transcriptional deregulation (Maze et al. 2015). These findings suggest that it would be advantageous for the nucleus to maintain a sufficient level of non-chromatin-bound H3.3 protein available for replication-independent incorporation. Thus, cells must elaborate a mechanism to maintain a given non-nucleosomal histone level available for incorporation, and yet also prevent these histones from being degraded. Indeed, the histone chaperone NASP can protect a reservoir of soluble histone H3-H4 dimers from chaperone (Hsc70 and Hsp90)-mediated autophagy (Cook et al. 2011). This pathway is not specific for H3.3 since depletion of NASP reduces the soluble level of both H3.3 and H3.1 (Cook et al. 2011). Moreover, NASP binding affinity to H3.3 is the lowest among human H3 variants and shows the highest specificity for canonical H3 histones (Osakabe et al. 2010). Therefore, a reduced level of soluble H3.3 detected after NASP depletion may not be due to degradation but may result from enhanced H3.3 incorporation into replicating DNA when H3.1 cannot be incorporated (Ray-Gallet et al. 2011). These data point to the existence of a mechanism for histone prevention from degradation which may be more specific for canonical histone H3.1 than for H3.3.

In paper I, we provide evidence for an H3.3-specific pathway which may prevent H3.3 from degradation by sequestration of a soluble pool and lead to the maintenance of available histones for incorporation into chromatin. H3.3 is recruited to PML bodies, which have been shown to be associated with the nuclear matrix (Sahin et al. 2014a; Guan and Kao 2015) and are resistant to non-ionic detergents (Stuurman et al. 1992). Accordingly, we show that the epitope-tagged H3.3 fraction enriched at PML bodies resists Triton X-100 and high salt extraction. Thus, non-incorporated H3.3 is brought to PML bodies where it may be sequestered and stored in an insoluble form. This is consistent with our results that depletion of PML leads to an increased soluble pool of H3.3 in the nucleoplasm in human cells. In contrary, our data in mouse cells show reduced soluble H3.3 in PML knock-out cells. This discrepancy could speculatively be explained by an ‘adaptive’ mechanism of regulation of an available soluble H3.3 acquired in Pml ko cells, which have never see the PML protein and yet show no phenotype in steady-state conditions in culture.
Interestingly, yeasts lack a PML gene homolog and have no structural equivalent to PML bodies (Quimby et al. 2006). There, excess of histones in the nucleoplasm has been shown to lead mitotic chromosome loss, increased DNA damage sensitivity and cytotoxicity (Meeks-Wagner and Hartwell 1986; Gunjan and Verreault 2003; Singh et al. 2010). This could conceivably be because histones in excess may be incorporated ectopically, thereby ‘overloading’ chromatin. In mammalian cells, loss of PML may elicit an enhanced level of available H3.3, which is abnormally loaded into the genome.

Indeed, in paper III, we find that H3.3 is incorporated into chromatin more readily in the absence of PML, and in particular within sites normally associated with PML in wt cells. Therefore, by sequestering a pool of soluble H3.3, PML bodies may regulate chromatin loading of H3.3, by restricting its access to specific regions. It would be interesting to determine which domain of the PML protein might be involved in the sequestration of H3.3, and whether an entire structure such as whole PML bodies are required. Another interpretation of these findings is that nuclear structural proteins such as PML or DEK (paper II), protect areas of the genome by maintaining their structural organization and do not necessarily act by ‘preventing’ genome overloading by H3.3. In fact, several studies show that destabilization of chromatin structure, through e.g. the generation of nucleosomal gaps (Ray-Gallet et al. 2011; Schneiderman et al. 2012), leads to H3.3 deposition by HIRA at those sites. This is reminiscent of our findings after DEK depletion in human cells (paper II) or PML loss in MEFs (paper III) even though we have not shown that chromatin organization is altered in these instances.

4.3. PML-dependent H3.3 loading on chromatin is necessary for maintenance of telomeric chromatin integrity

H3.3 localization at telomeres has been well established in mouse ES cells (Wong et al. 2009; Goldberg et al. 2010; Wong et al. 2010) (paper II). At these sites, H3.3 is loaded by ATRX/DAXX, a process that requires the association of PML bodies at telomeres (Goldberg et al. 2010; Lewis et al. 2010; Wong et al. 2010; Chang et al. 2013), presumably with the aim of bringing the K9me3 heterochromatin mark, as H3.3K9me3, to telomeres (Udagama et al. 2015). It has been proposed that PML loss in mouse ES cells leads to disruption of ATRX and H3.3 binding in telomeric regions, and induces dysfunctional telomere phenotype (Chang et al. 2013). In paper II, we show that after
DEK depletion, PML bodies together with ATRX are re-localized from telomeres to pericentric regions. This leads to re-localization of H3.3 from telomeric to pericentric heterochromatin and formation of a fragile telomere phenotype. These data suggest that association of PML bodies with telomeres is important for H3.3 deposition and thus maintenance of telomeric chromatin integrity, at least in mouse ES cells.

It has been suggested that telomere-associated PML bodies could be linked to the pluripotent state of ES cells (Chang et al. 2013). In mouse ES cells, induced cellular differentiation results in reduced association of PML bodies with telomeres and no co-localization between PML and telomeres have been detected in somatic cells (Chang et al. 2013). In agreement with this, in papers I-III we do not observe PML bodies at telomeres in non-pluripotent cells. However, this does not exclude the possibility that the PML protein or PML bodies may regulate loading of H3.3 at telomeres in these cells. In paper III, our ChIP-seq experiments show that both PML and epitope-tagged H3.3 are associated with telomeric repeats in wild-type MEFs. Strikingly, in Pml ko MEFs, we find a reduction of H3.3 at telomeres. This could be due to the loss of function from ATRX observed in these cells (paper III) reinforcing the view that H3.3 is loaded at telomeres by ATRX in a PML-dependent manner (Chang et al. 2013). From our data, we can conclude that PML is important for H3.3 incorporation at telomeres not just in pluripotent cells, but also in non-pluripotent cells. Brouwer and colleagues (2009) have found that in U2OS, HeLa, hematopoietic leukemia and MEF cells PML bodies are formed at telomeric DNA during interphase and dissociate from these sites after PML body formation. DAXX also has been detected at telomeres during PML assembly (Brouwer et al. 2009). This suggests that in non-pluripotent cells PML bodies are formed at telomeres where, and at which time, H3.3 may be incorporated through recruitment of DAXX.

Further corroborating this notion, a recent study shows that in normal human fibroblasts, ~10% of PML bodies associate with telomeres (Marchesini et al. 2016); knock-down of PML in these cells leads to marked genomic instability as a result of telomere dysfunction. This is consistent with the telomere dysfunction phenotype observed after loss of H3.3 due to either PML depletion in ES cells (Chang et al. 2013) or depletion of DEK, which leads to dissociation of PML bodies from telomeres (paper II). Therefore, PML is fundamental for maintenance of telomeric chromatin via ATRX/DAXX-dependent H3.3 loading. This process seems to be independent of species or pluripotency state.
The fact that, in our studies, we have not been able to detect PML at telomeres by immunostaining in unsynchronized human or mouse non-pluripotent cells may be due to a restricted time-window during which PML is associated with these sites during the cell cycle. It is also noteworthy that we did not specifically focus on identifying PML at telomeres during these observations, thus it remains possible that a putative low level PML enrichment at these sites (as we show by ChIP), was overlooked.

4.4. DEK function in H3.3 incorporation into chromatin

Using a reconstitution assay with native core histones purified from *Drosophila* cells and supercoiled plasmid DNA, DEK has been found to transfer histones to DNA, thereby forming mono- and dinucleosomes (Sawatsubashi et al. 2010). Histone chaperone activity has been also demonstrated for human DEK (Kappes et al. 2011). Moreover, DEK can be co-immunoprecipitated and co-localize with H3.3 in *Drosophila* salivary glands (Sawatsubashi et al. 2010). These data suggest the role of DEK in H3.3 deposition into chromatin. In paper II, we show that DEK co-localizes with PML bodies at telomeres (albeit not all) in mouse ES cells, and that its depletion leads to loss of H3.3 at telomeres and a fragile telomere phenotype. DEK, therefore, is important for proper H3.3 loading at these sites, and for genome stability. DEK may be involved in several ways in this process: (1) DEK can act as H3.3 chaperone and/or (2) DEK may be required for recruitment and/or anchoring of PML at telomeres. In agreement with the latter, PML bodies have been proposed to act as a platform for H3.3 loading at telomeres (Chang et al. 2013). Interestingly, DEK has been found to have binding specificity for telomere repeats (Antao et al. 2012) and for major histocompatibility complex promoters (Adams et al. 2003). PML is also found in association with these regions (Shiels et al. 2001; Ulbricht et al. 2012; Chang et al. 2013) suggesting a genomic link between DEK and PML. Using the PROSITE Scan database (Sigrist et al. 2013), we identified a SUMO1 recognition motif on DEK (paper II). Since PML interacts with protein partners via SUMO/SIM interactions (Sahin et al. 2014a), DEK may interact with PML bodies through sumoylation. However, it remains unclear whether DEK is able to deposit H3.3 in the mammalian genome, and whether this takes place at PML bodies. This will require further investigations.
DEK also colocalizes with PML bodies in human cells, although not at telomeres. DEK loss in these cells results in the detection of PML and epitope-tagged H3.3 (paper II) or endogenous H3.3 (Fig. 12; our unpublished data) at heterochromatic foci marked by H3K9me3. Using a combination of FRAP, immunofluorescence and knock-downs approaches, we show that H3.3 is incorporated in these regions by the ATRX/DAXX complex; however, this incorporation is not dependent on PML (paper II). Thus, H3.3 loading at these sites probably involves the known recognition of H3K9me3 by ATRX (Dhayalan et al. 2011; Eustermann et al. 2011; Iwase et al. 2011). Moreover, detection of PML bodies at these H3K9me3 sites in DEK-depleted cells raises the hypothesis that removal of DEK might elicit a re-localization of PML bodies and de novo associations with the genome. This would interestingly imply that DEK may play a role in the association of PML with chromatin – a hypothesis which could be testable by a ChIP-seq profiling of PML throughout the genome in the presence or absence of DEK. Additional ChIP-seq analyses of PML, H3K9me3 and H3.3 under these conditions would establish whether H3.3 is incorporated in heterochromatic foci formed prior to or after DEK depletion, in relation to PML anchoring.

Figure 12. DEK depletion leads to enrichment of endogenous H3.3 foci in H3K9me3 and DAPI-dense DNA regions. Cells were fixed 4 days after DEK depletion by siRNA and analyzed by anti-H3.3 and H3K9me3 immunofluorescence. Arrows point to sites of H3.3, H3K9me3 and DAPI-dense DNA co-localization (Delbarre and Collas, unpublished data).

Our findings further show that DEK controls H3.3 distribution in chromatin (paper II). We demonstrate that DEK depletion leads to HIRA-dependent wide-spread incorporation of H3.3 in human cells. Since DEK is a chromatin-bound protein promoting
heterochromatin formation (Kappes et al. 2001; Kappes et al. 2011), the loss of DEK may result in more accessible chromatin for HIRA-dependent H3.3 loading. Fractionation of chromatin into MNase-resistant (‘compact’) and MNase-sensitive (‘open’) compartments (as we have done in paper III) from cells with or without DEK would provide an indication of the influence of DEK on the extent of chromatin accessibility. HIRA has been found to incorporate H3.3 in more open chromatin where turnover of H3.3 is high (Fig. 6) (Goldberg et al. 2010; Pchelintsev et al. 2013; Huang and Zhu 2014). HIRA is also implicated in a nucleosome gap filling process by loading H3.3 at these sites (Ray-Gallet et al. 2011; Schneiderman et al. 2012). Therefore, DEK depletion may lead to faster histone exchange (Fig. 13, pathway 1), and/or to the creation of nucleosomal gaps (Fig. 13, pathway 2). A ChIP analysis of pan-H3 enrichment in the genome in H3.3-deficient cells with or without DEK would give an indication of whether nucleosome occupancy is reduced upon DEK depletion. If so, nucleosome gaps would be formed in DEK-depleted cells. On the contrary, unaltered nucleosome occupancy would suggest the possibility of a histone exchange process; this could in principle be examined by ChIP analysis of H3.1, H3.2 and H3.3 in cells with or without DEK. Further work is required to address these possibilities.

Figure 13. Potential mechanisms of H3.3 incorporation by HIRA in DEK-depleted cells: a model. In cells containing DEK, H3.3 loading is inhibited and H3.3 is recruited to PML bodies by DAXX. DEK depletion may lead to more open chromatin; thus H3.3 may be redistributed from PML to chromatin as a result of HIRA-dependent histone exchange (pathway 1). DEK loss may also lead to nucleosome ‘gaps’ which can be filled with H3.3-containing nucleosomes by HIRA (pathway 2).
A critical point on the effect of DEK depletion on the pattern of H3.3 deposition (paper II) is whether H3.3 phosphorylation state is altered after the loss of DEK. To be able to assess, by imaging, the distribution of H3.3 on chromosomes (and its enrichment at telomeres in particular) in DEK-depleted cells, we have relied on an antibody directed against H3.3 phosphorylated on serine 31 (H3.3S31ph). This approach was motivated by several factors: (1) investigating mitotic chromosomes provides sufficient resolution to distinguish telomeres, chromosome arms, pericentromeric and centromeric regions; (2) the anti-H3.3S31ph antibody is well suited for immunofluorescence and is highly specific to H3.3 (since other H3 variants do not harbor a serine in position 31); (3) H3.3, detected with this antibody has been shown to accumulate at telomeres in mouse ES cells (Wong et al. 2009). We found that after DEK depletion, in mitotic cells the H3.3S31ph immunofluorescence signal disappears from telomeres and appear on chromosome arms and pericentromeres. We cannot formally exclude, however, that the decrease in H3.3S31ph labelling at telomeres and increase at chromosome arms in DEK-depleted cells is not a mere consequence of H3.3S31 being dephosphorylated and phosphorylated, respectively, at these sites. DEK depletion could conceivably affect phosphatase activity towards H3.3S31ph; this remains yet to be tested. For this purpose, we overexpressed DEK in mouse ES cells. However, we did not detect any effect on H3.3 phosphorylation state (paper II) and the effect of DEK depletion remains unclear. Notwithstanding, taking into account the well-known chaperone activity of HIRA, and the fact that, to my knowledge, no role of HIRA on H3.3 phosphorylation was established, our observation that HIRA knockdown in DEK-depleted cells reduces H3.3S31ph staining on chromosome arms is most likely due to the absence of HIRA’s chaperone activity and H3.3 loading. It would be informative to repeat these experiments using a specific antibody to H3.3 that is, for this matter, not dependent on H3.3 phosphorylation (see paper III).

4.5. H3.3 deposition to maintain heterochromatin integrity

The work presented in this thesis provides additional evidence for an important role of H3.3 in the maintenance of chromatin integrity (paper II, III). Not only H3.3 contributes to preserving heterochromatin at telomeres – by bringing the K9me3 mark as H3.3K9me3 (Udugama et al. 2015), it also seems to be involved in maintaining a heterochromatic state of regions associated with PML (PADs), under conditions where PML is absent
Interestingly, we report an apparent substitution of H3K9me3 by H3K27me3 in PADs in Pml ko MEFs. This may be interpreted as an attempt to preserve a (repressed) heterochromatic state in these regions – a function directly or indirectly mediated by PML. We do not at present provide direct evidence that this increase in H3K27me3 is due to H3.3 enrichment per se; however, supporting this view, it correlates with an increase in H3.3-Flag. Moreover, importantly, knock-down of HIRA in Pml ko cells leads to a reduction of both H3.3 and H3K27me3 levels in PADs (paper III). Demonstrating a causal link between H3.3 and H3K27me3 in PADs in Pml ko cells would require showing that depletion of H3.3 would prevent the increase in H3K27me3. A sequential ChIP of H3.3 and H3K27me3 (and vice-versa) from mono-nucleosome preparations would further help to determine whether H3.3 itself is trimethylated on K27.

A question emerging from our work is whether PADs are conserved or divergent between cell types and species. It is noteworthy that megabase-size, gene-poor and heterochromatic regions referred to as lamin-associated domains (LADs) have been shown to associate with the nuclear lamina (Collas et al. 2014). Inasmuch as LADs are overall conserved between cell types and between mice and humans (Meuleman et al. 2013), the striking similarities in the genomic properties of LADs and PADs (Collas et al. 2014) (paper III) suggest that PADs may also show conservation between cell types. LADs and PADs may also overlap in parts of the genome, tentatively providing a functional link between PML bodies and nuclear lamins through their ‘chromatin anchor’ functions. Supporting this idea of conservation of PADs between cell types is our observation that in mouse ES cells, endogenous H3.3 levels are lower in regions corresponding to PADs in MEFs, than in non-PAD regions (paper III). Additional bioinformatics analyses will be able to assess the relationship between PADs and other large heterochromatic domains of the genome.

4.6. Potential implication of PML and DEK on genome organization and cell function

We present evidence for up-to-now uncharacterized chromatin domains associated with PML. A critical point that remains to be addressed is the functionality of PADs and their relationship to gene expression. Our RNA-seq studies show that genes in PADs are transcriptionally repressed in MEFs (paper III). The question then arises of whether
these genes also reside in PADs in other cell types in which they are expressed. For example, are olfactory receptor (Olfr) and vomeronasal 2 receptor (Vmn2r) genes, involved in olfactory function (Dulac and Torello 2003) localized in PADs in olfactory neurons? Are they expressed in these cells? What is their chromatin environment? Additional studies combining genomics and functional approaches in wild-type and Pml ko mice will be required to address the biological significance of PML association with the genome and its relationship to H3.3 deposition in chromatin.

Another important emerging issue is the impact of DEK on PADs. Indeed, DEK is found in a fraction of PML bodies in both human and mouse ES cells (in the latter, at telomeres), and removal of DEK results in the re-localization of PML from telomeres to pericentromeric regions in mouse ES cells. In human cells, DEK depletion leads to a re-modelling of the epigenetic environment of chromatin associated with PML bodies. These findings suggest that DEK influences either the association of PML with the genome (PADs) and/or the chromatin composition of PADs. It will be interesting to determine the spatial relationship between DEK and PML along the genome and to what extent DEK directly influences the positioning of PADs in various cell types (for instance in MEFs or neurons).

Collectively, the data presented in this thesis provides new important insights on the organization and dynamics of the mammalian genome and opens new perspectives to further investigations of nuclear and genome architecture.
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