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Drosophila Yemanuclein and HIRA Cooperate for De Novo Assembly of H3.3-Containing Nucleosomes in the Male Pronucleus

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Introduction

Assembly of octameric nucleosomes in eukaryotic chromatin is a stepwise process where deposition of a histone H3-H4 heterotetramer precedes incorporation of two H2A-H2B dimers [1]. While the bulk of de novo chromatin assembly occurs during genome replication and mainly involves canonical histone H3, alternative, replication-independent (RI) chromatin assembly pathways use the conserved histone H3 variant H3.3 [2,3]. Canonical (or replicative) H3s (H3.1 and H3.2 in mammals, H3.2 in Drosophila) are synthesized in early S phase and deposited at DNA replication forks by the trimeric CAF-1 (Chromatin Assembly Factor-1) complex [4]. In contrast, H3.3 is expressed throughout the cell cycle and is deposited at various genomic regions in a DNA-synthesis independent manner [3–8]. During the past decade, research on H3.3 has largely focused on the ability of this histone to be deposited at transcribed genes, opening the possibility that H3.3 could constitute an epigenetic mark of active chromatin [9–13]. Recent advances in the field have let emerge a more complex view of H3.3 biology. Although H3.3 is indeed enriched at transcribed gene bodies, it is now established that this histone is also deposited at various chromatin regions, such as regulatory elements, mammalian telomere repeats or satellite DNA blocks [5–7,14–18]. This surprising versatility of H3.3 could simply reflect its ability to be deposited in regions that are subjected to nucleosome depletion or rapid histone turnover [5,7,19].

In metazoa, H3.3 is also implicated in a variety of nuclear processes that specifically occur in germ cells and in early embryos [7,20–22]. In mouse spermatocytes, for instance, H3.3-containing nucleosomes are assembled on sex chromosomes during their inactivation and accumulate over the whole sex body [23].

Abstract

The differentiation of post-meiotic spermatids in animals is characterized by a unique reorganization of their nuclear architecture and chromatin composition. In many species, the formation of sperm nuclei involves the massive replacement of nucleosomes with protamines, followed by a phase of extreme nuclear compaction. At fertilization, the reconstitution of a nucleosome-based paternal chromatin after the removal of protamines requires the deposition of maternally provided histones before the first round of DNA replication. This process exclusively uses the histone H3 variant H3.3 and constitutes a unique case of genome-wide replication-independent (RI) de novo chromatin assembly. We had previously shown that the histone H3.3 chaperone HIRA plays a central role for paternal chromatin assembly in Drosophila. Although several conserved HIRA-interacting proteins have been identified from yeast to human, their conservation in Drosophila, as well as their actual implication in this highly peculiar RI nucleosome assembly process, is an open question. Here, we show that Yemanuclein (YEM), the Drosophila member of the Hpc2/Ubinuclein family, is essential for histone deposition in the male pronucleus. yem loss of function alleles affect male pronucleus formation in a way remarkably similar to Hira mutants and abolish RI paternal chromatin assembly. In addition, we demonstrate that HIRA and YEM proteins interact and are mutually dependent for their targeting to the decondensing male pronucleus. Finally, we show that the alternative ATRX/XNP-dependent H3.3 deposition pathway is not involved in paternal chromatin assembly, thus underlining the specific implication of the HIRA/YEM complex for this essential step of zygote formation.


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Author Summary

Chromosome organization relies on a basic functional unit called the nucleosome, in which DNA is wrapped around a core of histone proteins. However, during male gamete formation, the majority of histones are replaced by sperm-specific proteins that are adapted to sexual reproduction but incompatible with the formation of the first zygotic nucleosome. These proteins must therefore be replaced by histones upon fertilization, in a replication-independent chromatin assembly process that requires the histone deposition factor HIRA. In this study, we identified the protein Yemanuclein (YEM) as a new partner of HIRA at fertilization. We show that, in eggs laid by yem mutant females, the male pronucleus fails to assemble its nucleosomes, resulting in the loss of paternal chromosomes at the first zygotic division. In addition, we found that YEM and HIRA are mutually dependent to perform chromatin assembly at fertilization, demonstrating that they tightly cooperate in vivo. Finally, we demonstrate that the replication-independent chromatin assembly factor ATRX/XNP is not involved in the assembly of paternal nucleosomes. In conclusion, our results shed new light into critical mechanisms controlling paternal chromatin formation at fertilization.

Moreover, an insertion mutation in the mouse H3.3A gene induces male subfertility, among other phenotypes [24]. Certain lysine residues of H3.3 are also important for the establishment of heterochromatin during reprogramming in mouse zygotes [25]. Recently, knock-down experiments in Xenopus laevis demonstrated a specific and critical requirement of H3.3 during embryonic gastrulation [26]. In Drosophila, H3.3 deficient animals are viable but are both male and female sterile [27,28]. H3.3 is notably responsible for the strong paternal enrichment observed in male pronucleus in the crucian carp [40], confirming the widespread role of this histone chaperone in paternal nucleosome assembly at fertilization.

The Hir/HIRA complex is composed of a small number of proteins that are conserved between yeast and human. In S. cerevisiae, the Hir chromatin assembly complex includes the HIRA-related proteins Hrl1 and Hir2, Asf1 (Anti Silencing Factor 1), Hir3 and Hpc2 [41–43]. Hir3 is a poorly conserved protein related to Hip3 (S. pombe) and human CABIN1, but which does not seem to have an ortholog in Drosophila [43–45]. Hpc2 is functionally related to Hip4 in fission yeast and to the HIRA-associated proteins Ubinuclein 1 and Ubinuclein 2 (UBN1/UBN2) [8,44,46–48]. Interestingly, the strongest conservation between HIRA orthologs resides in a ~50 amino-acid domain called HIRD (Hpc2-Related Domain) or HUN (Hpc2-Ubinuclein-1) domain [44,48] and to a smaller domain called NHIRD [49]. In Drosophila, Yemanuclein (YEM; also named Yemanuclein-a [50,51]) is the only protein with a HIRD domain [44]. The yem gene has a strong ovarian expression and encodes a nuclear protein that accumulates in the germinal vesicle of growing oocytes [51]. Recently, a mutant allele of yem (yem1) has been characterized as a V479E replacement, which results in female sterility [52]. In this first report on YEM function, YEM was implicated in the segregation of chromosomes during the first female meiotic division but the sterility of mutant females suggested the existence of yet unknown roles for YEM [52]. In this paper, we have explored the implication of YEM in HIRA-dependent RI nucleosome assembly in the zygote. We show that the cooperation of YEM and HIRA in vivo is critical for the assembly of H3.3-containing nucleosomes in the male nucleus at fertilization.

Results

yem2 is a deletion allele of the yemanuclein gene

The original yem1 point mutation causes a single amino-acid replacement (V479E) in YEM protein (Figure 1A) [52]. This mutation induces female sterility but has no detectable effect on the level of yem transcripts in ovaries nor on the accumulation of YEM protein in the oocyte nucleus (or germinal vesicle, GV) (Figure 1B, 1C). To obtain a more severe mutant allele of yem, we mobilized a P-element inserted near the transcriptional start site of the yem gene (Figure 1A). One of the imperfect excisions of this P-element generated a 5180 bp deletion (named yem2) that spans the 5’ UTR and most of the coding region of yem. Accordingly, the yem2 allele induced female sterility in association with yem+ or with the large non-complementing deficiency Df(3R)3450 (Table 1). In yem2/ yem females, yem transcripts (corresponding to a region of the gene not covered by the yem2 deletion) were greatly reduced compared to yem+/yem females, and the YEM protein was not detected in the oocyte nucleus (Figure 1B, 1C). Finally, the female sterility of both yem mutant alleles was rescued by expressing a transgenic YEM protein tagged in its C-terminus with the Flag peptide (YEM-Flag) (Table 1). Taken together, these data suggest that yem2 is a null or at least a strong loss of function allele of yem.

YEM interacts with HIRA in vivo

The YEM protein has been previously detected in a HIRA complex purified from embryonic nuclear extracts [33], suggesting that it could represent the Drosophila ortholog of UBN1/Hpc2. To more directly test the interaction of HIRA and YEM, we performed co-immunoprecipitation experiments using functional Flag-tagged and GFP-Flag-tagged transgenic versions of YEM and HIRA proteins, respectively. We confirmed that, in ovarian protein extracts, HIRA was able to co-immunoprecipitate with
YEM, and vice versa (Figure 2A). In the same experiments, however, the ATP-dependent chromatin remodeling factor CHD1 was not detected in the HIRA immune complex, in contrast to what was previously reported [54]. Although the reason for this apparent discrepancy with the study by Konev et al. is not clear, it reinforces the fact that, in our experimental conditions, HIRA and YEM show reproducible and specific interaction, confirming that these proteins are subunits of a common complex.

HIRA and YEM were previously shown to display a remarkable and specific accumulation in the nucleoplasm of the GV throughout oogenesis [36,51]. Similarly, immunodetection of the Flag-tagged versions of HIRA and YEM recapitulates their endogenous accumulation in the GV, where both proteins co-localize (Figure 2B). The oocyte nucleus is a large nucleus that essentially contains nucleoplasm, as the oocyte chromosomes remain confined within a small, compact structure called the karyosome [55]. Surprisingly, we observed that HIRA-Flag accumulation in the GV was completely abolished in yem2/Df(3R)3450 mutant oocytes. Conversely, we found that YEM-Flag was undetectable in the GV of about half of null HiraHR1 mutant oocytes (Figure 2C). These effects could not be explained by reduced protein levels in mutant flies, as HIRA-Flag and YEM-Flag expression were apparently not affected in yem2/Df(3R)3450 and HiraHR1 mutants, respectively (Figure 2D). These results indicate that YEM and HIRA are mutually required for their localization or for their stabilization in the oocyte and suggest that these proteins interact prior to their release in the egg cytoplasm, after GV breakdown. Taken together, these results confirm that YEM and HIRA belong to the same complex in vivo.

YEM is required for paternal chromatin assembly at fertilization

The female sterility associated with yem mutations actually results from a maternal effect embryonic lethality phenotype. Indeed, eggs from yem1/Df(3R)3450 or yem2/Df(3R)3450 females (referred to as yem mutant eggs for simplicity) are normally fertilized and they initiate development, but the embryos systematically die before hatching (Table 1 and not shown). These features are reminiscent of the maternal effect embryonic lethality phenotype of Hira mutants, where embryos develop as non-viable gynogenetic haploids after the loss of paternal chromosomes during the first zygotic division [36,37,56].

### Table 1. Female sterility associated with yem mutations.

<table>
<thead>
<tr>
<th>Genotype of females</th>
<th>No. of eggs</th>
<th>No. of larvae</th>
<th>Hatch rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>w; yem1/Df(3R)3450</td>
<td>885</td>
<td>758</td>
<td>85.6</td>
</tr>
<tr>
<td>w; yem1/Df(3R)3450</td>
<td>984</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>w; yem2/Df(3R)3450</td>
<td>948</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>w; yem1/yem2</td>
<td>1109</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>w; yem-flagHPF1/ yw; yem1/Df(3R)3450</td>
<td>1444</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>w; yem-flagHPF1/yw; yem2/Df(3R)3450</td>
<td>1337</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>w; yem-flagHPF1/yw; yem-flagHPF1; yem1/Df(3R)3450</td>
<td>513</td>
<td>362</td>
<td>70.5</td>
</tr>
<tr>
<td>w; yem-flagHPF1/yw; yem-flagHPF1; yem2/Df(3R)3450</td>
<td>577</td>
<td>256</td>
<td>44.4</td>
</tr>
<tr>
<td>w; yem-flagHPF1/yw; yem-flagHPF1; yem1/Df(3R)3450</td>
<td>638</td>
<td>507</td>
<td>79.5</td>
</tr>
<tr>
<td>w; yem-flagHPF1/yw; yem-flagHPF1; yem2/Df(3R)3450</td>
<td>667</td>
<td>660</td>
<td>98.9</td>
</tr>
</tbody>
</table>

All yem mutant alleles are described in Figure 1. Df(3R)3450 is a large deficiency covering the yem locus. yem-flagHPF1 and yem-flagHPF1 are two independent insertions of the same transgene.

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We thus examined male pronucleus formation in *yem* mutant eggs. In wild-type eggs, shortly after fertilization, while maternal chromosomes complete meiotic divisions, the decondensing male nucleus is strongly and specifically stained with an antibody recognizing acetylated histone H4, a mark of newly assembled chromatin [36,37]. Strikingly, we observed that in *yem* mutant eggs, acetylated H4 was practically not incorporated in the male pronucleus (Figure 3A). At pronuclear apposition, male pronuclei in *yem* mutant eggs always appeared round and condensed (Figure 3B), in a way identical to the male nucleus in *Hira* mutants [36,37,57]. Paternal chromosomes subsequently failed to integrate the first zygotic division in *yem* eggs (Figure 3C), resulting in gynogenetic haploid development and embryonic lethality (Figure 3D). It should be mentioned however that exceptional gynogenetic development of adults can occur if the female pronucleus is diploid as the result of defective meiosis [52].

While the *yem-FLAG* transgene efficiently rescued *yem* female sterility, another insertion of the same construct (*yem-FLAG*Δ*HP16*) only restored fertility to very low levels, likely because of its weak expression (Table 1). Interestingly, in eggs laid by *yem*/*Df(3R)3450*; *yem-FLAG*Δ*HP16* females, the male pronucleus still appeared round and condensed but consistently incorporated...
significant levels of acetylated histone H4 (Figure 3B). This suggests that the level of maternal YEM protein is limiting for both nucleosome assembly and male pronucleus decondensation.

We have previously shown that HIRA-dependent nucleosome assembly in the male pronucleus exclusively uses the histone H3 variant H3.3 [36,37]. To observe H3.3 deposition in the male pronucleus, we used a previously described, maternally expressed Flag-tagged transgenic version of H3.3 (H3.3-Flag) [37]. In contrast to control eggs, H3.3-Flag was not incorporated in paternal chromatin of yem1/mutant eggs, similar to Hira mutant eggs (Figure 4A). However, the female pronucleus in yem eggs still incorporated low levels of H3.3-Flag during the first round of DNA replication, arguing that, like HIRA, YEM does not participate to the limited S phase deposition of H3.3 which occurs in replicating nuclei of early embryos [36] (Figure 4A). As a complementary approach, we analyzed the yem mutant phenotype using a commercially available monoclonal anti-H3.3 antibody. In wild-type fertilized eggs, the antibody specifically stained the decondensing male pronucleus, but not the maternal chromosomes, thus confirming its specificity for H3.3 (Figure 4B). In agreement with the results obtained with H3.3-Flag, no staining was detected above background when Hira and yem mutant eggs were stained with the anti-H3.3 antibody (Figure 4C). Altogether, these results demonstrate the critical requirement of YEM for the assembly of H3.3-containing nucleosomes on paternal DNA.

Although mutant yem1/Df(3R)3450 and yem2/Df(3R)3450 adults were viable, survival rates were reduced for yem2/Df(3R)3450 individuals (Table S1) indicating that YEM also functions in somatic cells. Interestingly, the partial lethality of yem2 mutant individuals was not aggravated when combined with the HiraH3 null allele. Thus, HIRA and YEM do not have redundant functions but, instead, are obligate partners not only for male pronucleus chromatin assembly but presumably also for other somatic RI nucleosome assembly processes.

HIRA and YEM cooperate for their localization in the male pronucleus

Consistent with its critical role in paternal chromatin assembly, maternally expressed HIRA is recruited to the male nucleus shortly after fertilization in both Drosophila and mouse [37,38]. Strikingly, while robust HIRA-Flag staining is observed in the decondensing male nucleus in control eggs, HIRA-Flag was not detected in eggs from yem1 and yem2 females (n>20; Figure 5D). Thus, YEM is required for the recruitment or for the stabilization of HIRA in the male nucleus. As expected, maternal YEM-Flag was also detected in the decondensing male nucleus before pronuclear apposition (Figure 5B). However, in contrast to the homogeneous distribution of HIRA-Flag in the male nucleus, YEM-Flag appeared also enriched in a small number of discrete foci of unknown nature (Figure 5A). We verified that these foci localized neither to the centromeres nor to the telomeres of the male pronucleus (Figure 5C). Interestingly, the formation of these YEM-Flag foci appeared largely independent of HIRA, whereas the rest of YEM-Flag was not detected in a large majority of Hira mutant eggs (Figure 3B). Thus, with the exception of these discrete regions, our experiments demonstrate that HIRA and YEM are interdependent for their localization within the male pronucleus and for paternal chromatin assembly.

Drosophila ATRX/XNP is not involved in paternal nucleosome assembly

Several groups have recently established that in mammalian cells, RI H3.3 deposition is mediated by at least two distinct protein complexes. HIRA and its partners are involved in the enrichment of H3.3 at active genes and at upstream regulatory elements of both active and repressed genes [6]. In contrast, ATRX, a member of the SNF2 family of ATP-dependent chromatin remodeling factors and the histone chaperone DAXX (Death-Associated protein) are essentially responsible for the enrichment of H3.3 nucleosomes at heterochromatin loci [6,58–60]. In Drosophila, the ATRX homolog XNP (or dATRX) colocalizes with H3.3 throughout the chromatin of somatic cells [16]. To investigate the potential involvement of this chromatin remodeler in the assembly of paternal nucleosomes in the newly fertilized egg, we first determined its distribution in oocytes and eggs using a specific antibody recognizing both XNP isoforms [16]. Interestingly, XNP was found to accumulate in the oocyte nucleus, in a way remarkably similar to HIRA and YEM (Figure 6A). However, XNP was not observed in the decondensing male nucleus at fertilization (n>20) and the protein remained absent from early cleavage nuclei until their migration to the embryo periphery, at the syncytial blastoderm stage (Figure 6B and not shown). In addition, we observed that chromatin assembly in the male nucleus occurred normally in eggs from xnp2/xnp3 mutant females (n>20; Figure 6C). Finally, females homozygous for the semi-lethal allele xnp2, which abolishes the expression of the long XNP isoform [61], produced a limited amount of eggs that nevertheless hatched (not shown). We conclude that dATRX/XNP is most likely not involved the assembly of paternal nucleosomes at fertilization.

Discussion

In human cells, the HIRA core complex is composed of at least three subunits, including HIRA, UBN1 and CABIN1 [43]. This complex is functionally involved in a large diversity of cellular and developmental processes that require dynamic histone turnover or de novo assembly of nucleosomes, independently of DNA synthesis. Although the HIRA complex mediates the deposition of the highly conserved H3.3 histone variant, its subunits display a comparatively weak overall conservation in animals. For instance, Drosophila does not seem to have any CABIN1 homolog and the highest conservation between UBN1 and YEM is mainly restricted to the small HRD domain. Despite this poor conservation, our work establishes Yemanuclein as a bona fide ortholog of Ubiquinolin, by demonstrating its physical interaction with the HIRA histone chaperone and its critical requirement for H3.3 deposition during male pronucleus decondensation.

Paternal chromatin assembly is a major function of YEM

In contrast to the knock-out of the Hira gene in mouse, which is zygotic lethal in early embryos [62], null mutants of Drosophila Hira are viable but homozygous females are completely sterile [36]. This indicates that only the maternal contribution of Hira is essential, at least to form the male pronucleus. Our characterization of a null yem2 allele allowed us to reach the same conclusion for YEM. Remarkably, the phenotype of the male pronucleus in eggs laid by yem mutant females appeared indistinguishable to what we previously reported for Hira mutants. In both cases, RI deposition of H3.3-containing nucleosomes is practically abolished, typically preventing the full decondensation of the male nucleus and its integration into the zygotic nucleus. Thus, YEM and HIRA are equally required to assemble paternal nucleosomes at fertilization. This unique and major function of the HIRA complex is most likely conserved in animal groups where histones, and notably H3 and H4, are replaced with SNBPs in sperm. This is for instance the case of mammals, where protamines package
about 95% and 85% of mouse and human sperm DNA, respectively [30,32]. In fact, HIRA has been previously detected in the decondensing male nucleus at fertilization in mouse, which incorporates H3.3 before the first round of DNA replication [38,39]. We thus expect Ubinuclein1/2 to be also involved in paternal chromatin assembly in mammals. In apparent contradiction with this prediction, a transgene expressing human UBN1 in the female germline could not rescue the sterility of yem mutant females (Figure S1 and not shown). However, this absence of complementation of YEM and UBN1 can be explained by the strong divergence of these orthologous proteins at the primary sequence level and it suggests that UBN1 can only function within its native, human HIRA complex. The apparent lack of a CABIN1 homolog in Drosophila also underlines the central role played by the HIRA-UBN1/YEM pair in the assembly of paternal nucleosomes. Interestingly, while the implication of HIRA and UBN1 for RI deposition of H3.3 in vivo was recently demonstrated in human cells, CABIN1 seemed to play only an auxiliary role in this context [63]. Possibly, CABIN1 could be important for human-specific functions of the HIRA complex, such as the formation of senescence-associated heterochromatin foci [45,64].

HIRA and YEM are interdependent to target the male nucleus

We had previously shown that HIRA specifically accumulates in the sperm nucleus shortly after its delivery in the egg cytoplasm [37]. Here, we have established that maternally expressed YEM similarly accumulates in the male nucleus at fertilization and until pronuclear apposition. Strikingly, we have also shown that HIRA and YEM are mutually dependent for their targeting to the male nucleus, strongly suggesting that these proteins physically interact during the assembly of paternal nucleosomes. However, nothing is known about the mechanism responsible for their rapid and specific localization in the fertilizing sperm nucleus, which is
delivered in the cytoplasm of the gigantic egg cell. We had previously established that the HIRA-dependent assembly of paternal nucleosomes occurs after the removal of sperm protamines [36]. This opens the simple possibility that the HIRA complex could recognize exposed sperm DNA immediately after the removal of SNBPs. Interestingly, pioneer work on YEM by Aït-Ahmed et al. had established that this maternal protein was able to bind DNA in vitro [51]. This property could be important to efficiently target the HIRA complex to sites of de novo nucleosome assembly in the decondensing male nucleus. This hypothesis has recently received indirect experimental support in human cultured cells [63]. In their study, Ray-Gallet et al. established that HIRA, UBN1 and CABIN1 were all individually able to bind DNA in vitro and they proposed that this remarkable property could allow the HIRA complex to target naked DNA for H3.3 deposition. Accordingly, this HIRA-dependent nucleosome gap-filling mechanism has been shown to participate in the maintenance of genome integrity [63], but could also be employed, at the genome-wide scale, for de novo assembly of paternal chromatin at fertilization.

Finally, the observation that YEM accumulates in discrete nuclear regions in both the male nucleus (this study) and the oocyte karyosome [52] opens the possibility that YEM could perform additional roles not related to nucleosome assembly.

**Paternal chromatin assembly is specifically performed by the HIRA-YEM complex**

Despite its expression in the female germline, we found that *Drosophila* ATRX/XNP is not targeted to the male nucleus and does not seem to play any role in male pronucleus formation. Among the 17 SNF2 type chromatin remodelers present in *Drosophila* [16], the Chromodomain-helicase-DNA-binding protein 1 (CHD1) is the only one that has been implicated in the remodeling of paternal chromatin at fertilization [21,54]. In contrast to *Hira* and *yem*, mutations in *chd1* do not drastically affect H3.3 incorporation in paternal chromatin but still severely compromise the decondensation of the male nucleus, which appears aberrant in shape [21,54]. In contrast to the HIRA/CHD1 interaction reported by Konev et al. [54], we could not...
detect any interaction between these proteins in ovaries, using experimental conditions that permitted co-immunoprecipitation of HIRA and YEM. Our results thus suggest that the role of CHD1 in the male nucleus is distinct from the nucleosome assembly process mediated by the HIRA complex.

Although the implication of the HIRA histone chaperone in paternal chromatin assembly was firmly established a few years ago, it has remained unclear until now if this highly specialized RI assembly process also involved other subunits of the HIRA complex or other histone deposition pathways. In fact, we have previously reported that the histone chaperone ASF1 [65], which is known to interact with both the CAF1 and HIRA complexes, was actually absent from the decondensing male nucleus [36]. Although the role, if any, of ASF1 in paternal chromatin assembly awaits a proper functional characterization, we do not expect this histone chaperone to be directly involved in the assembly of

Figure 5. HIRA and YEM are interdependent for their recruitment to the male pronucleus. (A) YEM localizes to the decondensing male nucleus. Confocal images of eggs from yem-Flag[HPF6] yem[2] homozygous females (upper panels) with the male nucleus (arrows) magnified in lower panels. YEM-Flag (green) is detected throughout the decondensing male nucleus but also accumulates in a small number of nuclear foci (arrowheads). Note that YEM-Flag is no longer detected at pronuclear apposition (right panels). Bar: 10 μm. (B) Hira mutations affect the general distribution of YEM-Flag in the male nucleus. Eggs from females of the indicated genotype were stained with anti-Flag antibodies and imaged as in (A). For each male nucleus, the presence of YEM-Flag (whole nucleus and/or foci) was evaluated and each category is represented as a percentage of the total number (n) of observed pronuclei. (C) YEM-Flag foci in the male nucleus do not colocalize with telomerases or centromeres. Upper panels: Nuclear foci of maternally expressed YEM-Flag (red arrow) do not localize to the cluster of telomeres marked with the paternal telomere marker GFP-K81 [69] (green arrow). Lower panels: YEM-Flag nuclear foci (red arrows) do not colocalize with paternal centromeres (green arrows) in the male nucleus. (D) Confocal sections of male pronuclei in eggs laid by females of the indicated genotype (HIRA-Flag is shown in green, DNA in red) (n > 20). In wild-type eggs, HIRA-Flag accumulates in the decondensing male nucleus. In eggs from yem mutant females however, HIRA-Flag is not detected.

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nucleosomes on paternal DNA. Accordingly, ASF1 has been previously shown to be dispensable for direct de novo RC or RI histone deposition in Xenopus egg extracts [66].

The complete failure of the male nucleus to assemble its chromatin in Him or yem mutant eggs demonstrates that no other nucleosome assembly machinery can substitute for the HIRA-YEM complex in this peculiar context. However, the functional requirement of H3.3 itself in this process is not known. In Drosophila, H3.3 is not absolutely required for survival but it is essential for both male and female fertility [27,28]. Viability of His3.3A; His3.3B double null mutants could be explained by the fact that, in the absence of H3.3, canonical H3 can be assembled in a RI manner [28]. Although the mode of RI deposition of replicative H3 in these mutants is not known, it opens the possibility that HIRA could use canonical H3 in certain critical circumstances, such as a limiting availability of H3.3. This compensatory mechanism, however, is apparently not possible in Drosophila spermatocytes, where H3.3 is required for the correct segregation of chromosomes during meiotic divisions, underlining the importance of this variant for sexual reproduction [28]. Similarly, future work should aim at determining whether H3.3 is specifically required for the assembly of paternal nucleosomes at fertilization.

Figure 6. XNP is not involved in paternal chromatin assembly. (A) The dATRX/XNP protein (green) is expressed in the female germline and accumulates in the germinal vesicle (arrow) of the oocyte, here in a stage 10 egg chamber. (B) At fertilization (left panel), XNP is not observed in the male nucleus (inset) whereas it is detected in the nuclei of blastoderm embryo. (C) Confocal images of eggs or embryos from xnp mutant females stained for anti-H4act (green) and DNA (red). Left: in eggs from xnp mutant females, assembly of paternal chromatin seems to occur normally, as revealed by the strong incorporation of acetylated H4 in the male nucleus (inset). Middle: a cycle 1 embryo in prometaphase with the still separated sets of maternal and paternal chromosomes. Note the stronger anti-H4act staining of paternal chromosomes (arrowhead) relative to maternal chromosomes, as in wild-type embryos (see Figure 3C). Right: normal cleavage divisions in a syncytial embryo.
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The Germinal Vesicle: A storage compartment for maternal nuclear proteins?

Both HIRA and YEM proteins, which are presumably expressed from germinal nurse cells, display a remarkable accumulation in the oocyte nucleus during oogenesis [36,51]. Most of the volume of the large germinal vesicle is devoid of DNA as the maternal genome is tightly packaged within the karyosome. The presence of HIRA and YEM in the nucleoplasm of the GV is thus not related to nucleosome assembly. However, the fact that HIRA and YEM are mutually dependent for their accumulation in the GV suggests that they are stored in this compartment as a complex. In contrast to the null alleles, point mutations do not affect HIRA/YEM localization in the GV, suggesting that the mechanisms controlling their recruitment to the GV or to the male pronucleus are distinct. This could reflect the fact that the HIRA complex is active in the male pronucleus where these proteins are in a chromatin environment in contrast to their nucleoplasm distribution in the GV. Whether or not this transient accumulation of HIRA/YEM in the GV plays any role in the maturation of the complex before paternal chromatin assembly at fertilization remains to be tested. Interestingly, it has been proposed that in human cells, formation of senescence-associated heterochromatin foci by HIRA requires its prior localization to promyelocytic leukemia nuclear bodies, suggesting that these structures could participate in the formation of the HIRA complex before its translocation to chromatin [48,67]. It should be mentioned, however, that dATRX/XNP also accumulates in the GV despite its dispensability for paternal chromatin assembly. A recent study [68] reported the presence of several nuclear proteins in the GV with no known function in the oocyte, suggesting that this structure could serve as a storage compartment for a large number of nuclear proteins.

In conclusion, our characterization of Drosophila Yemanuclein demonstrates that this protein is a functional partner of HIRA in vivo. It also establishes that HIRA and YEM directly cooperate in the male nucleus for the genome-wide replacement of sperm protamines with H3.3-containing nucleosomes. The specific requirement of the HIRA complex in this unique developmental chromatin assembly process implies the existence of specific properties not shared with other H3.3-deposition pathways. In this regard, future work should explore the potentially conserved DNA binding property of the HIRA complex [51,63] and its potential role in targeting the fertilizing sperm nucleus in animals.

Materials and Methods

Flies

Flies were grown in standard conditions at 25°C. The w1118 stock was used as a wild-type control in all experiments. The Hira+ and HiraB1 alleles and the Hira-flag transgenic constructs have been described earlier [36,37]. For the construction of the Hira-GFP-FLAG fusion gene, the eGFP coding sequence was inserted between the Hira and Flag tag sequences of HiraHR1 stock has been kindly provided by Stefan Heidmann. The D(3R)3450 deficiency, the P[E(Flytrans)2]EY23024 insertion and the sof2 and sof1 mutant alleles [61] were obtained from the Bloomington Drosophila Stock Center.

Generation of the yem deletion allele

The yem+ mutation was isolated after standard remobilization of the P[E(Flytrans)2]EY23024 element and selected for its non-complementation of the yem− chromosome. yem+ is a 3180 bp deletion from position +2 in the 5′ UTR (positions 29495416 to 29495806 in the genome), uncovering the first 5 exons and part of exon 6 of the yem gene. Note that we only refer in this study to the original gene model [31] identified as RA in Flybase (Flybase ID: FBtr0065413) and not to the recently predicted longer RB transcript (see Flybase.org).

RT–PCR

Total RNAs were extracted with the Trizol method (Invitrogen) from at least 50 whole adults, ovaries or carcasses. Reverse transcription was performed using oligo(dT) primers and the SuperScript First-Strand Synthesis system for RT-PCR (Invitrogen). For the yem and RP49 PCR reactions, the following primers were used YEMAPRIMER15/YEMAPRIMER16 and RP49FWD/RP49REV (see primers section).

Transgenic constructs

yem-flag. The yem EcoRI genomic fragment in bluescript vector [51] was digested with Nhel and XbaI. This fragment was replaced by a PCR amplification product with primers OA37 and OA38, bearing the Flag tag sequence in 3′ of yem. Next, the resulting vector was digested with EcoRI and XbaI and the yem-flag fragment was inserted into the Casper vector. Finally, SV40 polyadenylation signals were added to the previous construct as a Xbal-Pol fragment from the pCasper[AUG-flag] plasmid [71]. The resulting transgenesis construct is called HPF for HoloProtein flanked with Flag. HPF1 (chromosome X), HPF16 (chromosome 2 and HPF3 (chromosome 3) are independent insertions of HPF. The yem-flagHPF1 yem+ chromosome was obtained by meiotic recombination. yem-flagHPF3 yem− homozygous females (used in Figure 5) are fully fertile (not shown).

pUASP-Ubn1-68F. A human Ubn1 cDNA [47] was cloned into a BamHI site of the pUASP-attB vector [69]. Transgenic lines were established using the plbo-C31 integrase method [72,73] in the M(3xP3-RFP-attP)ZH-60Fb attP platform in polytene region 86F.

Primers

YEMAPRIMER2: TGGGAAAACCGCGACAGTGYEMAPRIMER9: GGCGATTTGTGCGCTGATGYEMAPRIMER15: GATTCCTTCTCCTCGCTGTYEMAPRIMER16: CTGCAGGTTACACCTCAAT... (see primers section).

Immunofluorescence

Eggs were collected, dechorionated, devitellinized and fixed in methanol as described [56]. Eggs were then rehydrated in TBS-Triton 0.15% and incubated with primary and secondary antibodies at the indicated dilution. Finally, eggs were incubated in a 2 mg/ml RNase A solution for 1 h at 37°C and were mounted in a mounting medium (DAKO S3023) containing 5 µg/ml propidium iodide. For anti-YEM AS2 antibody staining, ovaries were dissected in PBS-Triton 0.15% and were immediately incubated with the antisera without fixation, stained with DAPI and mounted, as described [74]. For other experiments, ovaries were...
were dissected in PBS-Triton 0.1% and fixed at room temperature in 4% PFA in PBS for 25 minutes. Ovaries were then stained with propidium iodide and mounted as described above. Slides were observed under an LSM 510 META confocal microscope (Zeiss). Images were treated with LSM image browser, Image J or Photoshop CS2 (Adobe).

We used the following antibodies: AS2 anti-YEM antibody (1/100; [51,74]), M2 monoclonal anti-Flag antibody (1:500 in ovaries; 1:1000 in embryos; Sigma), anti-polyacetylated histone H4 (1:200; Millipore 06-589), monoclonal anti-H3.3 (H3F3B) (1:300, Abnova), anti-XNP [16] (1:5000) and anti-UBN1 (1:200) [75]. Secondary antibodies were Alexa488 goat anti-mouse or goat anti-rabbit (1:1000, Invitrogen) and Cy3 donkey anti-rabbit (1:300, Millipore).

**Western blots**

50 µl of ovaries were homogenized in lysis buffer (15 mM Hepes [pH 7.6]; 10 mM KCl; 5 mM MgCl2; 0.5 mM EDTA; 0.5 mM EGTA; 350 mM Sucrose; 1 mM DTT) with protease inhibitors (Halt Protease Inhibitor Single Use Cocktail, Thermo Scientific). The following antibodies were used: M2 anti-Flag (1:1000; Sigma), anti-Tubulin (1/1000; Sigma), Peroxydase-coupled goat anti-mouse (1:10000; Beckman).

**Immunoprecipitations**

For co-immunoprecipitation experiments, we essentially used the protocol described in Jäger et al., 2001 [76] with some modifications as indicated. A hundred ovaries were dissected manually in 250 µl lysis buffer on ice. Lysis buffer was as described [76] to the exception of the protease inhibitors. In our conditions, Roche tablets of EDTA-free protease inhibitor cocktail were used (not carrying a balancer chromosome) were counted separately and their rate to total population was calculated (in every cross, [Hu+] progeny is expected to be 33% of total). For most of the crosses, this percentage exceeds 33%, showing normal viability of the yem[+]/u insertion allele and of the yem+ allele. The yem+ allele is viable but shows lower survival rate than yem+. This sub-viability can be rescued with two yem-flag insertions, showing that it is indeed a specific effect of the yem mutation.

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**Author Contributions**

Conceived and designed the experiments: GAO OA-A BL. Performed the experiments: GAO AA REM MC LMS-T BH OA-A BL. Analyzed the data: GAO PC OA-A BL. Contributed reagents/materials/analysis tools: HG. Wrote the paper: GAO OA-A BL.


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