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Epigenetic and replacement roles of histone variant H3.3 in reproduction and development

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ABSTRACT The nucleosomal organization of eukaryotic chromatin is generally established during DNA replication by the deposition of canonical histones synthesized in S phase. However, cells also use a Replication Independent (RI) nucleosome assembly pathway that allows the incorporation of non-canonical histone variants in the chromatin. H3.3 is a conserved histone variant that is structurally very close to its canonical counterpart but nevertheless possesses specific properties. In this review, we discuss the dual role of H3.3 which functions as a neutral replacement histone, but also participates in the epigenetic transmission of active chromatin states. These properties of H3.3 are also explored in the light of recent studies that implicate this histone and its associated chromatin assembly factors in large scale, replication-independent chromatin remodeling events. In particular, H3.3 appears as a critical player in the transmission of the paternal genome, from sperm to zygote.

KEY WORDS: *H3.3, epigenetic, sperm chromatin remodeling, histone chaperone*

Introduction

The organization of chromatin in eukaryotic cells is remarkably conserved. The basic unit of chromatin, the nucleosome, is constituted by a hetero-octamer of histones that are wrapped with about 146bp of DNA. The structural properties of nucleosomes can be modulated by a large variety of post-translational modifications (PTMs) of histone proteins. The combinatorial complexity of these modifications is at the origin of the "histone code" hypothesis, which proposes that histone PTMs participate, along with other epigenetic marks such as DNA methylation, in the functional organization of the genome (Jenuwein and Allis, 2001). Nucleosomes can also be modulated by the incorporation of histone variants that differ from the major, canonical histones synthesized during S phase. Histone variants differ from their canonical counterpart at the level of the primary sequence. These differences can range from a few amino-acid positions (e.g. H3.1 vs H3.3) to large protein domains (e.g. H2A vs macroH2A) and usually confer specific properties to nucleosomes. In contrast to canonical histones that are devoted to Replication Coupled (RC) chromatin assembly, histone variants are expressed throughout the cell cycle and are thus available, at least theoretically, in nucleosome assembly pathways that occur in a Replication-Independent (RI) manner (Henikoff and Ahmad, 2005; Sarma and Reinberg, 2005). For this reason, histone variants are also called "replacement" histones. Finally, certain variants are preferentially or specifically expressed in certain tissues, such as the testis specific histone H3 variant, for instance (Witt *et al.*, 1996).

The combination of PTMs and histone variant creates a wide diversity of nucleosomes. This variability is important to determine the properties of the chromatin fiber at a local and regional level, with respect to essential aspects of DNA metabolism, such as replication, transcription, heterochromatin formation, repair, condensation or kinetochore formation.

In this article, we focus on the function of the histone H3.3 variant during development and reproduction. A main feature of H3.3 is its association with transcriptionally active chromatin and its potential role in the epigenetic transmission of active chromatin states. These properties are at the origin of a growing interest for H3.3 over the past few years. However, recent studies in various model organisms have revealed unexpected roles for this variant, particularly during sexual reproduction. We will discuss the respective importance of replacement and epigenetic roles of this histone, in the context of its diversity and evolutionary history, and in the light of its interactions with nucleosome assembly machineries.

Abbreviations used in this paper: MSCI, meiotic sex chromosome inactivation; MSUC, meiotic silencing of unsynapsed chromatin; PTM, post-translational modification; RC, replication coupled; RI, replication independent; SCR: sperm chromatin remodeling; SNBP, sperm nuclear basic protein.

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The histone H3.3 family of proteins

Genes encoding canonical histones are usually organized in tandem, multi-copy clusters and have no introns. Replicative histone mRNAs are not polyadenylated. Instead, translation is tightly regulated by the binding of SLBP (Stem Loop Binding Protein) and U7 snRNP to the 3' end of the histone RNAs (Jaeger *et al.*, 2005). This peculiar genomic organization and transcriptional regulation allows a massive production of canonical histones at the beginning of the S phase and ensures the synthesis of stoichiometric quantities of each protein. On the contrary, histone variant genes are regular genes that are represented by a single or a few copies and are scattered throughout the genome. In addition, they often possess introns and their polyadenylated mRNAs are expressed throughout the cell cycle (Fig. 1).

In mouse, thirteen canonical *H3* genes are present in the genome, encoding two versions of canonical H3: H3.1 and H3.2. They differ by a single amino acid in position 96 (Graves *et al.*, 1985). The functional relevance of having two different replicative H3s is unclear (Hake and Allis, 2006). In *Drosophila*, the histone gene cluster on the left arm of chromosome 2 contains twenty-three copies of each *H1*, *H2A*, *H2B*, *H3* and *H4* genes (Fig. 1). All the *Drosophila* histone *H3* genes encode the same H3 protein, identical to mammalian H3.2.

Histone H3 variant types include centromeric H3 variants (CenH3s), H3.3 and testis specific H3 in mammals (Fig.1). CenH3s form a highly divergent family of histone H3 variants that are characterized by an H3-like histone fold domain and a variable N-terminus tail (reviewed in Ahmad and Henikoff, 2002a; Dalal *et al.*, 2007). In mouse, human and *Drosophila*, two *H3.3* genes (*H3.3A* and *H3.3B*) encode the same conserved protein, but the transcripts have distinct untranslated regions (Akhmanova *et al.*, 1995; Frank *et al.*, 2003; Krimer *et al.*, 1993).

H3.3 is one of the most conserved proteins and appears to be present in all eukaryotes (Malik and Henikoff, 2003). H3.3 differs from H3.2 (mouse) or H3 (*Drosophila*) by only four amino acids at positions 31, 87, 89 and 90 (Fig. 2). The residue at position 31 sits in the N-terminal tail of the protein while positions 87, 89 and 90 are located in the α2 helix of the histone fold domain (Fig. 2). In spite of the great sequence similarity between H3.3 and H3, it has been proposed that these residues could account for specific properties of H3.3 proteins. In vertebrates, the serine in position 31 (H3.3S31p) can be phosphorylated and this PTM is detected on metaphase chromosomes, at specific sites bordering centromeres, unveiling a possible role of this mark during cell division (Hake *et al.*, 2005). H3.3S31p also exists in the urochordate *Oikopleura dioica* and is detected during mitosis and oogenic meiosis (Schulmeister *et al.*, 2007). In addition, a potentially phosphorylable threonine residue is found in position 31 in *C. elegans* and *A. thaliana* H3.3, respectively, but this is not the case for other members of the family (Fig. 2).

The residues in positions 87, 89 and 90 are necessary and sufficient to exclude canonical H3 from RI assembly pathways in *Drosophila* (Ahmad and Henikoff, 2002b), suggesting that they could directly or indirectly mediate the interaction of H3 and H3.3 with their specific histone chaperone. In vertebrates and *Drosophila*, the residues at positions 87, 89 and 90 are S, V and M in H3, and A, I and G in H3.3, respectively. Interestingly however, the identities of the residues found at these positions in H3 and H3.3 vary between species but distinguish H3 from H3.3 (Fig. 2) (Malik and Henikoff, 2003). It has been proposed that these three residues could participate in histone-histone interaction stability: nucleosomes assembled with H3.3 may have different intrinsic stability properties than those assembled with canonical H3 (Hake and Allis, 2006).

In the nematode *C.elegans* at least two different H3.3 proteins are encoded (namely, His71 and His72) (Ooi *et al.*, 2006). *His71* and *His72* individually mutated animals are viable, suggesting that these genes are functionally redundant. In the protist *Tetrahymena thermophila*, two H3.3 proteins have also been characterized (H3.3 and H3.4) (Cui *et al.*, 2006). These two versions present differences to *Tetrahymena* canonical H3 on three of the four characteristic positions but, in addition, present 8 non-conserved amino acid differences. Moreover, these proteins

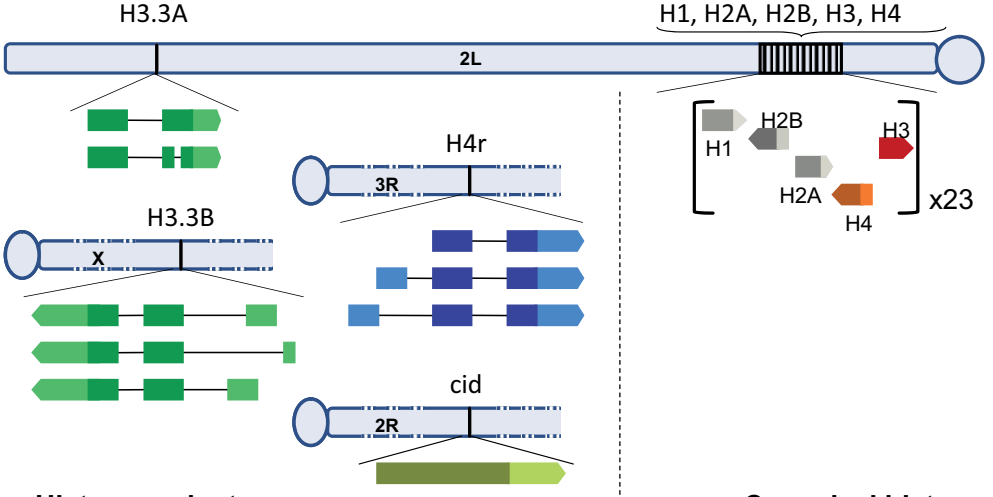

Fig. 1. Genomic organisation of histone *H3* and *H4* genes in *Drosophila*. Drosophila melanogaster *chromosomes are represented and histone gene locations are showed.* H3.3A, H3.3B, H4r *and* Cid *are single-copy genes and their corresponding transcripts are shown. Known or putative introns are represented as thin lines. Coding regions are shown in darker colors. Lighter color boxes represent untranslated regions. Canonical histones H1, H2B, H2A, H3 and H4 are encoded by multi-copy genes in the histone gene cluster of chromosome 2L. Note that Flybase predicts a* H3.3A *transcript that would result in a shorter protein. Although supported by independent EST sequences, the signification of this transcript remains to be investigated. Gene annotations are from Flybase (http://flybase.bio.indiana.edu).*

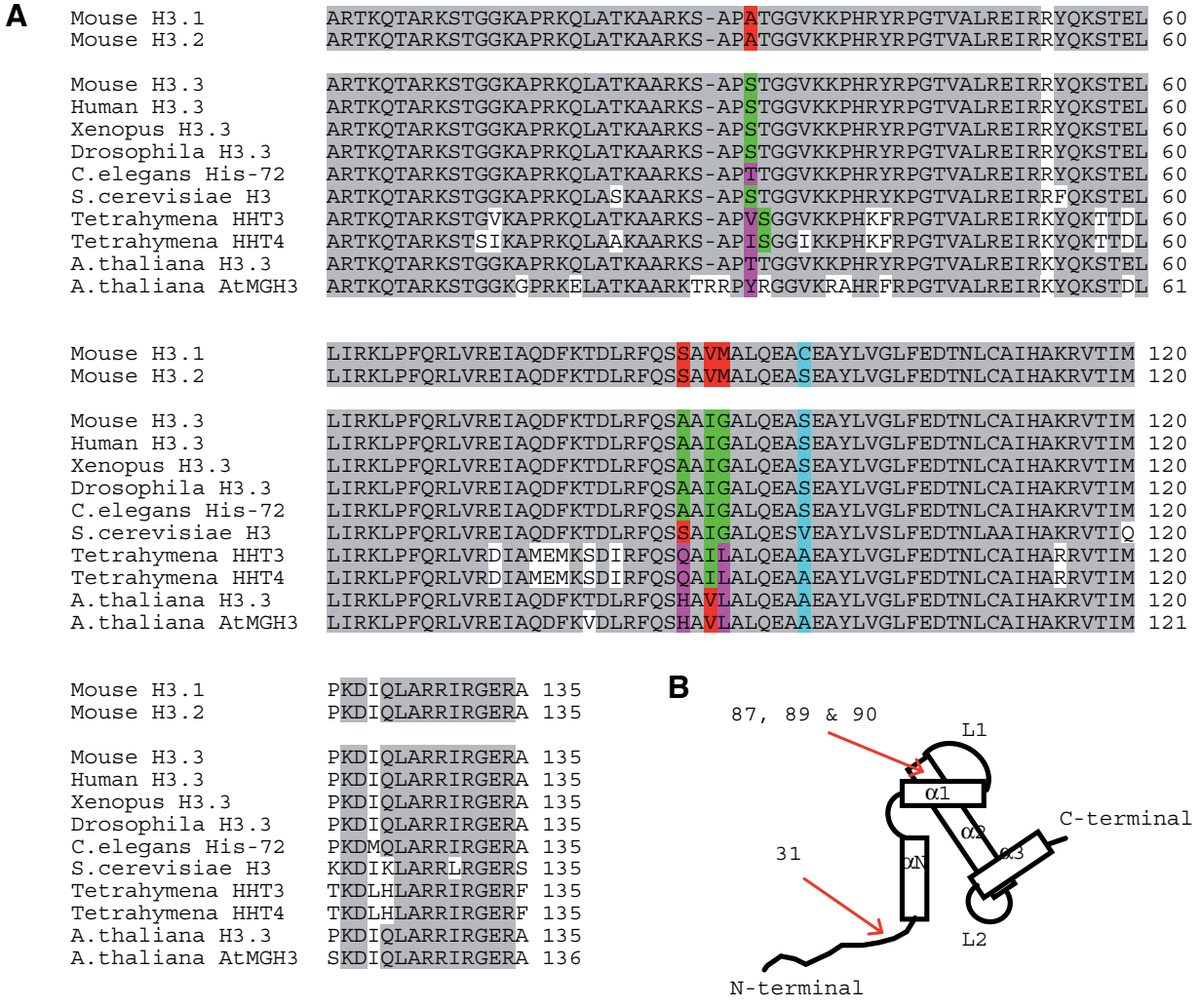

Fig. 2. Sequence alignment of H3.3 proteins. (A) *CLUSTAL alignement of H3 and H3.3 histones. Accession numbers are from GenBank:* M. musculus *H3.3A (NP_032236.1),* H. sapiens *H3.3A (NP_002098.1),* X. tropicalis *H3.3A (NP_001091902),* D. melanogaster *H3.3A (NP_523479.1),* C. elegans *His-72 (NP_499608.1),* S. cerevisiae *H3 (NP_009564.1),* T. thermophila *HHT3 (XP_001008397.1),* T. thermophila *HHT4 (XP_001008400.1) and* A. thaliana *H3.3 (NP_195713.1),* M. musculus *H3.1 (NP_038578.2) and H3.2 (NP_473386.1), and* A. thaliana *AtMGH3 (NP_173418.1). Alignments were performed with EMBL-EBI ClustalW2 software. Significantly conserved amino-acid residues are shaded in grey. In positions 31, 87, 89 and 90, amino-acids from canonical H3s are shaded in red, those from H3.3 family are shaded in green and those not fitting these categories are shaded in purple. The single residue that differentiates mouse H3.1 and H3.2 and differs among the H3.3 family is shaded in cyan.* **(B)** *Residues 31, 87, 89 and 90 are positioned on a schematic representation of nucleosomal H3 protein.*

differ from vertebrate H3.3 by twenty-two and twenty-five amino acids respectively, which accounts for the diversity of the H3.3 family across evolutionary divergent species. In plants, the diversification of H3.3 proteins seems to be even more accentuated: for instance, *Arabidopsis thaliana* has eight non-centromeric RI H3 variants (Okada *et al.*, 2005). The greater diversity of H3.3 proteins in certain groups, such as plants, for instance, opens the possibility that new H3.3 functions have emerged during evolution. Indeed, an evolutionary scenario proposes that H3.3 has independently arisen at least four times in plants, animals, ciliates and apicomplexans (Malik and Henikoff, 2003). Interestingly, a single version of H3 exists in ascomycetes (yeasts) and it is of the RI type (see Fig. 2). Since both RI and RC H3 versions are present in basidiomycetes, it has been proposed that canonical H3 genes have been lost in ascomycetes (Malik and Henikoff, 2003).

Epigenetic and replacement roles of H3.3 in somatic cells

Replacement roles of H3.3

The fact that expression of *H3.3* genes is not linked to S phase has been known for decades (Wu *et al.*, 1982). Because of this property, a simple expected function of H3.3 is to replace H3 whenever nucleosome assembly takes place independently of DNA synthesis, hence the term “replacement” variant. In an alternative view to this neutral replacement role of H3.3, the deposition of H3.3 can confer specific properties to the nucleosomes that are functionally important for the establishment of epigenetic marks (see the next section). A simple example of neutral replacement is provided by differentiated cells, after their exit from the cell cycle. In the absence of DNA replication and S

phase histone gene expression, differentiated cells are expected to rely on replacement histones for the assembly of new nucleosomes. Indeed, during the differentiation of various cell types, *H3.3* transcripts are abundant whereas replication dependent *H3* transcripts are no longer detected (Brown *et al.*, 1985; Krimer *et al.*, 1993; Pantazis and Bonner, 1984). The replacement of replicative H3 with H3.3 has been also observed at the protein level during the course of cell differentiation in vertebrates (Bosch and Suau, 1995; Pina and Suau, 1987; Urban and Zweidler, 1983; Wunsch and Lough, 1987). The underlying mechanism responsible for this H3.3 enrichment in chromatin of differentiated cells is not clear. It has been proposed that a general mechanism of nucleosome turnover allows the slow incorporation of histone variants in the chromatin in absence of DNA replication (Grove and Zweidler, 1984). This process is probably critical for the maintenance of a normal nucleosome density in long-lived cells as H3.3 nucleosomes compensate for the loss of old H3 nucleosomes.

Another example of H3.3 deposition that fits well into this type of neutral replacement is a recently described "repair" mechanims of heterochromatin in human cells after treatment with histone deacetylase inhibitors (Zhang *et al.*, 2007b). Exposure to these drugs triggers the recruitment of Heterochromatin Protein 1 (HP1) to sites of altered pericentric heterochromatin. This recruitment occurs independently of DNA replication and is mediated by the deposition of H3.3 by the histone chaperone HIRA at these sites (Zhang *et al.*, 2007b). The authors proposed that this mechanism could participate in the maintenance of centromere integrity and kinetochore formation. Interestingly, HIRA is also involved in the formation of SAHF (Senescence Associated Heterochromatin Foci) in human cells (Zhang *et al.*, 2007a; Zhang *et al.*, 2005). These cytological markers of cellular senescence are condensed domains of facultative heterochromatin that notably contain the macroH2A histone variant and HP1 proteins (Adams, 2007). The implication of HIRA in this process strongly suggests that H3.3 is also involved, although this remains to be formally demonstrated (Adams, 2007; Zhang *et al.*, 2007a). If it is the case, it could establish H3.3 as a key actor for the remodeling of heterochromatin in different biological situations, a property that is clearly not related to its role as a mark of active chromatin.

Although the need for H3.3 in non-dividing cells is expected, it is less clear whether H3.3 can actually replace H3 in cycling cells. A recent study in the protist *Tetrahymena thermophila* addressed this point through elegant genetic analyses (Cui *et al.*, 2006). In this organism, replacement or "minor" H3s are represented by two similar RI histone genes, called *H3.3* and *H3.4*, that are probably the result of a recent duplication event (Cui *et al.*, 2006) (Fig. 2). When both canonical *H3* genes were knocked-out, the expression of *H3.3* with a *H3* gene promoter was able to partially rescue the growth defect associated with the loss of RC H3 histones. This result indicates that the growth phenotype is mainly the consequence of an inadequate amount of histone protein rather than a specific absence of H3. However, H3.3 cannot fully replace H3 as rescued cells display a slight growth reduction and a small micronuclei phenotype. Thus, in *Tetrahymena*, H3 must have some intrinsic properties not shared with H3.3 (Cui *et al.*, 2006). These same authors also directly tested the function of minor H3s by generating cells with both *H3.3* and *H3.4* genes deleted. Surprisingly, minor H3s appear not essential for cell growth but only for the production of viable conjugation progeny. In addition, in the absence of minor H3s, Transcription-Coupled (TC) nucleosome assembly is apparently abolished without causing any obvious growth problem. These surprising phenotypes indicate that minor H3s seem to contribute to still unknown functions related to sexual reproduction.

Availability of H4 for RI nucleosome assembly?

Nucleosome assembly is initiated by the deposition of H3 along with H4 on DNA to form a (H3-H4)2 tetramer. This implies that H4 is made available at stoichiometric levels with H3.3 throughout the cell cycle, in order to be deposited through RI chromatin assembly pathways. Surprisingly, the problem of the source of H4 for RI assembly has received little attention. Interestingly, RI H4 genes encoding a H4 identical to canonical H4 have

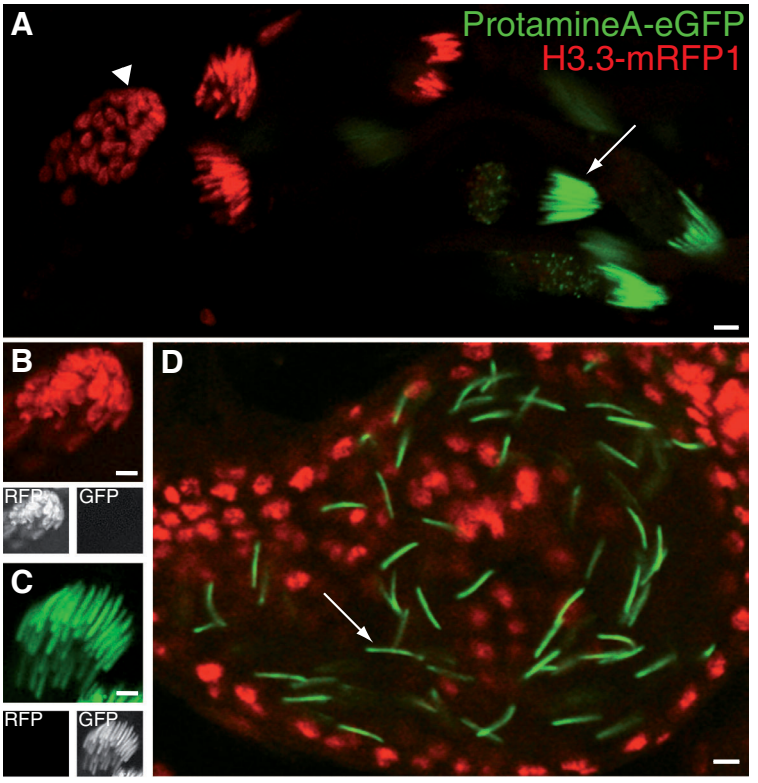

Fig. 3. H3.3 was not detected in *Drosophila* sperm. *Confocal images of fixed testes from males expressing both* H3.3-mRFP1 *and* ProtamineA-EGFP *transgenes. H3.3-mRFP1 is expressed from the* Drosophila H3.3A *gene promoter.* **(A)** *In this testis, groups of spermatid nuclei at different stages of differentiation (from left to right) are visible. Arrowhead points a group of spermatids with round nuclei and strong H3.3-mRFP1 fluorescence. Arrow indicates a group of elongated spermatid with strong ProtamineA-GFP fluorescence.* **(B)** *Close-up of a group of round spermatid nuclei.* **(C)** *Close-up of a group of elongated spermatid nuclei. In (B,C), small panels show the same nuclei with only RFP (left) or GFP (right) respective fluorescence.* **(D)** *A seminal vesicle containing mature gametes with strong ProtamineA-EGFP fluorescence (arrow). All bars, 5 μm. The mRFP1 (monomeric Red Fluorescent Protein1) protein is described in Campbell* et al., *2002. The* ProtamineA-EGFP *transgene is described in Jayaramaiah Raja and Renkawitz-Pohl (2005).*

been reported in *Drosophila*, nematodes and mammals (Akhmanova *et al.*, 1996; Gendron *et al.*, 1998; Poirier *et al.*, 2006). In *Drosophila*, the single copy *His4r* gene contains two introns, its mRNA is polyadenylated and it is expressed independently of DNA synthesis. In addition, it is preferentially expressed in adult, non-dividing cells, like *H3.3* genes (Akhmanova *et al.*, 1996). *H4r* might thus serve as a source of H4 for RI assembly processes. Once assembled, however, this protein is expected to behave identically to its RC counterpart. The role of *H4r* can thus only be explained by its RI expression profile. Another possible way of providing H4 to RI assembly pathways could be by recycling already assembled histones, by storing H4 expressed during S phase as pre-deposition complexes, or by allowing a certain level of transcription outside S phase. In this regard, it has been reported that the replicative histone H3.1 is deposited at sites of DNA repair, indicating that deposition of canonical histones is not absolutely coupled to S phase, at least for H3.1 (Polo *et al.*, 2006). Similarly, in *Tetrahymena*, H3 is specifically used for nucleosome assembly at sites of DNA synthesis associated with meiotic recombination (Cui *et al.*, 2006). The functional analysis of RI H4 genes should help distinguish between these possibilities.

H3.3 as an epigenetic mark of active chromatin

Opposed to the neutral replacement of H3 with H3.3 is the observation that H3.3 deposition does not occur homogeneously in the genome but instead correlates with regions of high transcriptional activity (Ahmad and Henikoff, 2002b; Chow *et al.*, 2005; Cui *et al.*, 2006; Janicki *et al.*, 2004; Mito *et al.*, 2005; Schwartz and Ahmad, 2005; Wirbelauer *et al.*, 2005).

It has been proposed that the passage of the RNA polymerase complex displaces nucleosomes, a situation that potentially creates a need for deposition of histones in a RI manner (Li *et al.*, 2007; Schwabish and Struhl, 2004). This Transcription Coupled deposition of H3.3 has been directly observed *in vivo* on *Drosophila* polytene chromosomes, throughout large transcription units such as the induced HSP70 genes, indicating that H3.3 deposition is associated with transcriptional elongation (Schwartz and Ahmad, 2005). Other studies have led to a similar conclusion based on the analysis of the distribution of H3.3 nucleosomes at high resolution by chromatin immunoprecipitation (ChIP) (Mito *et al.*, 2005; Wirbelauer *et al.*, 2005). However, this methodology also revealed an enrichment of H3.3 at the promoters of active genes, suggesting that chromatin remodeling associated with transcriptional initiation is also responsible for H3.3 deposition (Chow *et al.*, 2005). Finally, some studies found an enrichment of H3.3 at regulatory sites of active but also silent genes, such as the beta-globin locus control region in chicken or Polycomb Response Elements in *Drosophila* (Jin and Felsenfeld, 2006; Mito *et al.*, 2007; Nakayama *et al.*, 2007). These observations point to the possible existence of two distinct roles of H3.3 linked with gene activity. A first role for H3.3 in TC deposition is to compensate for the eviction of nucleosomes by the RNA polymerase complex in the body of highly transcribed genes (Schwartz and Ahmad, 2006). Another role links H3.3 to a continuous process of histone turnover that maintains accessibility of regulatory elements to their cognate factors (Henikoff, 2008).

In addition to its preferential incorporation at sites of active chromatin, H3.3 is enriched with PTMs typically associated with gene activity, such as methylation of lysine 4 among other marks (Hake *et al.*, 2006; McKittrick *et al.*, 2004; Mito *et al.*, 2005). How these PTMs are established on H3.3 and their importance in conferring an epigenetic role to this variant are crucial questions (Loyola and Almouzni, 2007). A recent study proposed that non-nucleosomal H3 and H3.3 carry a distinct set of modifications before their deposition, which in turn determine their final PTMs in nucleosomes (Loyola *et al.*, 2006).

The potential role of H3.3 in the epigenetic memory of active gene states has been recently studied in nuclear transfer experiments of *Xenopus* oocytes (Ng and Gurdon, 2008). Inheritance of active gene states of donor somatic nuclei is observed in embryos after nuclear transfer. For instance, about half of the embryos obtained after the transfer of a donor somite cell nucleus expressing the muscle-specific gene *MyoD* still express this marker in animal and vegetal regions, which do not differentiate into muscle (Ng and Gurdon, 2005). The authors found that this epigenetic memory of an active gene state correlates with the presence of H3.3 in its promoter. Importantly, this epigenetic memory can persist through 24 cell divisions in the absence of transcription (Ng and Gurdon, 2008). This finding supports a model where the H3.3 epigenetic mark is faithfully transmitted during DNA replication rather than through a mechanism involving the reactivation of transcription at each cycle. However, it is also compatible with the dynamic replacement model proposed by S. Henikoff (2008). Importantly, Ng and Gurdon found that the lysine 4 of H3.3 was required for the epigenetic memory, suggesting that the sole presence of the histone variant on promoter is not sufficient for the inheritance of the active gene state, but also requires the presence of specific PTMs. Functional studies, including formal genetic analyses of *H3.3* genes, are now required to progress on these fascinating aspects of chromatin function.

Functions of H3.3 in sexual reproduction

Besides its general replacement and epigenetic roles in somatic cells, several recent studies have highlighted the implication of H3.3 in chromatin remodeling mechanisms unique to the germline (Ooi and Henikoff, 2007). Although different in nature and function, these processes all require extensive RI nucleosome disassembly/reassembly at the genome or chromosome level.

Meiotic sex chromosome inactivation (MSCI)

The pachythene phase of the first meiotic prophase in mammalian males is characterized by the formation of synapses between chromosome pairs in preparation of recombination. Only the non-homologous X and Y chromosomes partially escape this process and are separated in a specific chromatin domain, the “XY body”. In this domain, the sex chromosomes are transcriptionally silenced in a process called MSCI (Turner, 2007). Unsynapsed autosomal chromatin is also silenced in a similar mechanism called MSUC (Meiotic Silencing of Unsynapsed Chromatin). A recent study discovered that both MSCI and MSUC depend on an extensive nucleosome replacement mechanism involving the deposition of the H3.3 variant (van der Heijden *et al.*, 2007). To which extent H3.3, and its chaperone HIRA, are critical for this process is yet unknown, but it is interesting to note that male mice with an impaired *H3.3A* gene have reduced fertility (Couldrey *et*

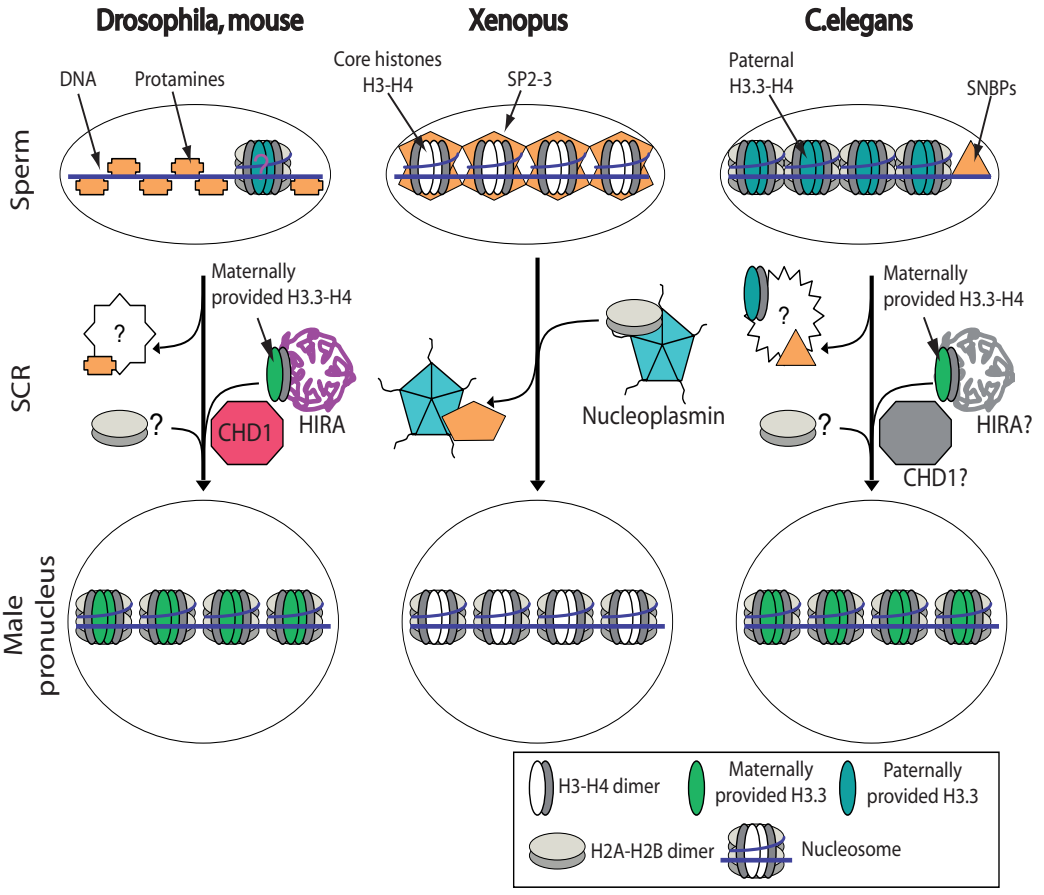

Fig. 4. Comparison of SCR in different animal models. *Schematic illustrations of sperm chromatin, SCR and male pronucleus chromatin in* Drosophila*, mouse,* Xenopus *and* C. elegans. Drosophila *and mouse present a protamine-based sperm chromatin structure although small levels of core histones could remain associated to DNA.* Xenopus *sperm chromatin is organized in nucleosome-like structures where core histones H3 and H4 are associated with sperm specific nuclear basic proteins SP2-3. Whether these core H3s are canonical H3 or H3.3 variants is not known.* C. elegans *sperm chromatin is probably organized with nucleosomes containing H3.3 although sperm-specific Small Nuclear Basic Proteins (SNBPs) are present as well. During SCR, a yet unknown factor removes protamines in* Drosophila *and mouse, and histone chaperone HIRA deposits maternally provided H3.3 and H4. In* Xenopus*, nucleoplasmin exchanges SP2-3 for H2A-H2B thereby reconstituting nucleosomes in male pronucleus. In* C. elegans*, unknown factors participate in the exchange of paternally provided H3.3 and SNBPs with maternally provided H3.3.*

al., 1999). MSCI represents a case of chromosome wide, RI chromatin remodeling that is involved in silencing. Along with the implication of H3.3 in sperm chromatin remodeling at fertilization (see below), this developmental process indicates that H3.3 can be deposited at large genomic regions that are depleted in nucleosomes. In *C. elegans*, a mechanism presenting similarities with MSCI is responsible for the silencing of the X chromosome during male meiosis (reviewed in Ooi and Henikoff, 2007). In the absence of a homologous counterpart, the X chromosome is silenced. Similar to the situation in mouse, it correlates with enrichment in silent PTMs such as H3K9me2 (Reuben and Lin, 2002). However, in the nematode, H3.3 is surprisingly depleted from the silent X chromosome, suggesting that, in contrast to MSCI, silencing does not involve chromosome wide RI nucleosome assembly (Ooi and Henikoff, 2007; Ooi *et al*., 2006).

Spermiogenesis

After the completion of meiosis, spermatids undergo a complex differentiation process called spermiogenesis, which results in the production of mature gametes. Marking features of this maturation include the formation of a motile flagellum, the elimination of excess cytoplasmic materials and the dramatic rearrangement of the nuclear architecture. In many species, spermiogenesis is in fact the only differentiation process where nuclei loose, in a reversible manner, their nucleosome-based chromatin to a totally different structure. Indeed, histones are first replaced with transition proteins and then with Sperm Nuclear Basic Proteins (SNBPs) during the condensation phase of spermatid nuclei. SNBPs include testis specific histone variants but also non-histone proteins such as protamine-like proteins and protamines (Caron *et al*., 2005; Govin *et al*., 2004; Lewis *et al*., 2003; Poccia and Collas, 1996). The sperm chromatin structure is highly diverse in animals, even between species of the same animal group (Frehlick *et al*., 2006). In general, sperm chromatin is highly condensed and thus not compatible with DNA replication or transcription (Poccia and Collas, 1996). Like other core histones, H3.3 is expressed in the male germ line. In *Drosophila*, only the histone *H3.3A* gene is strongly expressed in testis (Akhmanova *et al*., 1995) and the protein is detected in nuclei at all stages of spermatogenesis, with the exception of late spermatid and mature sperm nuclei (Akhmanova *et al*., 1997; Bonnefoy *et al*., 2007). Because these studies relied on immunofluorescence techniques, the possibility remained that H3.3 epitopes were not accessible in highly condensed spermatid and sperm nuclei. However, the use of a *H3.3-mRFP1* expressing transgene confirms that H3.3 is eliminated from the spermatid nuclei, just before the deposition of protamines (Fig. 3). This situation is in clear contrast with the case of the nematode *C. elegans* that retains H3.3 in mature sperm nuclei (Ooi *et al*., 2006), illustrating the diversity of sperm chromatin architecture and composition in animals. Although the bulk of sperm chromatin in *Drosophila*, mouse or humans is packaged with protamines, it also retains a variable proportion of histones (Caron *et al*., 2005; Dorus *et al*., 2006; Poccia and Collas, 1996; Raja and Renkawitz-Pohl, 2005). Consequently, it has been proposed that histones, and, possibly, H3.3, could play a role in transmitting epigenetic information

through the male gamete (Ooi and Henikoff, 2007). In mammals, similarly, it has also been proposed that paternal imprinting control regions could escape the histone/protamine exchange and would remain organized in nucleosomes in mature sperm (Delaval *et al*., 2007). The emergence of global ChIP approaches should help determining the putative role of H3.3 in the chromatin landscape of the male gamete.

In *Drosophila*, almost all the transcription required for spermiogenesis occurs in primary spermatocytes (Fuller, 1993). Thus, the abundance of H3.3 in the male germline also probably reflects the high level of transcription that takes place in these cells. Another possible role for H3.3 in spermatid nuclei could be related to its "nucleosome destabilizing" property. Indeed, nucleosomes containing H3.3, alone or in synergy with the H2A.Z variant, are more prone to loose H2A/H2B dimers in salt-disruption experiments, than regular nucleosomes (Jin and Felsenfeld, 2007). Similarly, assembly and disassembly of nucleosomes containing the mammalian variant H2A.Bbd occur more efficiently in association with H3.3 than with H3 (Okuwaki *et al*., 2005). If this were true in the context of *in vivo* chromatin, it would be interesting to see if it has any role in facilitating the replacement of nucleosomes with transition proteins and protamines during spermatid differentiation.

In flowering plants, the structure of the male gamete chromatin is poorly known. Recently however, a pollen specific *H3* gene called *AtMGH3* has been identified in *Arabidopsis*, along with eight *H3.3* genes (Okada *et al*., 2005). Although AtMGH3 is quite distantly related to animal H3.3 (see Fig. 2), this histone has the same amino-acid substitutions at position 87, 89 and 90 than those found in plant *H3.3* genes. Moreover, this gene was found to exhibit RI expression in male gametic cells (Okada *et al*., 2005). AtMGH3 is present in the chromatin of the male gamete and, similarly to the situation found in *C. elegans*, this H3 variant is removed from the zygote nucleus in a RI manner (Ingouff *et al*., 2007). Interestingly, *AtMGH3* mutants do not seem to display any phenotype, probably indicating a redundant role with other H3 variants (Okada *et al*., 2005).

Male pronucleus formation

The formation of the male pronucleus at fertilization implies the removal of SNBPs followed by *de novo* assembly of paternal nucleosomes, a process called SCR (Sperm Chromatin Remodeling) (Fig. 4). An essential, although largely overlooked aspect of SCR, is the fact that paternal chromatin assembly takes place independently of DNA synthesis (Nonchev and Tsanev, 1990; Poccia *et al*., 1984). The recent discovery that H3.3 was specifically deposited in the decondensing sperm nucleus in *Drosophila* and mouse confirmed the RI nature of this conserved process (Loppin *et al*., 2005; Torres-Padilla *et al*., 2006; van der Heijden *et al*., 2005). In these two model species, the sperm chromatin is essentially packaged with protamines (see previous section). Thus, the RI reassembly of H3.3 containing nucleosomes on paternal DNA is a genome wide process. The male pronucleus is in fact the only nucleus to undergo whole genome RI chromatin assembly during development. The specific use of the H3.3 variant in SCR is remarkable, in particular for those species where large pools of maternally expressed histones, including H3 and H3.3, are stored in the egg. In *Drosophila*, for instance, early development is under strict maternal control and zygotic transcription begins when several thousands nuclei have already assembled their chromatin (Foe, 1993). By analyzing transgenic fly lines expressing tagged versions of H3 or H3.3, we have shown that H3.3, and not H3, is deposited during SCR (Loppin *et al*., 2005). SCR is thus under the control of a specific nucleosome assembly machinery that specifically uses H3.3, despite the availability of both histone types in large quantities. Thus, SCR is clearly a process where H3.3 deposition is not determined by the simple unavailability of H3, but by its proper nucleosome assembly pathway.

In *C. elegans* and *Arabidopsis*, H3.3 histones are present at apparently high levels in the male gamete, in contrast to mouse or *Drosophila*. Surprisingly however, these paternal histones are also removed at fertilization, before the first zygotic DNA replication (Ingouff *et al*., 2007; Ooi *et al*., 2006). In *C. elegans* (this is not known for *Arabidopsis*), a RI deposition of maternally expressed H3.3 is observed during SCR, as in *Drosophila* or mouse. The functional signification of this apparent replacement is not clear. Mass spectrometry analysis of *C. elegans* sperm has revealed the presence of SNBPs similar to invertebrate protamines (Chu *et al*., 2006), suggesting that maternal H3.3 replaces the removed SNBPs. In this case, paternal H3.3 would be removed along with SNBPs before global deposition of maternal H3.3. It is thus difficult to imagine any epigenetic role for paternal H3.3, at least for the bulk of it. More probably, the persistence of high levels of H3.3 in sperm could only reflect the vast diversity of sperm chromatin types in animals (Poccia and Collas, 1996). In their recent finding that H3.3 was the support for the epigenetic memory of active gene states in nuclear transfer experiments, Ng and Gurdon (2008) pointed the importance of H3.3 lysine 4 in this phenomenon. Indeed, a mutant form of H3.3 with a glutamic acid in position 4 interfered with the epigenetic inheritance. It is interesting to note that maternal H3.3 incorporated during *Drosophila* or mouse SCR is not methylated on lysine 4 (Loppin *et al*., 2005; van der Heijden *et al*., 2005), thus reinforcing the view that SCR is essentially a neutral replacement process. Accordingly, in *Drosophila*, the paternal H3.3 enrichment is lost after a few nuclear cycles as the chromatin accumulates H3 nucleosomes at each S phase (Bonnefoy *et al*., 2007). In this case, the perpetuation of a putative H3.3 "barcode", as proposed by Hake and Allis (2006), is not observed. Whether the distinction between methylated and non-methylated forms of H3.3 is involved here remains to be established.

Roles of nucleosome assembly machineries in the deposition of H3.3

The HIRA nucleosome assembly pathway

Although the implication of the CAF-1 complex in RC chromatin assembly was established long ago (Smith and Stillman, 1989), the identification of assembly factors able to deposit histones in the absence of DNA synthesis received attention only recently. HIRA belongs to the HIR family of proteins whose funding members are the budding yeast Hir1p and Hir2p proteins (Spector *et al*., 1997). These two proteins are orthologs to the N- and C-terminus of HIRA proteins, respectively (Lamour *et al*., 1995). HIRA proteins are characterized by the presence of seven WD-repeats known to assemble into a secondary structure called a Beta propeller (Smith *et al*., 1999). In mouse, *Hira* is an essential

gene and knocked-out embryos die early in development with a complex phenotype that has been interpreted as resulting from precocious cell differentiation (Meshorer *et al.*, 2006; Roberts *et al.*, 2002). It is the finding that HIRA had histone binding properties that fuelled its functional characterization *in vitro* (Lorain *et al.*, 1998). The nucleosome assembly activity of HIRA was initially characterized from *Xenopus* egg extracts, and found to be specific for a DNA synthesis-independent assembly pathway (Ray-Gallet *et al.*, 2002). The subsequent purification and characterization of proteins interacting with H3.1 and H3.3 in human cells established a first link between H3.3 and HIRA (Tagami *et al.*, 2004). HIRA and the two largest CAF-1 subunits were specifically found in the H3.3 and H3.1 complexes, respectively, hence confirming the existence of distinct assembly pathways defined by their dependence on DNA synthesis, assembly factors and preferential histone H3 type.

SCR: a challenging task for RI nucleosome assembly machineries

The *in vivo* function of *Hira* received an unexpected highlight from the characterization of *sésame* (*ssm*), its first mutant allele in *Drosophila*. Embryos produced by homozygous mutant *ssm* females are haploid and develop with the sole, maternally derived, chromosome set. The loss of paternal chromosomes occurs at the first embryonic mitosis and is the consequence of a defect in male pronucleus formation (Loppin *et al.*, 2000). In *Drosophila*, SCR classically involves the rapid replacement of two closely related protamines with maternally provided histones (Bonnefoy *et al.*, 2007; Raja and Renkawitz-Pohl, 2005; Rathke *et al.*, 2007). Moreover, *Drosophila* SCR is a RI process that specifically involves the H3.3 variant (Loppin *et al.*, 2005). In *ssm* eggs, SCR is defective: although protamines are normally removed, the sperm derived nucleus does not incorporate histones. As a consequence, the male pronucleus does not fully decondense and does not replicate its DNA (Bonnefoy *et al.*, 2007; Loppin *et al.*, 2001; Loppin *et al.*, 2005). In *Drosophila*, HIRA is thus critical for the RI chromatin assembly of the whole paternal genome and specifically assembles H3.3 containing nucleosomes (Loppin *et al.*, 2005). In addition, *Drosophila* HIRA has also been implicated in H3.3 deposition at a regulatory site near a variegating *white* transgene inserted near centromeric heterochromatin (Nakayama *et al.*, 2007). Histone exchange at this site is dependent on the binding of the GAGA factor-FACT complex. In *ssm* flies, the silencing of this *white* transgene is enhanced, indicating that HIRA is involved in counteracting the spreading of heterochromatin in this locus (Nakayama *et al.*, 2007). Surprisingly, homozygous flies with a null allele of *Hira* are viable and female sterility is the only associated phenotype (Bonnefoy *et al.*, 2007). This surprising result indicates that any function of HIRA not related to SCR is dispensable in *Drosophila*. In mouse, the zygotic lethality of *Hira* knocked-out embryos does not allow testing the requirement of maternal HIRA for SCR. However, considering that mouse HIRA actually localizes to the decondensing male pronucleus and that SCR involves the massive deposition of H3.3 in this species (Torres-Padilla *et al.*, 2006; van der Heijden *et al.*, 2005), this critical function of HIRA is most probably conserved. In fact, HIRA is expected at least to play this role in a majority of species whose sperm contains non-nucleosomal chromatin. Some species, like the frog *Rana catesbeiana*, for instance, do not seem to contain protamines or protamine-like SNBPs but instead retain core histones in the sperm chromatin (Frehlick *et al.*, 2006). It is thus possible that HIRA is not required for SCR in these species. Similarly, *Xenopus* sperm chromatin retains H3 and H4 whereas H2A and H2B are replaced with protamin-like proteins. Since nucleoplasmin, a histone chaperone for H2A and H2B is necessary and sufficient for *Xenopus* SCR *in vitro* (Philpott and Leno, 1992; Philpott *et al.*, 1991), it suggests that this process does not actually require a H3/H4 RI assembly factor such as HIRA (Fig. 4).

The specific *Hira* mutant phenotype observed in *Drosophila* could result from a function of HIRA related to some peculiar features of SCR, rather than from a general RI nucleosome assembly defect. At least, we know that the removal of protamines itself does not seem to depend on HIRA because these SNBPs are normally removed in *Hira* mutant eggs (Bonnefoy *et al.*, 2007). The recent discovery that another nucleosome assembly factor, CHD1, was important for male pronucleus formation in *Drosophila* shed a new light on this process (Konev *et al.*, 2007). CHD1 (Chromo-ATPase/Helicase-DNA-binding protein 1) is an ATP-dependent nucleosome remodeling factor of the SNF2-like family of proteins, which is characterized by the presence of two chromodomains (Brown *et al.*, 2007; Hall and Georgel, 2007; Marfella and Imbalzano, 2007; Woodage *et al.*, 1997). *In vitro*, CHD1 facilitates the transfer of histones from the NAP-1 histone chaperone to DNA and allows the assembly of regularly spaced nucleosomes (Lusser *et al.*, 2005). *Drosophila* adults with no functional CHD1 survive but are sterile. In females, the sterility results from a 100% maternal effect embryonic lethality. As in embryos produced by *Hira* mutant females, the male nucleus in *chd1* mutant eggs is unable to participate in the formation of the zygote (Konev *et al.*, 2007). In contrast to *Hira* mutant eggs, where the male nucleus is always spherical and devoid of histones, the male nucleus in *chd1* mutant eggs adopts various shapes and histones are detected (Konev *et al.*, 2007) (G.A.O and B.L unpublished observations). Notably, H3.3 is detected in the paternal chromatin of *chd1* mutant eggs (Fig. 5), indicating that at least some HIRA-dependent histone deposition occurs in the absence of this motor protein. Thus, CHD1 could synergize with HIRA for the very rapid and massive RI nucleosome assembly activity required for SCR or could participate in the regular spacing of nucleosomes on paternal DNA.

Although the exact function of CHD1 at fertilization remains to be determined, it is remarkable that SCR, a process that occurs once in the life cycle and in a single nucleus, represents a critical task for at least two different nucleosome assembly factors. Understanding how these proteins are orchestrated *in vivo* for RI assembly over a whole genome is a fascinating question for future research.

Multiple assembly pathways involved in H3.3 deposition?

Although the functional characterization of H3.3 in metazoans awaits formal genetic analysis, it is now clear that this histone variant is involved in a variety of chromatin remodeling mechanisms. Whether these mechanisms rely on different nucleosome assembly pathways largely remains to be investigated. The fact that H3.3 is deposited independently of DNA synthesis is a major property that distinguishes it from H3, although at least one exception has been reported in the *Xenopus* oocyte where H3 seems to be RI deposited by a dynamic histone exchange process (Stewart *et al.*,

2006). Several lines of evidence indicate that H3.3 is deposited during S phase. In *Drosophila* cultured cells, overexpressed H3.3 is deposited at sites of DNA replication (Ahmad and Henikoff, 2002b). In early *Drosophila* embryos, during the rapid nuclear cleavages and before the onset of zygotic transcription, we have observed a relatively weak and uniform deposition of H3.3 in the chromatin of all nuclei that we interpret as S phase deposition (Bonnefoy *et al.*, 2007). Interestingly, this H3.3 deposition does not depend on the presence of the HIRA protein, opening the possibility that the CAF-1 complex could be responsible for the bulk of H3.3 nucleosome assembly during early *Drosophila* development. In this peculiar developmental context, where both H3 and H3.3 are stored in the egg and are thus available in large quantities, the RC assembly machinery seems to allow some deposition of the RI variant despite the fact that H3 is preferentially deposited (Bonnefoy *et al.*, 2007). More generally, the different models accounting for the propagation of epigenetic states through cell divisions also imply the deposition of H3.3 at DNA replication forks (Eitoku *et al.*, 2007; Hake and Allis, 2006; Henikoff *et al.*, 2004; Polo and Almouzni, 2006). However, the simple hypothesis that HIRA could participate in this task is challenged by our observations in fly embryos and thus deserves a real investigation.

Asf1 (Anti Silencing Factor 1) is a conserved histone chaperone involved in both RC and RI assembly pathways (reviewed in (De Koning *et al.*, 2007; Eitoku *et al.*, 2007; Mousson *et al.*, 2007). Several recent studies have showed that Asf1 interacts with a single H3-H4 dimer (Agez *et al.*, 2007; Antczak *et al.*, 2006; English *et al.*, 2005; Mousson *et al.*, 2005) suggesting that Asf1 could function in distributing H3-H4 or H3.3-H4 dimers to CAF-1 and HIRA, respectively. In addition, Asf1 plays a critical role for the unwinding of DNA replication forks by disrupting (H3-H4)2 tetramers (Natsume *et al.*, 2007) and by interacting with the putative replicative helicase MCM2-7 (Groth *et al.*, 2007). However, Asf1 is not directly involved in *de novo* RI or RC histone deposition in *Xenopus* egg extracts (Ray-Gallet *et al.*, 2007). Similarly, Asf1 is not detected in the decondensing male nucleus during *Drosophila* SCR (Bonnefoy *et al.*, 2007).

Interestingly, while TC assembly of H3.3 nucleosomes is well established, the histone chaperone responsible for this deposition remains elusive. In *Drosophila*, adults devoid of HIRA are viable suggesting that HIRA is not critical for TC assembly (Bonnefoy *et al.*, 2007). In addition, the absence of HIRA only causes a slightly delayed growth in chicken cells (Ahmad *et al.*, 2005). Spt6 and FACT are histone binding proteins that are involved in the reassembly of nucleosomes after the passage of the RNA polymerase II and thus represents interesting candidates for TC H3.3 deposition (Adkins and Tyler, 2006; Andrulis *et al.*, 2000; Belotserkovskaya *et al.*, 2004; Kaplan *et al.*, 2000). In addition to its role in SCR mentioned above, the CHD1 assembly factor has also been shown to affect H3.3 deposition in *Drosophila* blastoderm embryos suggesting its participation in TC assembly (Konev *et al.*, 2007).

The diversity of RI chromatin assembly processes should thus be reflected by the implication of various assembly factors, depending both on the model species as well as on the developmental or cellular processes considered. Understanding how these different factors

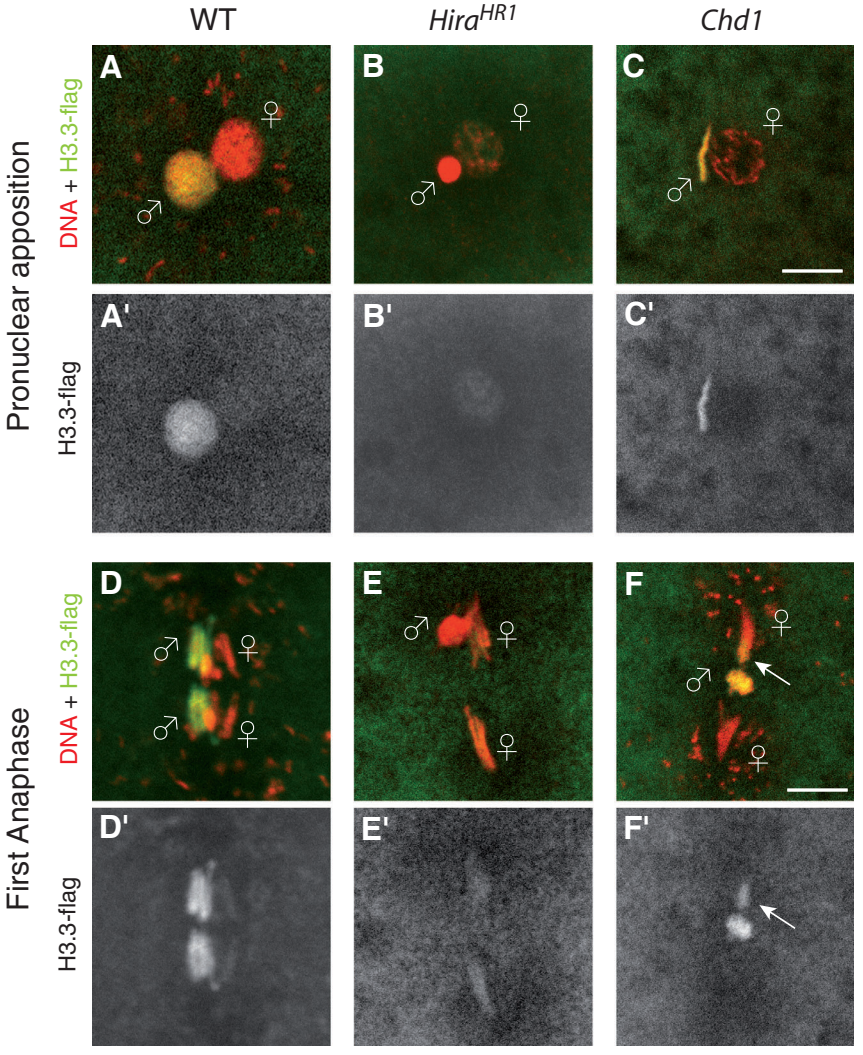

Fig. 5. HIRA and CHD1 are involved in *Drosophila* SCR. *Confocal images of eggs at the pronuclear apposition stage* **(A,B,C,A′,B′,C′)** *or embryos at the first zygotic anaphase* **(D,E,F,D′,E′,F′)** *stained for DNA (red) and with an anti-FLAG peptide antibody to detect maternally expressed H3.3-FLAG (green or gray).* **(A,A′)** *In eggs from wild type females, H3.3-FLAG is detected in the male pronucleus.* **(B,B′)** *In eggs laid by mutant* Hira^{HR1}/Hira^{HR1} *females, H3.3-FLAG is not detected in the abnormally condensed male nucleus.* **(C,C′)** *In eggs laid by* chd1[1]/Df(2L)Exel7014 *females (with no functional CHD1 protein), the male nucleus is aberrant in shape but stains for H3.3-FLAG (see also Konev* et al., *2007).* **(D,D′)** *During the first zygotic anaphase, paternal chromosomes still contain high levels of H3.3-FLAG.* **(E,E′)** *In* Hira^{HR1} *mutant eggs, the male nucleus is excluded from the first spindle that contains only maternal chromosomes.* **(F,F′)** *In* chd1 *mutants eggs, the male nucleus is occasionally incorporated in the first mitosis but paternal chromosomes (stained with H3.3-FLAG) segregation is defective (arrow). Wild-type males were used to fertilize females of indicated genotypes. All females used in these crosses contained a copy of a* H3.3-FLAG *transgene (Loppin* et al., *2005). DNA positive dots visible in (A,D,F) are* Wolbachia *endosymbiotic bacteria.*

cooperate and interact on the nucleosome assembly line will certainly need the forces of both biochemical and *in vivo* approaches.

Conclusion

Two levels of complexity challenge the dynamic nature of eukaryotic chromatin. The first level is common to most cells and includes the invariable remodeling events associated with the cell cycle, from DNA replication to cellular senescence. The diversity of remodeling processes that occur during development represents a second level of complexity, which is best illustrated by the dramatic reorganization of chromatin associated with the transmission of paternal DNA from one generation to another. The universal ability of eukaryotic cells to assemble nucleosomes independently of DNA replication drives this versatility. The H3.3 histone variant is at the heart of RI nucleosome assembly mechanisms. Being very close to its RC counterparts at the primary sequence level, H3.3 fulfills a neutral replacement role supported by its constitutive expression. In addition, the biochemical characterization of the H3.3 deposition pathway, the association of this variant with active PTMs, as well as its dynamic distribution over the genome have paved the road to establish a role in the epigenetic transmission of active chromatin states. Finally, developmental and genetic studies have unveiled unexpected roles for H3.3 or associated assembly factors in chromatin remodeling events essential for sexual reproduction. In this regard, the evolution of new functions for RI nucleosome assembly factors could be the key for the diversification of H3.3 roles. These different aspects of H3.3 biology must be considered to understand the evolutionary forces that shaped this histone and perpetuated it as one of the most conserved proteins in life.

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