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Report

A new role for *hth* in the early pre-blastodermic divisions in Drosophila

Lara Salvany, Silvia Aldaz,† Elise Corsetti and Natalia Azpiazu*

Centro de Biología Molecular "Severo Ochoa"; CSIC-UAM; Universidad Autónoma de Madrid; Madrid, Spain; and Instituto de Biología Molecular de Barcelona; CSIC; Barcelona, Spain

†Present address: MRC-Laboratory of Molecular Biology; Cambridge, UK

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In drosophila, the preblastodermic syncytial nuclear divisions occur very fast. In this short period of time chromosomes must condense, segregate and decondense, in conditions governed by maternally provided RNAs and proteins. In this report, we show that the Homothorax (Hth) transcription factor is maternally provided and that its function is necessary for the proper assembly of the centric/centromeric heterochromatin during preblastodermic divisions. Embryos lacking the hth maternally-derived transcript, show abnormal localisation of the centromeric CID protein, and aberrant chromosomal segregation. In this syncytial context, Hth presumably acts together with its partner Extradenticle (Exd) and the RNA PolII, to facilitate transcription of satellite repeats. The transcripts derived from these sequences are needed for the correct assembly of the centric heterochromatin.

Introduction

The first mitotic divisions in drosophila take place in a common cytoplasm and are governed by the maternally inherited collection of RNAs and proteins, that help the young embryo develop during the fast syncytial divisons. During the first mitotic divisions there is no transcription of zygotic genes. Whatever products are needed during this period of embryonic development are present in the common cytoplasm of the dividing syncytium. In the past years, we have been studying the developmental functions of the homeobox containing genes *homothorax* (*hth*) and *extradenticle* (*exd*) of drosophila. They encode proteins of the TALE-homeodomain subfamily and share a high degree of homology with their vertebrates counterparts: the Pbx and Prep-Meis family of proto oncogenes.[1-3] The Hth and Exd products are intimately associated with each other and frequently work as a functional unit. The nuclear import of Exd is regulated by Hth,[4] so that in the absence of Hth, Exd remains in the cytoplasm, where it is supposed to be inactive. In the presence of Hth both proteins associate and translocate into the nucleus to regulate downstream genes. In all the previous reports, it appears that the Hth and the Exd proteins act together forming a complex. However, it has been shown that *exd* has a maternal component,[5] whereas *hth* appears to have only a zygotic function.[3,6] This suggests that in early development Hth and Exd may have independent functions or that a possible maternal *hth* activity has passed unnoticed. In this study we characterize a new function of *hth* in early embryonic development. We show that in the pre-blastodermic embryo there is uniform distribution of an *hth* isoform, encoded by a maternal transcript. We also find that, in pre-blastodermic embryos, satellite DNA sequences that comprise the centromeric regions are being transcribed. We find that Hth colocalize with RNA Polymerase II in the transcriptional pre-initiation complex, and demonstrate that the transcription of centromeric satellite repeats shows a 25 fold reduction in syncytial embryos mutant for *hth*. We also show an enrichment of this satellite DNA in the chromatin immunoprecipitated with both anti-Hth and anti-RNAPolII antibodies. Thus, we suggest that Hth (presumably together with Exd) forms a physical association with the RNA Polymerase II that facilitates the transcription of satellite DNA sequences located in the centric/centromeric region. We propose that the transcription of those sequences is an essential step for centric/centromeric heterochromatin assembly in the pre-blastodermic embryo.

*Correspondence to: Natalia Azpiazu; Centro de Biologia Molecular "Severo Ochoa" CSIC-UAM, C/ Nicholas Cabrera; 1, 28049; Madrid, Spain; Tel.: 34.9.11.96.4697; Email: nazpiazu@cbm.uam.es

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Results

Distribution of hth transcripts and protein in the syncytial embryo. In the course of our studies on *hth* we have found a pre-blastodermic distribution that was previously unnoticed, and have studied the functional requirements of *hth* during the early syncytial divisions. The genomic region of *hth* extends for approx. 100 Kb., giving rise to three classes of transcripts, the long and two short ones[7] (Flybase). Two groups of proteins encoded by these transcripts can bind to Exd via the HM domain[8] (Fig. 1A), but only the long ones, and the RG isoform, contain the

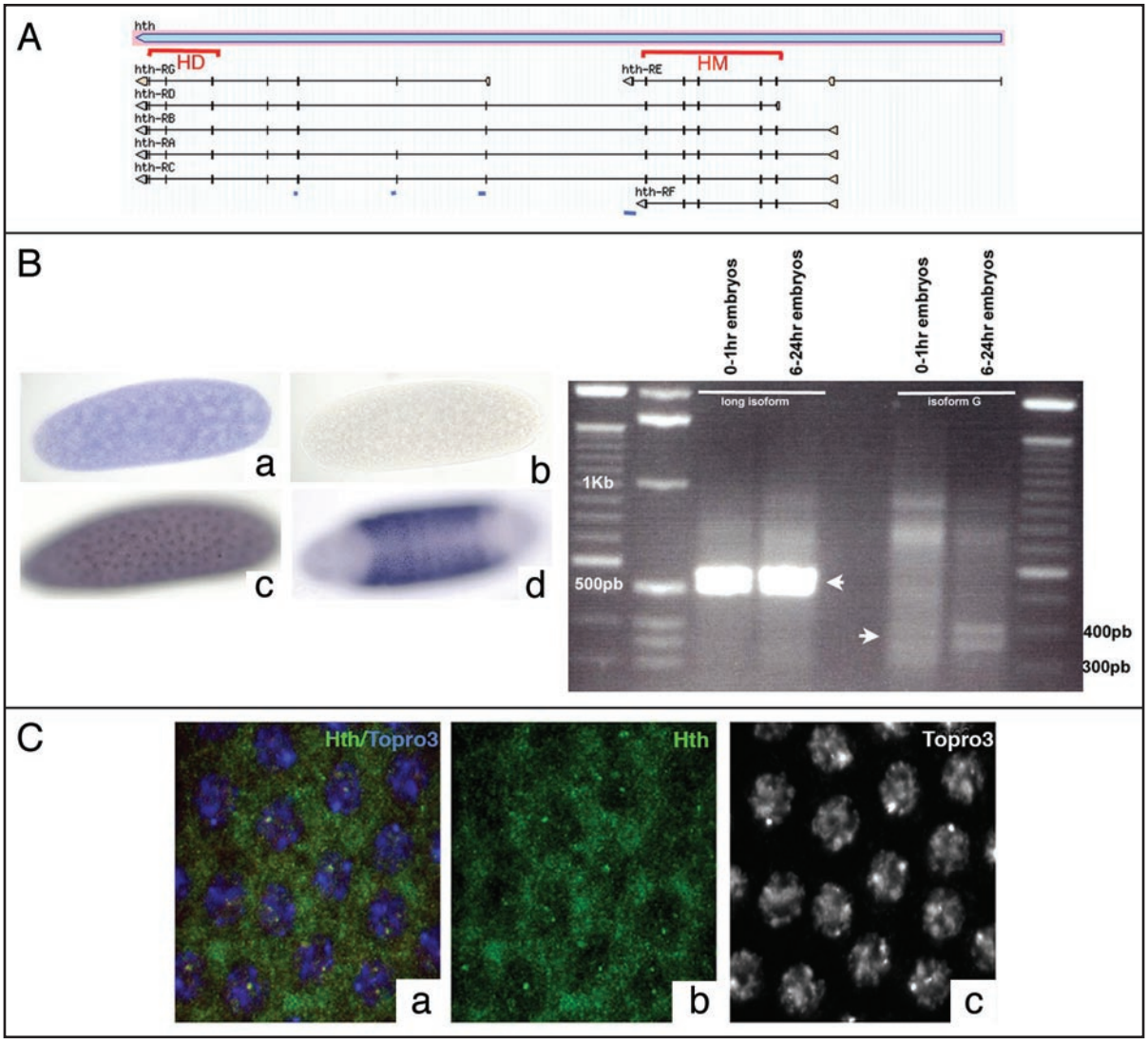

Figure 1. Genomic region of hth and distribution of the different *hth* mRNAs and protein in pre-blastoderm embryos. (A) Seven different mRNAs are transcribed in the genomic region, which extends for more than 100 Kb. The mRNAs can be grouped into three families: the long mRNAs, that code for a full-length protein, and that comprises hth-RA, hth-RB, hth-RC and hth-RD, and two types of short mRNAs: one that comprises hth-RE and hth-RF and that code for a short protein without the HD, and another (hth-RG) hat code for a short protein without the HM domain. The conserved Exd binding domain (HM) is encoded by the first 2–6 exons, whereas the homeodomain (HD) is encoded by exons 11–13. The blue bars indicate the exons used to generate the specific probes. (B) In all panels anterior is to the left. (Part a) Wild type pre-blastoderm embryo stained with a probe against the 5' part of the *hth* mRNA. The signal is detected all over the embryo. (Part b) Embryo as in A stained with a probe against the RE/RF *hth* mRNAs. No signal is observed. (Part d) The first expression of the short isoform is detected after cellularization in the central region of the embryo. (Part c) Wild type embryo stained with an anti-Hth antibody. The protein is detected in the nuclei of the syncytium. RT-PCR with primers that amplify either the long *hth* RNAs or the RG isoform (see M&M). Total RNA was extracted either from 6–24 hrs or from 0–1 hr. old embryos. The long isoforms are present in both types of embryos (arrow), whereas the RG isoform is only present in the old embryos (6–24 hrs). Note the low levels of the RG isoform. (C, Parts a–c) Interphase nuclei stained with anti-Hth (green) and topro3, a general marker for DNA (white). A punctate pattern of Hth distribution is observed inside the nuclei.

homeodomain (HD) able to bind DNA (Fig. 1A). The distribution of the long and some short transcripts has been reported to be very similar during embryonic and larval stages.[7] Particularly, an early pre-blastodermic distribution has not been detected. However, we have found that the long transcript, but not the RG isoform, is ubiquitously distributed in the early syncytial drosophila embryo (Fig. 1B, Part a, and RT-PCR), just as has been reported for *exd*.[5] Consistently, we also detect the presence of the Hth protein at this early stage, which can only be of maternal origin. We used an anti-Hth antibody to study in detail the localization of the protein during the first stages of embryonic development. The antibody has been previously reported[9] and was tested to be specific for all the Hth products. During interphase-early prophase the Hth protein localizes to the nuclei in a punctuate pattern (Fig. 1C, parts a and b). We also detect high levels of Hth protein in the cytoplasm, which is unspecific and can be also detected when stained with the pre-immune serum (Fig. 1C, Parts a and b; Suppl. Fig. 3). After the nuclear membrane breaks down and the

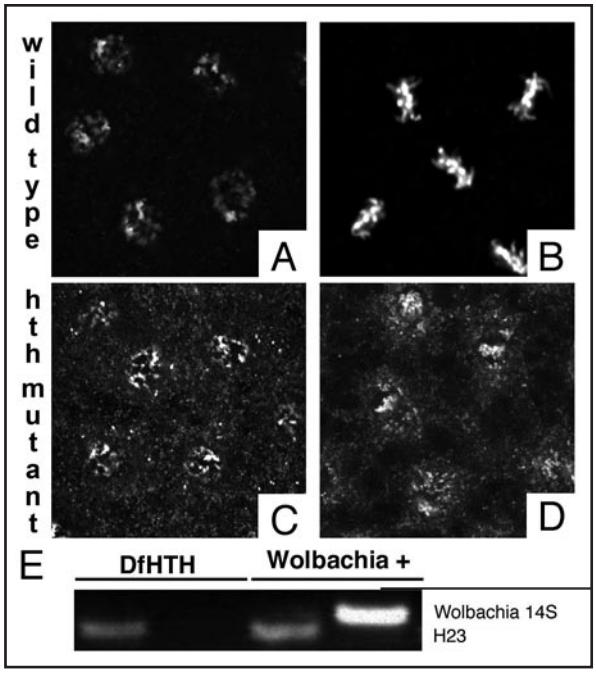

Figure 2. Aberrant mitotic divisions in *hth* mutant nuclei (A) Wild type nuclei of a cycle 7 embryo in prophase stained with topro3. (B) Wild type metaphase nuclei of an embryo in the same cycle and stained as in (A). (C) *hthmat* mutant nuclei in early prophase of cycle 7 stained with topro3 (white). Note the small pieces of chromatin surrounding the nuclei. (D) Magnification of a cycle 7 *hthmat* mutant embryo in early metaphase stained with topro3 (white). The metaphase plate is not well formed, and pieces of broken chromatin are dispersed around the nuclei. (E) PCR done with genomic DNA using specific probes for the 14S ribosomal DNA of Wolbachia (WolbachiaFW: 5'-CCT GAT TCG CGT TAC GGC AGT G-3', WolbachiaRV: 5'-GGT ACT TGA TAA GTT GCA CCA CC-3'). The *dfhth* stock shows no contamination with Wolbachia, whereas a contaminated fly stock specifically amplifies the Wolbachia 14S DNA. The H23 locus was used as a control for the presence of genomic DNA in the PCRs.

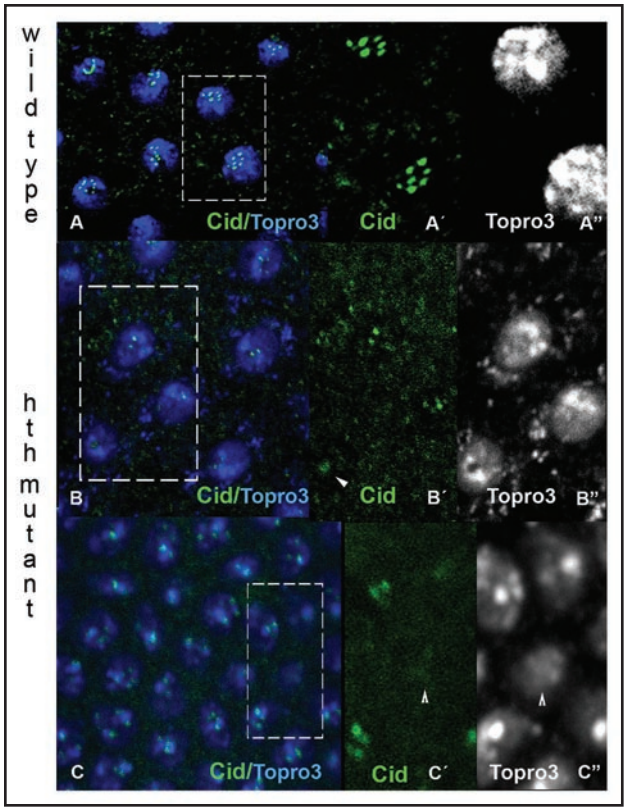

Figure 3. Abnormal CID localization in *hth* mutant nuclei. The embryos are stained with anti-CID (green) and topro3 (blue, white). (A–A") Wild type interphase nuclei in which CID localizes to the centromeres. (B–B") *hthmat* mutant nuclei in interphase. CID staining is weaker than in wild type nuclei (compare A' with B'), and is observed in less dots than in the wild type. In some nuclei CID is present but with a more diffuse distribution (arrowhead in B'). (C) Older syncytium of the same genotype and stained as in (B). Some nuclei have lost much of their genetic material, as judged by the weak staining with topro3 (arrowhead in C"), and do no have any CID protein (arrowhead in C').

mitotic spindle is formed, the Hth protein is no longer detected in the chromatin (not shown).

Phenotype of embryos lacking the maternal hth function. To investigate if the syncytial distribution of *hth* has a function during early embryogenesis, we generated maternal germ line clones mutant for *hth* and examined embryos that are deficient for the maternal function (*hthmat*$^-$). By doing so, we generate syncytia that have all the necessary maternal components for their proper development, but lack the Hth product. We used a deficiency (which we called *dfhth*) that eliminates all *hth* isoforms but the G one, and does not eliminate any other annotated gene (see Materials and Methods), and an *hth* allele (*hth*^{P2}) previously reported to be nearly null.[6] We got similar results with both *hth* alleles. The fact that in the *dfhth* mutants the hth-RG isoform is still present should not interfere with our results, because hth-RG is not present in the early syncytial embryos, and therefore does not have any function during the first mitotic divisions (see Fig. 1, RT-PCR).

The first result is that *hthmat*$^-$ embryos develop very abnormally, and only about 8–9% of them are able to differentiate larval cuticle. The few that secrete cuticle exhibit segment fusions and loss of structures similar to those reported in previous publications for *exdmat*$^-$.[5] These observations indicate that *hth* codes for maternal products, which are required for an early function. We have studied in detail the abnormalities we observe in *hthmat*$^-$ mutant embryos in an attempt to find out the primary defect caused by the mutation. We have also examined *exdmat*$^-$ and found that they exhibit identical phenotypes, and therefore we consider that Exd and the long Hth protein are involved in the same function during the early syncytial divisions.

Most of the *hthmat*$^-$ mutant embryos arrested development before nuclear division 10, that is, before the nuclei reach the surface of the embryo and cellularisation begins.[10] We detected DNA fragmentation with the fragments dispersed around the dividing nuclei (Fig. 2B–D), as soon as cycle 5. During mitosis, those mutant nuclei show abnormal mitotic figures, like abnormal metaphase plates (Fig. 2D). We decided to look at the centromeres, because they are the key structures for chromosome segregation.[11,12] The centromeric heterochromatin can be recognized by the presence of a histone H3 variant known as centromere

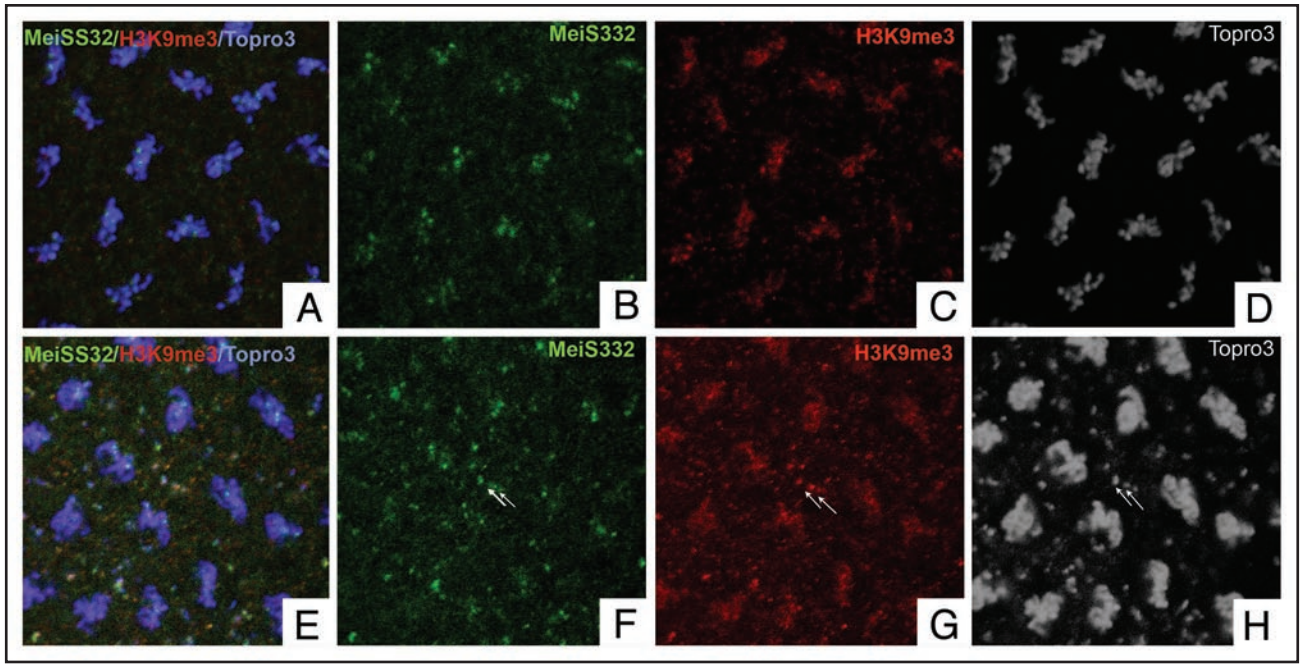

Figure 4. Distribution of MeiS332 and H3K9me3 in *hthmat*⁻ mutant nuclei. Nuclei are stained with anti-MeiS332 (green), anti-H3K9me3 (red) and topro3 (blue, white). (A–D) Nuclei in metaphase show accumulation of MeiS332 in the pericentromeric region (B), where H3K9me3 shows also higher accumulation (C). Some H3K9me3 is also observed along the chromosomal arms. (E–H) Mutant nuclei in metaphase show abnormal MeiS332 (green) and H3K9me3 (red) distribution in the chromosomes. Note that MeiS332 and H3K9me3 are also associated with the small pieces of chromatin that surround the nuclei (arrows).

identifier (CID) in drosophila[13,14] and CENP-A in other organisms.[15] We used an antibody against CID to check the distribution of the protein in both wild type and *hthmat*⁻ mutants. In wild type nuclei, CID can be detected in all stages of the cell cycle always associated with the centromeres (Fig. 3A). Eight individual dots, corresponding to the eight centromeres, can be seen in nuclei stained for CID (see Fig. 3A, inset A'). In contrast, CID distribution in *hthmat*⁻ mutant embryos is diffuse and in some nuclei it is difficult to detect a clear signal at the centromeres (Fig. 3B and C inset B'C'). The staining with topro3 indicates that some nuclei have lost much chromosomal material, which may include the centromeric regions, as pointed by the weak CID staining (Fig. 3C' and C"). A similar distribution of CID is observed in the hth^{P2} mutant syncytia (Suppl. Fig. 2G–I).

Centric heterochromatin assembly in hth mutant embryos. To analyse if the absence of Hth influences the assembly of the centric/centromeric heterochromatin, we studied the distribution of histone 3 methylated on Lys 9 (H3K9me3) and of MEI-S332 in the mutant embryo. MEI-S332 is the founding member of a class of protective proteins known as Shugoshins.[16] Human and yeast Shugoshins ensure that the cohesin does not prematurely dissociate from mitotic centromeres.[17-19] The drosophila Shugoshin Mei-S332 binds the cohesin complex at the centric heterochromatin in metaphase and dissociates from the chromosomes prior to the onset of anaphase, thus allowing the separase to release the cohesion of sister chromatides.[20-22] We performed double stainings MeiS332/H3K9me3 in wild type and *hth* mutant syncytia. In a wild type metaphase, the Mei-332 protein localizes at the centric heterochromatin (Fig. 4A–C), and H3K9me3 is distributed along the chromosomal arms with some co-localization with MeiS332. In *hthmat*⁻ mutants, Mei-S332 and H3K9me3 localization in metaphase are abnormal, and do not concentrate in the dots observed in wild type syncytia (Fig. 4E and G), suggesting an aberrant assembly of the centric heterochromatin, and probably of other heterochromatin structures. Both marks are also associated with broken pieces of chromatin in the yolk surrounding the dividing nuclei (Fig. 4E–G, arrows). These data suggest that in mutant embryos the centric heterochromatin is not well formed, and presumably breaks in the process of sister chromatide separation.

Transcription of heterochromatic repeats in hth mutant syncytia. In yeast, it has been shown that the transcription of repetitive DNA sequences by the RNAPolII, and the subsequent process of these transcripts by the RNAi pathway, is essential for the assembly of centric/centromeric heterochromatin and for centromere function.[23-28] In drosophila, it was previously reported that *Argonaute-2* (*AGO2*), a component of the RNA-induced silencing complex (RISC), is necessary for the correct formation of centric/centromeric heterochromatin.[29] We therefore investigated if centromeric DNA sequences are transcribed in the young drosophila embryo. The 359-bp repeats of the complex 1.688 satellite DNA family extend across the centromere ot the X chromosome in drosophila.[30,31] To investigate if these sequences are transcribed in the syncytial drosophila embryo, we performed FISH experiments using the 359-bp repeat as a probe (see Materials and Methods). As shown in Figure 5, 1.1 E–G, the 359-bp transcript can be detected in the early dividing nuclei, always associated with the RNAPolII.

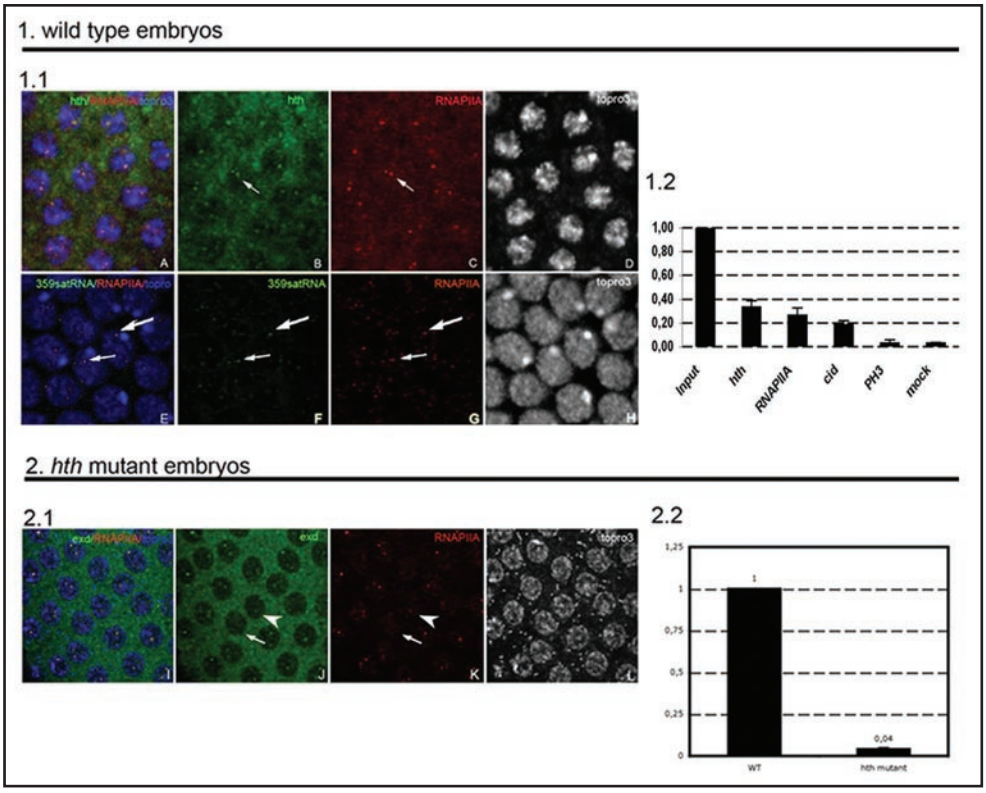

Figure 5. FISH of satellite RNA, distribution of RNAP IIA, ChIP and real-time RT-PCR using primers of satellite repeats. (1) Wild type syncytia (1.1) The nuclei are stained with anti-Hth (green), anti-RNA Polymerase IIA (red) and topro3 (white) in (A–D), and in (E–H), green represents nascent 359 pb. satellite RNA. (A–C) Wild type nuclei showing the colocalization of the RNAP IIA with Hth in the pre-initiation complexes (arrow). (E–G) Wild type nuclei showing colocalization of the nascent satellite RNA (green) and the RNAP IIA (red, arrows). (1.2) ChIP analysis of wild type young syncytia (0–2 hrs.) with antibodies anti-CID, anti-Hth, anti-RNAP IIA, and anti-H3S10ph amplifying the inmunoprecipitated sequences with specific primers for the 359-pb. satellite sequence. The chromatin precipitated with anti-CID, anti-Hth and anti-RNAP IIA shows a similar enrichment (20–30%) when compared with the input DNA. The chromatin inmunoprecipitated with anti-H3S10ph is similar to the mock. (2) *hth* mutant syncytia. (2.1) *hthmat*-nuclei showing the distribution of Exd (green) and RNAP IIA (red). They colocalize. Some nuclei show a wild type distribution of Exd and the RNAP IIA (compare C with K), whereas in others Exd and the RNAP IIA are not detectable in the nucleus (J and K, arrow) or show a faint distribution (J and K, arrowhead). (2.2) The table shows the result of the RT-PCR done with total RNA extracted from 0–2 hrs. old embryos and amplied with primers of the 359-pb repeats of the 1.688 satellite DNA family. In *hthmat*-mutants the transcription of the repeats is 25 fold reduced.

Because both Exd and Hth are reported transcription factors, we wondered if they could mediate the transcription of the satellite sequences in the context of a dividing syncytium. We first compared the nuclear localisation of the Hth protein in relation with that of RNA Polymerase IIA (RNAP IIA), which participates in the formation of the transcriptional pre-initiation complex.[32,33] In interphase nuclei both proteins colocalize in well-defined dots (Fig. 5, 1.1 A–C). The colocalization of both proteins suggests that Hth may facilitate the transcription of the satellite repeats by the RNAPolII. To further test this hypothesis, we performed ChIP experiments using antibodies against the RNAPolII, Hth and the CID products, and amplify the DNA fragments with specific probes for the 359-pb repeat, and for a labial enhancer region, known to be bound by Hth, as a positive control.[34] As shown in Figure 5, 1.2, there is a similar enrichment of the satellite repeat in chromatin precipitated with the CID, RNAPolII and Hth (3.5–4%) when compared with the input DNA. This enrichment is similar to the one observed with the labial enhancer region (Suppl. Fig. 1). We also performed quantitative RT-PCR with RNA extracted from 2 h staged wild type embryos, and with RNA from *hthmat*⁻ embryos, which we amplify with the specific probes for the 359-pb repeat. The results are shown in Figure 5, 2.2. A decrease of 25 fold in the transcription of the 359-pb repeat is observed in syncytia mutant for *hth* when compared with wild type ones. All together, our results indicate that the maternally deposited Hth transcription factor facilitate the transcription, via RNAPolII, of centromeric repeats, which are 25 fold reduced in the mutant syncytia.

The incomplete penetrance of the phenotypes observed in the *hth* mutant syncytia could be due, at least in part, to the presence of the maternally deposited Exd protein, the known partner of Hth in many of its biological functions. We decided to check the distribution of Exd, and of the RNAPII, in nuclei lacking the maternal component of *hth*. As shown in Figure 5, 2.1 I–K, both proteins always colocalize inside the mutant nuclei. Some of the nuclei show a wild type distribution of them (compare Fig. 5, 1.1 B and C with 2.1 J and K), whereas in others, both Exd and the RNAPII are missing (Fig. 5, 2.1 J and K, arrow) or show a faint distribution (Fig. 5, 2.1 J and K, arrowhead). Our observations suggest that the wild type distribution of the RNAPII in some of the mutant nuclei is due to the presence of Exd in those, and explains, at some degree, the partial rescue of the *hth* mutant phenotype. This result also shows that the nuclear import of Exd is not dependent upon Hth in the syncytial embryo (see discussion).

Distribution of histone marks in hth mutant embryos. We have also examined the possibility that the requirement for Hth-mediated transcription of non-coding DNA repeats extends to other heterochromatin regions. To test this, we looked at the distribution of the heterochromatin protein 1 (HP-1), which is known to provide a foundation for a self-assembly mechanism of heterochromatin.[35] We detect a strong and punctate distribution of HP-1 in *hth* mutant embryos, which is never observed in wild type syncytia (compare Fig. 6B with E). A similar distribution

of HP1 has been previously reported for *AGO2* mutants,[29] and suggests that *hth* have a more general role in heterochromatin assembly during the syncytial divisions.

Phosphorylation of histone H3S10 in euchromatic regions has been implicated in counteracting heterochromatisation, so that reduced levels of H3S10ph results in spreading of heterochromatin markers to ectopic locations.[36,37] We checked if the lack of correct heterochromatin assembly observed in *hth* mutants could also influence the degree at which histone H3S10 gets phosphorylated. To do so, we stained wild type and mutant syncytia with an antibody that recognizes the phosporylated state of Ser 10 in H3 (anti-H3S10ph). In wild type syncytia, anti-H3S10ph staining is first observed in late prophase, by the time the DNA starts to condense (not shown).[38] H3S10ph staining peaks during metaphase/anaphase and lasts until late anaphase/telophase, where a gradual loss of H3S10ph is observed on the chromosomes.[38] No H3S10ph staining is detectable in interphase and early prophase nuclei. Embryos derived from females with *hth* germ line clones show high levels of H3 phosphorylation at Ser10 in metaphase, anaphase and telophase, like the wild type ones. However, unlike in wild type embryos, nuclei maintain the phosphorylated state of the H3 at Ser 10 in interphase (Fig. 6G and H). All together this results suggest that, in the absence of *hth,* the chromatin changes its higher-order structure to a more "open euchromatic state" marked by the ectopic presence of H3S10ph.

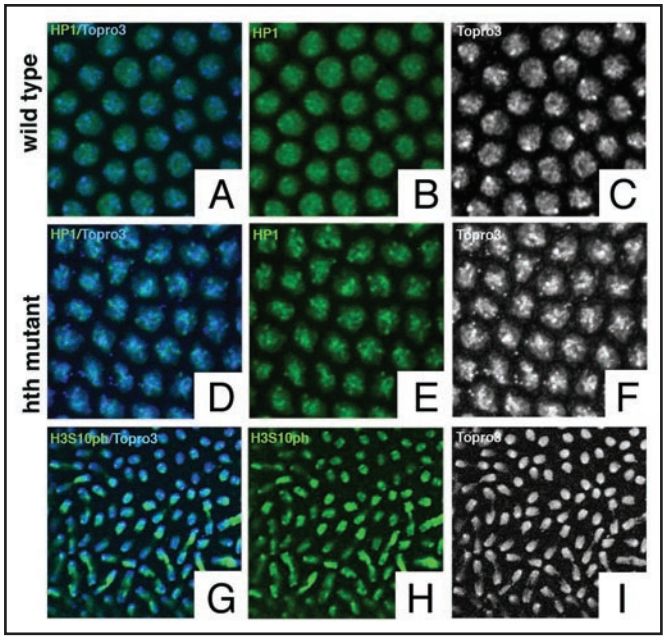

Figure 6. Distribution of H3S10ph and HP1 in *hth* mutant nuclei. The nuclei are stained with anti-HP1 (green in A, B, D and E), anti-H3S10ph (green in G and H), and topro3 (white). (A–C) Wild type nuclei show homogeneous distribution of HP1 with some higher accumulation in heterochromatin domains (marked by dark topro3 staining). (D–F) *hth* mutant nuclei show higher levels of HP1 protein, with a more punctate distribution, similar to what was previously reported for *AGO2* mutants (see text). (G–I) *hth* mutant syncytium showing and ectopic distribution of H3S10ph (green) in interphase and telophase nuclei (see text for details).

Discussion

The first mitotic divisions in drosophila take place in a common cytoplasm and are governed by the maternally inherited collection of RNAs and proteins. During this period the divisions occur very fast and there is no need of zygotic transcription.[10,39] In this study we show for the first time that *hth* is maternally deposited and that Hth (and its partner Exd) are essential for the proper development of the syncytium. Without hth/exd function chromosomes do not segregate normally, and correct embryonic development is impeded.

Normal chromosomal segregation during cell division requires the correct assembly of the centromeres. Centromeres in drosophila are embedded in heterochromatin, and assemble in different satellite DNA sequences.[40,41] In this work we report for the first time transcription of the 359 pb satellite region of the centromeric heterochromatin of chromosome X in the syncytial embryos depending on the RNAPolII. We have also demonstrated that this transcription is dependent on the homeobox containing gene *hth*. In *hth* mutant syncytia, the 359-pb transcripts are reduced 25 fold with respect to their wild type siblings.

hth mutant syncytia show abnormal distribution of the centromeric CID protein. The altered distribution of CID in those mutants, and the abnormal chromosome segregation observed, suggests that, as described in yeast,[23-28] the transcription of centromeric repeats in the drosophila syncytium is necessary for the centromeres to function. The aberrant CID distribution is probably due to a defect in the centric/centromeric heterochromatin assembly (as visualized in the mutant by abnormal HP1 and MeiS332 distribution) that also compromises centromere function. Without fully functional centromeres chromosomal segregation is compromised, and the embryo ends up loosing much of its genetic material.

We observed an abnormal distribution of HP1 in *hth* mutant syncytia. This is consistent with an early role of *hth* in heterochromatin assembly. We detect HP1 in the nucleus as early as nuclear cycle 6–7 (not shown) suggesting that heterochromatization begins early in the development of the embryo. In the absence of *hth*, the assembly of heterochromatin is impaired (at least in part), and the chromatin seems to acquire a more "open euchromatin" state, which is now marked with ectopic phosphorylation of H3 in Ser10.

Despite the severe phenotypes observed in our mutants, a low percentage of them are able to develop until late stages of embryogenesis. We believe that this is due to a partial rescue by the maternal component of *exd*. The amount of maternal Exd that enters the nucleus in a *hthmat*$^-$ embryo becomes critical to determine until when the embryo is going to develop. Because this amount is variable we get a high variability of phenotypes. The fact that Exd translocates to the nucleus in the absence of Hth suggests that, at least in the syncytial embryo, Hth is not required for the nuclear translocation of Exd. This is also true in the cellular blastoderm (st. 5), where Hth is nuclear and Exd remains in the cytoplasm. The statement that Hth is required for the nuclear import of Exd should therefore be re-examined.

Transcription factors are known to be key regulators of gene expression, but a specific role in the transcription of satellite sequences has not been hitherto reported. We have demonstrated

that *hth* has a role in the correct assembly of centric/centromeric heterochromatin during the early syncytial divisions of drosophila, and probably of other heterochromatin structures. Without it (and its partner Exd), chromosomal segregation fails to occur properly and the chromatin acquires a more "open" euchromatic-like conformation. This new function of known transcription factors interacting with the RNA Polymerase II in pre-blastodermic embryos to facilitate transcription of non-coding DNA repeats opens new avenues of research about the formation and spreading of heterochromatin.

Materials and Methods

Drosophila strains. The following drosophila strains were used in this work to analyse mutant phenotypes or generate loss of function clones: exd^{Y012}FRT18D/FM7,[5] ovo^{D2}FRT18D/Y;FLP38,[5] w^+;P(ovo-FLP.R)M1B;MKRS/TM2 (Bloomington), and FRT82$Bovo^{D1}$/ßtub85De/TM3,Sb (Bloomington). We used the Df(3R)*hth* from Exelisis (6158), which eliminates almost all *hth* transcripts (hth-RG remains in the deficiency), to generate the *FRT82*hth^{Df}*/TM3* recombinant. The hth^{P2} allele was described in.[6]

Generation of female germ line clones. To generate *exd* female germ line clones exd^{Y012}FRT18D/FM7 females were crossed with ovo^{D2}FRT18D/Y;FLP38 males.[5] After 48 hours the progeny was heat shocked for 2 hours at 37°C. Virgin females of the genotype exd^{Y012}FRT18D/ovo^{D2}FRT18D were collected and crossed with Y/FM7 males. The embryos laid by the females were collected and fixed for antibody staining. For the *hth* germ line clones, the following stock was generated: P(ovo-FLP.R)M1B/Cyo;FRT82 hth^{Df}/TM2. Virgin females of the genotype P(ovo-FLP.R) M1B/Cyo; FRT82 hth^{Df}/TM2 were crossed to FRT82$Bovo^{D1}$/ßtub85De/TM3,Sb males. Females of the genotype P(ovo-FLP.R) M1B;FRT82Bhth^{Df}/FRT82$Bovo^{D1}$ were collected and crossed with FRT82 hth^{Df}/TM3 males. The eggs laid by those females were treated as described above.

Immunostaining of embryos. Embryos were collected for 2 hours, dechorionated and immediately fixed in a mixture of 5% formaldehyde and heptane for 20 minutes at room temperature. The aqueous phase was removed and methanol added. The vitelline membrane was removed by vigorous shaking and the embryos were washed in methanol several times. They were subsequently rehydrated and blocked in 10% BSA. The incubation with primary antibody was done overnight in PBT (PBS, 0.1% Tween). The antibodies used were: anti-Hth 1:500 (rabbit), anti-CID (gift from S. Henikoff) 1:200 (rabbit), anti-Mei-S332 (gift from T. Orr-Weaver) 1:2,500 (guinea pig), anti-H3S10ph (Cell Signalling) 1:200 (rabbit), anti-HP1 (Hybrydoma Bank) 1:20 (mouse), anti-H3K9me3 (Millipore) 1:400 (rabbit), and anti-RNAP IIA (Covance) 1:50 (mouse). Washes were performed in PBT, and the appropriate fluorescent secondary antibody was added for 1 hour at room temperature. Following further washes in PBT, topro3 was added for 15 mins., washed again, and the embryos were mounted in Vectashield (Vector Laboratories). Images were taken in a confocal laser MicroRadiance microscope (Leica) and subsequently processed using Adobe Photoshop.

In situ hybridisation of embryos. Specific digoxigenin-labelled probes for the two types of *hth* RNAs where synthesized as described.[42] For the long RNAs a probe spanning exons 7 to 11 was used, whereas for the short RNAs we used a probe that hybridizes to exon 8 of hth-RE, which is not present in the long RNAs (see Fig. 1A). In situ hybridisation was performed as described before,[43] and the embryos were mounted in Permount (Fischer Scientific).

Antibody and FISH staining. PCR was performed with the primers described below that amplify the 359-bp repeats of the 1.688 satellite subfamily. The PCR fragment was subsequently cloned in the p-GEM-T easy vector and transcribed using the bio-labeling kit of Roche. The FISH experiment was preformed as described in[44] with some modifications. The first antibody was incubated o/n in PBT (PBS, 0.1% tween) and washed 3X20 min. in PBT before the secondary antibody was added together with the AB Complex (Vectacstain, Vector Laboratories). They were together incubated at RT for 45 mins. After 3X15 min. washes with PBT the embryos were incubated with tyramide-FITC for 15 mins. Following further washes in PBT, topro3 was added for 15 mins. washed again, and the embryos were mounted in Vectashield (Vector Laboratories). Images were taken in a confocal laser MicroRadiance microscope (Leica) and subsequently processed using Adobe Photoshop.

Real-time RT-PCR. RNA was extracted from syncytial embryos using the GE Healthcare extraction kit. The real-time PCR was performed in the presence of SYBR Green on a Roche LightCycler 480 with the following pair of primers: 5'-TAT TCT TAC ATC TAT GTG ACC-3' and 5'-GTT TTG AGC AGC TAA TTA CC-3' to amplify the 359 pb repeats of the 1.688 satellite subfamily. To normalize, we used primers for bcd 5'-AAG GGT CTG GAC AAG AGC TG-3' and 5'-AAG GCT CTT ATT CCG GTG CT-3'.

Chromatin immunoprecipitation (ChIP). Chromatin immunoprecipitations assays were carried out with DNA obtained from 0.52 g of *D. melanogaster* 2 hours staged embryos as previously described,[45] with some minor modifications. Homogenized embryos were sonicated 5 times (10 seconds continuous pulses at 7 amplitude microns power) in a MSE Soniprep 150 sonifier with a microtip probe at 4°C, with 30 s cooling on ice between pulses, yielding DNA fragments mostly between 200 and 700 bp. For immunoprecipitation, rabbit anti-Homothorax (1:25), mouse anti-RNAPIIA (1:25), rabbit anti-CID (1:25), and rabbit anti-H3S10ph (1:30) were used. Immunoprecipitated DNA was used for Real-Time PCR amplification with primers for the 359 bp repeats of the 1.688 satellite subfamily (the same primers described above) using a Roche Light Cycler equipment and accessories as described.[46] The data are presented as the amount of DNA enrichment normalized with the input (100% value).

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/SalvanyCC8-17-Sup.pdf

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