Cell Reports
Article

Heterochromatin Reorganization during Early Mouse Development Requires a Single-Stranded Noncoding Transcript

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http://dx.doi.org/10.1016/j.celrep.2013.08.015

SUMMARY

The equalization of pericentric heterochromatin from distinct parental origins following fertilization is essential for genome function and development. The recent implication of noncoding transcripts in this process raises questions regarding the connection between RNA and the nuclear organization of distinct chromatin environments. Our study addresses the interrelationship between replication and transcription of the two parental pericentric heterochromatin (PHC) domains and their reorganization during early embryonic development. We demonstrate that the replication of PHC is dispensable for its clustering at the late two-cell stage. In contrast, using parthenogenetic embryos, we show that pericentric transcripts are essential for this reorganization independent of the chromatin marks associated with the PHC domains. Finally, our discovery that only reverse pericentric transcripts are required for both the nuclear reorganization of PHC and development beyond the two-cell stage challenges current views on heterochromatin organization.

INTRODUCTION

Widespread changes in the organization of chromatin occur during cellular differentiation and genome reprogramming (Fraser and Bickmore, 2007; Hemberger et al., 2009; Meister et al., 2011). The most striking example of genome-wide chromatin reorganization is observed upon fertilization. At this time two specialized gametes, the sperm and the oocyte, fuse to form the zygote, and the chromatin configuration of each of the parental genomes is extensively modified in order to reestablish totipotency (Albert and Peters, 2009; Burton and Torres-Padilla, 2010; Feng et al., 2010; Gill et al., 2012; Seisenberger et al., 2013). This developmental transition is of key importance. On the one hand, the memory of parental origin, which is essential for mechanisms of imprinting, needs to be preserved (Surani, 2001). On the other hand, the equalization of the two parental genomes is needed for domains that behave similarly in the subsequent cellular divisions. Constitutive heterochromatin (Brown, 1966) represents a paradigm for such domains and has an important role in safeguarding nuclear architecture, genomic stability, and proper chromosome segregation during cellular division (Bernard et al., 2001; Festenstein et al., 1999; Maison et al., 2010; Martínez-A and van Wely, 2011; Misteli, 2010; Peters et al., 2001; Verdaasdonk and Bloom, 2011). In the mouse genome, constitutive heterochromatin is composed of two distinct regions, the centric and pericentric domains (Choo, 2000). Centric heterochromatin, which comprises minor satellite repeats, is associated with a specific histone H3 variant, CENP-A, which is important for centromere organization and kinetochore assembly (Amor et al., 2004; Boyarchuk et al., 2011). Pericentric heterochromatin (PHC) flanks centric heterochromatin and consists of arrays of AT-rich major satellite repeats (up to several megabases in length). It is associated with transcriptional repressive epigenetic marks such as H3K27me1 (Martens et al., 2005), H4K20me3 (Schotta et al., 2004), and H3K9me2/3 (Guenatri et al., 2004; Rea et al., 2000), and is bound by heterochromatin protein 1 (HP1) (Lachner et al., 2001; Maison et al., 2002). In most somatic interphase cells, PHC is organized in nuclear domains termed chromocenters, in which the major satellite DNA from different chromosomes cluster together, while the minor satellite repeats of each chromosome remain as separate entities (Guenatri et al., 2004). In contrast to the situation in somatic cells, in the zygote, paternal and maternal pericentric DNA is spatially organized around nucleolar precursor bodies (NPBs), forming partial rims or spherical patches (Martin et al., 2006; Probst et al., 2007). A large-scale nuclear reorganization

leads to a somatic-like conformation only at the end of the two-cell stage, after the transcriptional activation of the embryonic genome (Aoki et al., 1997; Bouniol et al., 1995; Houlard et al., 2006; Probst and Almouzni, 2008).

During early preimplantation stages, the two parental PHC domains are associated with different histone posttranslational modifications and chromatin proteins. Whereas the maternal PHC is enriched in most of the typical repressive marks, the paternal PHC is devoid of H3K9me3 and HP1 (Arney et al., 2002; Martin et al., 2006; Santos et al., 2005; van der Heijden et al., 2005). Instead, it is enriched in the histone variant H3.3 and in Polycomb group (PcG) proteins (Puschendorf et al., 2008; Santenard et al., 2010). These differences are only resolved later, after the appearance of chromocenters, at around the four- to eight-cell stage transition (Merico et al., 2007; Puschendorf et al., 2008). A key question thus concerns how the distinct parental PHC domains undergo major nuclear rearrangements and become equalized in order to fulfill their function as constitutive heterochromatin.

In a previous study, we revealed the importance of a burst of pericentric transcription at the two-cell stage for the reorganization of PHC into chromocenters (Probst et al., 2010). Remarkably, pericentric transcription from the parental genomes shows a strand-specific spatiotemporal regulation (Probst et al., 2010). The forward strand of major satellites is mostly transcribed from the paternal genome, suggesting a differential transcriptional competence of the distinct parental PHC domains. Although this initial work underlined the crucial role of major satellite transcripts in PHC organization, the relative contribution of replication and transcription to this reorganization, and how the distinct parental PHC domains are affected remained unclear. Most importantly, how noncoding RNAs (ncRNAs) operate in this process remains to be determined.

In the present study we investigate, using mouse preimplantation embryos, the link between the asymmetry of paternal and maternal PHC domains and the dynamics of their replication and transcription. By interfering with these processes, we demonstrate that, unlike major satellite transcription, replication is dispensable for the acquisition of a somatic-like heterochromatin configuration. Using parthenogenetic embryos, we show that major satellite RNAs are essential for the reorganization of PHC, irrespective of their parental origin. Finally, using methods to specifically knockdown either one of the two major satellite transcripts, we show that only reverse RNAs are essential for clustering of pericentric satellites into chromocenters at the late two-cell stage. Our data point to an unsuspected role for major satellite transcripts in PHC organization and suggest that these transcripts operate independently of canonical double-stranded RNA mechanisms, such as those found in fission yeast.

RESULTS

Different Impact of Replication and Transcription on PHC Reorganization

Considering that epigenetic marks can influence replication timing (Göndör and Ohlsson, 2009; Martin et al., 2006), we asked whether the replication dynamics of the two parental PHC domains could be influenced by the asymmetry in chromatin marks found at the two-cell stage (Mayer et al., 2000). We performed staged pulse labeling of mouse embryos with 5-ethynyl-2′-deoxyuridine (EdU, a deoxynucleotide precursor incorporated into DNA during replication; Wossidlo et al., 2010) and estimated the duration of S phase to be 7–9 hr (Figures 1A and S1A–S1D), as previously reported (Artus and Cohen-Tannoudji, 2008; Molls et al., 1983; Streffer et al., 1980). By comparing the EdU patterns with DNA fluorescent in situ hybridization (FISH) for major satellites (Figures S1C and S1D), we determined that the replication of major satellite DNA begins at ~35–37 hr post-human chorionic gonadotropin injection (phCG, corresponding to mid S phase) and is completed after 38 hr phCG (corresponding to late S phase), long after the replication of euchromatin. Next, to distinguish the replication dynamics of the two parental PHC domains, we combined EdU pulse labeling with immunodetection of Ring1B and H3K9me3, respectively marking paternal and maternal PHC in two-cell embryos (Puschendorf et al., 2008). In >80% of the nuclei of embryos in mid S phase, the EdU signal within PHC domains colocalized predominantly with the Ring1B-decorated heterochromatin (Figures 1B and S2A). In contrast, in embryos in late S phase, EdU showed no marked difference between the parental PHC domains, indicating that both paternal and maternal PHC undergo replication at this time (Figures 1B and S2A). Thus, as previously observed in mouse zygotes (Bouniol-Baly et al., 1997; Ferreira and Carmo-Fonseca, 1997), the heterochromatin domains of paternal origin initiate replication during mid S phase, about 2–3 hr earlier than their maternal counterparts. However, as assessed in these experiments, both paternal and maternal PHC domains complete their replication in a similar time frame.

Given that paternal PHC is both replicated (Figure 1B; Aoki et al., 1997; Bouniol-Baly et al., 1997) and transcribed earlier (Figure S2B; Probst et al., 2010) than its maternal counterpart, we asked how interfering with these processes impacts the dynamics of PHC reorganization. Late zygotes placed in medium containing aphidicolin (an inhibitor of DNA replication) arrest at the G1/S border of the two-cell stage. Moreover, reorganization of their PHC domains at 48 hr is incomplete (Probst et al., 2010), without a significant impact on major satellite transcription (Figures S3A and S3B). Here, we directly compared embryos arrested by replication block with embryos arrested after knockdown of major satellite RNA at 72 hr phCG (a time point at which embryos should have cleaved to the four-cell stage; Figure 2A). As expected, the lack of EdU incorporation in aphidicolin-treated embryos confirmed an efficient replication block, whereas EdU incorporation after knockdown of major satellite RNA demonstrated that these embryos replicate their DNA (Figure 2B). DNA FISH (at 72 hr or 96 hr phCG) in the latter embryos shows that in ~80% of the nuclei, a portion of the major satellites fail to cluster into chromocenters (Figures 2B, S3C, and S3D). Surprisingly, in aphidicolin-treated embryos, in contrast to the situation at 48 hr phCG (Probst et al., 2010), ~70% of the nuclei have achieved a complete reorganization of major satellites in foci resembling chromocenters (Figure 2B). To strengthen this observation, we arrested embryos injected with locked nucleic acid (LNA) gapmers or grown in the presence of α-amanitin (an RNA Polymerase II inhibitor that reduces the levels of major satellite transcripts; Figure S3E) in G1/S with aphidicolin (Figures 2A

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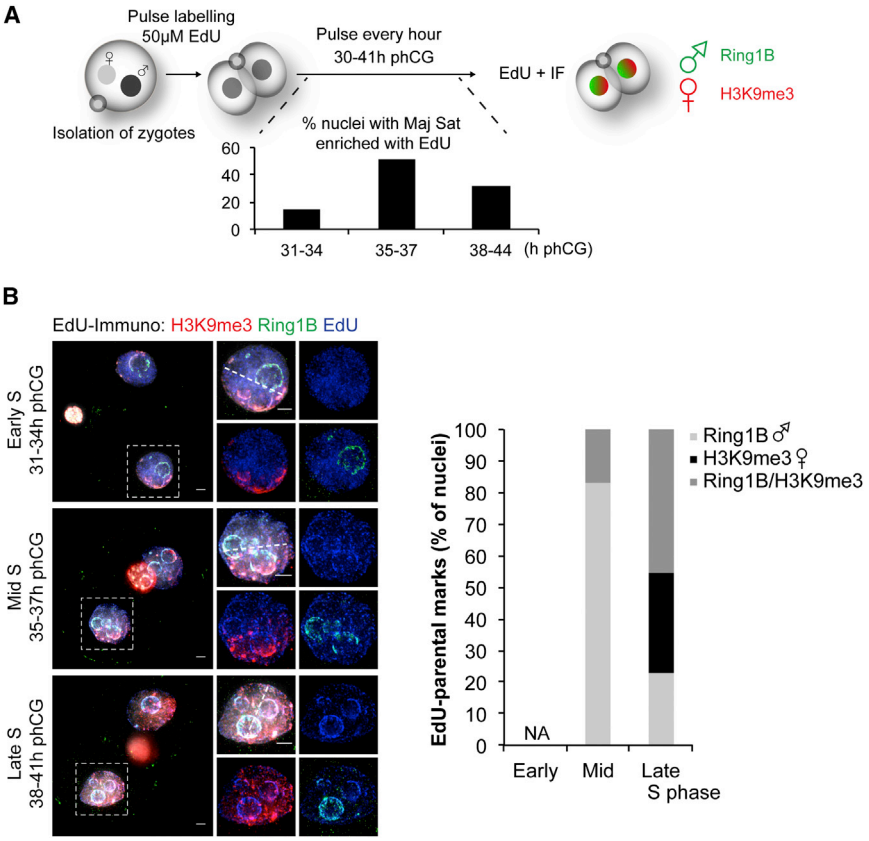

Figure 1. Major Satellites Are Replicated during Mid–Late S Phase of the Two-Cell Embryo and Show Different Parental Replication Dynamics

(A) Shortly after completion of the first cleavage, embryos were pulsed every hour from 30 to 41 hr phCG by culturing in the presence of EdU for 1 hr. Immediately after the pulse, embryos were collected and processed for EdU detection together with either DNA FISH for major satellites or immunostaining for H3K9m3 or Ring1B. The graph represents the percentage of nuclei in which major satellites are labeled with EdU at different time frames within S phase of the two-cell stage.

(B) Two-cell embryos were pulsed with EdU at different time points during S phase. Representative embryos stained for H3K9me3 (red), enriched at the maternally derived genome and Ring1B (green), enriched at the paternally derived genome, followed by EdU click-iT (blue) are depicted. DNA is counterstained with DAPI (gray). Scale bar represents 5 μm. The graph represents the replication timing of paternal and maternal heterochromatin. Replicating heterochromatin regions were assigned to maternal or paternal domains on maximum-intensity projections by their enrichment in H3K9me3 and Ring1B, respectively.

See also Figures S1 and S2.

and 2C). Embryos treated with aphidicolin and injected with control gapmers behave similarly to embryos treated only with aphidicolin, in which the majority of nuclei show a complete reorganization of major satellites into chromocenters (Figure 2C). In contrast, embryos arrested in G1/S and either injected with LNA gapmers Maj1/2 or treated with α-amanitin fail to reorganize their PHC domains into chromocenters (Figure 2C). Based on these data, we propose that, unlike transcription, replication is not essential for the reorganization of major satellites, but rather contributes to the appropriate timing of chromocenter formation.

Paternal and Maternal Heterochromatin Domains Are Differentially Affected by the Knockdown of Pericentric Transcripts

Given the parental asymmetry in epigenetic marks between PHC domains, we wondered whether the subset of major satellites that remain associated with NPBs upon major satellite RNA knockdown originate from one parent only. To address this issue, we collected two-cell stage embryos injected with control or major LNA-DNA gapmers and carried out DNA FISH for major satellites simultaneously with immunostaining for Ring1B and H3K9me3 to distinguish between paternal and maternal genomes, respectively (Figure 3A). We confirmed that the injection of LNA-DNA gapmers does not interfere with marks associated with either paternal or maternal heterochromatin (Figures S4A and S4B). Furthermore, DNA FISH on arrested embryos showed that in approximately half of the nuclei with ring-like major satellites, the satellites are predominantly localized in the Ring1B-enriched regions and hence are of paternal origin (Figures 3B–3D and S4C). Most of the remaining nuclei that contain ring-like major satellites do not show a parental asymmetry, localizing to both maternal and paternal nuclear regions. Only ~18% of the nuclei show rings exclusively labeled with maternal-specific marks. A distinct dynamics of reorganization of both parental PHC domains could partly explain these differences. To assess this, we performed DNA FISH for major satellites together with immunostaining for H3K9me3 (Figures 3E and S4D). We found that during late G2 phase (50 hr phCG) of control two-cell embryos, the few remaining ring-like major satellites are predominantly localized in regions that are not enriched for H3K9me3 and hence are of paternal origin (Figure 3E and S4D). This suggests that paternal PHC domains complete their reorganization later than their maternal counterparts. We thus conclude that even though maternal domains cannot completely reorganize in the absence of major satellite transcripts, paternal PHC domains are more sensitive to the knockdown of these transcripts.

Major Satellite Transcripts Are Essential for Complete Reorganization of Both Parental Heterochromatin Domains

Given that knockdown of major satellite transcripts has a greater impact on the capacity of paternal PHC to reorganize into chromocenters, we asked whether the parental origin and/or the lack

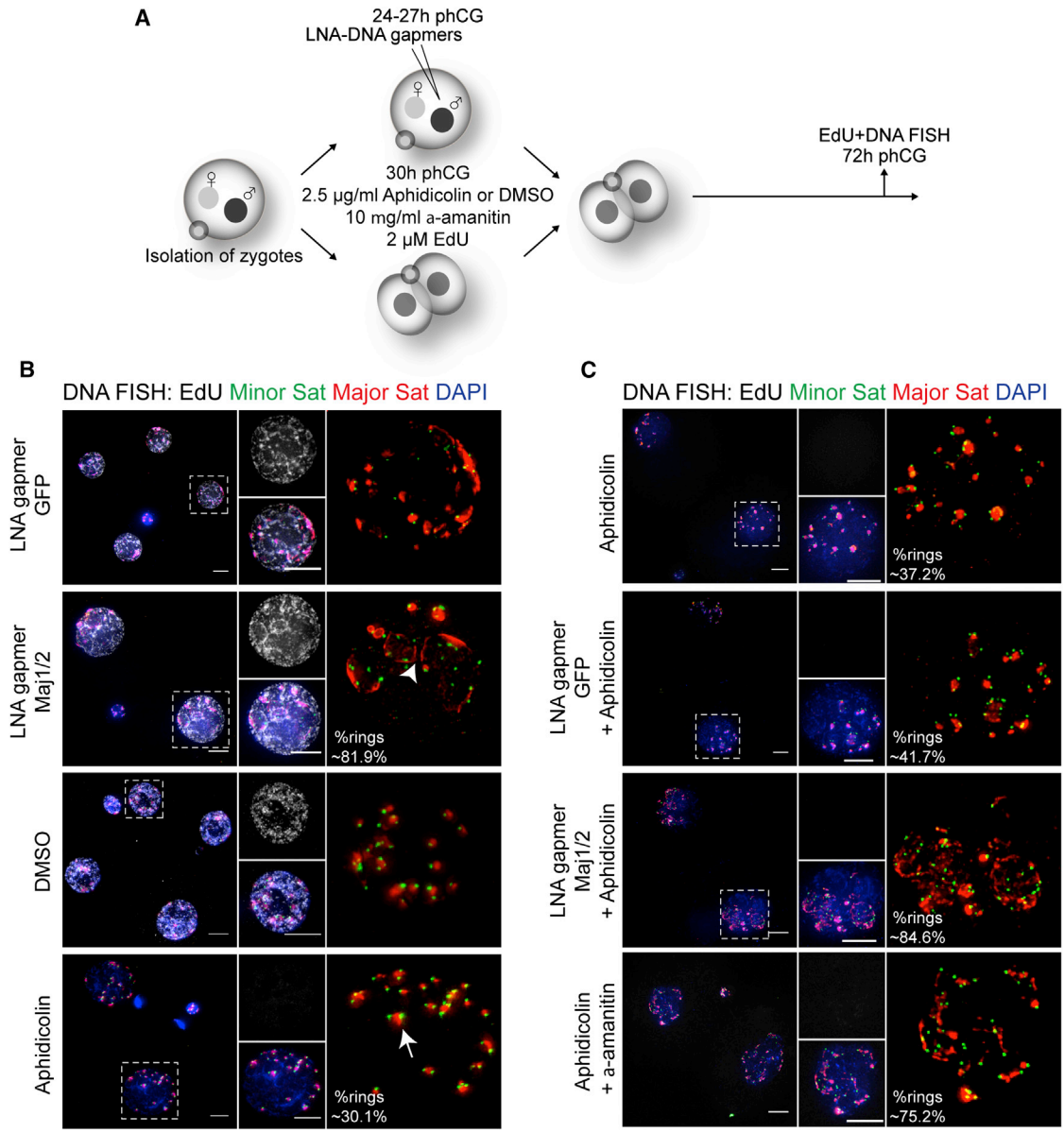

Figure 2. PHC Reorganization Requires Major Satellite Transcription, but Not Progression through Replication

(A) Zygotes were collected and divided into two groups. The first group was injected between 24 and 27 hr phCG with LNA-DNA gapmers directed against GFP or a combination of forward and reverse major transcripts. The second group was placed in medium supplemented with 2.5 µg/ml aphidicolin or DMSO at 30 hr phCG. Both groups of embryos were cultivated in medium supplemented with 2 µM EdU from 30 hr phCG. Embryos were collected at ~72 hr phCG and EdU incorporation was detected together with DNA FISH. At this time point, control embryos already cleaved to four cells.

(B) Control, aphidicolin-treated, and microinjected embryos were collected at ~72 hr phCG and processed for EdU revelation and DNA FISH. Panels with respective enlargements show EdU staining (gray), as well as the predominant patterns of DNA FISH signals for minor (green) and major (red) satellites. Arrowhead indicates ring-like major satellites; arrow indicates major satellite DNA reorganized in chromocenters. The proportion of nuclei containing ring-like major satellites is displayed on the enlarged panels ($p < 0.01$ for aphidicolin versus LNA Maj1/2, Student's t test). DNA was counterstained with DAPI (blue). Scale bar represents 10 µm.

(C) Aphidicolin-treated, microinjected embryos under aphidicolin treatment and α-amanitin/aphidicolin-treated embryos were collected at ~72 hr phCG and processed for EdU revelation and DNA FISH. Panels with respective enlargements show EdU staining (gray), as well as DNA FISH for minor (green) and major (red) satellites. The proportion of nuclei containing ring-like major satellites is displayed on the enlarged panels. Binomial tests were applied to assess the significance of the differences observed between the treatments (all treatments compared with aphidicolin: not significant (NS) for LNA-GFP; $p < 1.0 \times 10^{-11}$ for LNA Maj1/2; $p < 1.0 \times 10^{-06}$ for α-amanitin). DNA was counterstained with DAPI (blue). Scale bar represents 10 µm.

See also Figures S2 and S3.

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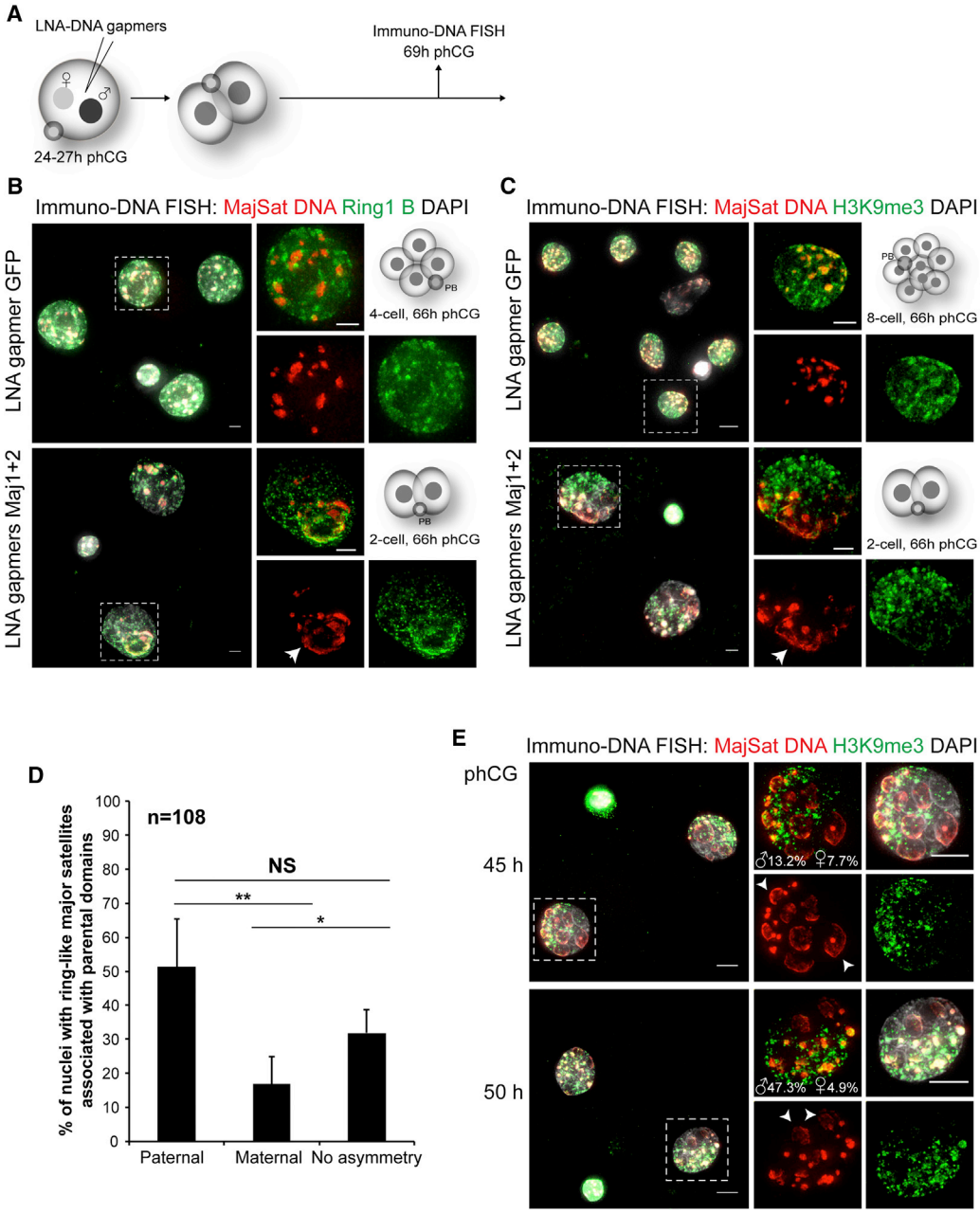


Figure 3. Knockdown of Major Satellite Transcripts Impacts the Reorganization of Paternal and Maternal Heterochromatin in Two-Cell Embryos Differently

(A) Zygotes were injected between 24 and 27 hr phCG with LNA-DNA gapmers targeting either GFP or forward and reverse major satellite transcripts. Embryos were collected at ~69 hr phCG and processed for immuno-DNA FISH.

(B) Microinjected embryos were stained for Ring1B (green, specifying the paternal heterochromatin) and processed for DNA FISH revealing major satellites (red).

(C) Similarly to (B), embryos were stained for H3K9me3 (green) to reveal the maternal PHC. DNA was counterstained with DAPI (gray). Scale bar represents 5 μm.

(D) Within the population of embryos with ring-like PHC domains, we assessed the percentage of two-cell nuclei in which the rings were of either paternal or maternal origin, based on the H3K9me3/Ring1B parental asymmetric staining. Error bars represent the SD from four independent experiments; *$p < 0.05$, **$p < 0.01$, Student's t test.

(E) Two-cell embryos were collected at 45 and 50 hr phCG and stained for H3K9me3 (green, specifying the maternal genome) and processed for DNA FISH revealing major satellites (red). Arrowheads point to ring-like structures of major satellites. The percentage of nuclei containing ring-like major satellites enriched (maternal, ♀) or devoid (paternal, ♂) of H3K9me3 is indicated on the enlarged panels. Binomial tests were applied to test the significant increase in the presence of exclusively paternal ring structures between the two time points (paternal versus maternal: NS for 45 hr phCG, $p < 1.0 \times 10^{-12}$ for 50 hr phCG). DNA was counterstained with DAPI (gray). Scale bar represents 10 μm.

See also Figure S4.

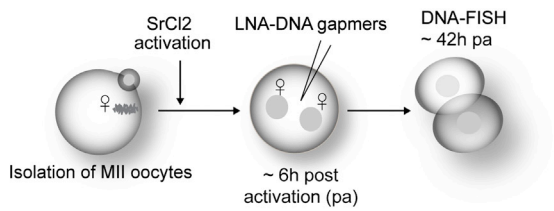
of somatic heterochromatin marks are responsible for the developmental arrest. We thus generated diploid parthenogenetic embryos, in which the complete genome is contributed by the oocyte and therefore all PHC domains are enriched in repressive histone modifications and devoid of PcG proteins (Puschendorf et al., 2008). In parthenotes, the expression of the forward strand of major satellites is low, while the burst of the reverse strand during the late two-cell stage is maintained, albeit at lower levels (Probst et al., 2010). We isolated unfertilized oocytes, activated them for parthenogenetic development, and knocked down both forward and reverse major satellite transcripts by microinjecting LNA-DNA gapmers (Figure 4A). We collected injected parthenotes at ~42 hr postactivation (pa) to process them for DNA-FISH. Similar to the observation in embryos, parthenotes that received major LNA-DNA gapmers arrested at the two-cell stage with a significantly higher frequency than control injected parthenotes (Figure 4B). Moreover, pericentric domains in arrested parthenotes failed to organize into chromocenters (in ~67% of the nuclei; Figure 4C). Thus, major satellite transcripts are required for the reorganization of both paternal and maternal PHC, irrespective of their distinctive chromatin marks.

Forward and Reverse Major Satellite Transcripts Play Distinct Roles during Preimplantation Development

The low levels of forward major satellite RNA in parthenotes, in which the reorganization of PHC still occurs, prompted us to investigate the exact contribution of the forward and reverse transcripts. To that end, we microinjected zygotes before the onset of zygotic genome activation with LNA-DNA gapmers specifically targeting either the forward or the reverse transcripts (Figure 5A). We confirmed the efficient strand-specific knockdown of major satellite transcripts by both RNA FISH (Figures 5B and S5A) and quantitative RT-PCR (qRT-PCR; Figures 5C, S5B, and S5C). Under these conditions, depleting one transcript does not affect the levels of the complementary strand, suggesting that they are independently regulated. Despite the prominent burst of forward-strand expression observed during the two-cell stage, embryos injected with LNA-DNA gapmers targeting the forward transcripts did not show an increased frequency of developmental arrest compared with control embryos (Figure 5D). In contrast, injection of LNA-DNA gapmers targeting the reverse transcripts resulted in developmental arrest at the two-cell stage at a high frequency (Figure 5D). These results demonstrate a crucial role for reverse transcripts in development progression. However, it remained possible that even though forward transcripts are dispensable for development progression, they could be necessary for PHC clustering. We thus examined the organization of PHC in injected embryos at ~69 hr phCG by DNA FISH. Embryos injected with gapmers targeting forward transcripts organize their major satellites in chromocenters in a manner similar to that observed for noninjected and control GFP-injected embryos (Figure 5E). This is in sharp contrast to embryos depleted of reverse transcripts, in which some of the pericentric satellites remain organized around NPBs in ring-like structures and distinct from control counterparts at the same stage (Figure 5E, compare with Figures 2B and S3C).

We next tested the importance of the distinct strands of major satellite transcripts in parthenotes, which lack the burst in

A



B

LNA DNA gapmer	n_o of experiments	arrested in 2-cell (%)	n_o of parthenotes
Non injected	5	2.4	167
LNA GFP	5	40.8	120
LNA Maj1+2	5	86.4	140

Fisher's Exact Test:
p=6.79e-15 compared to LNA GFP

C

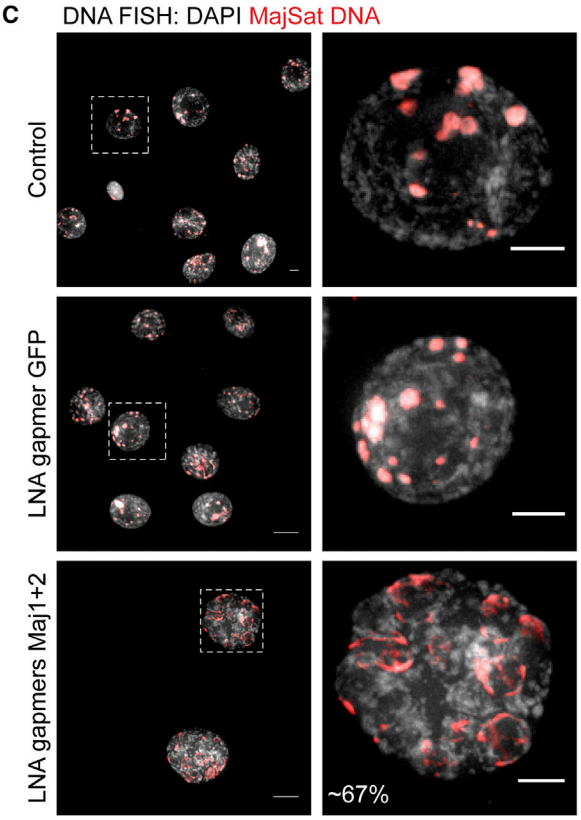
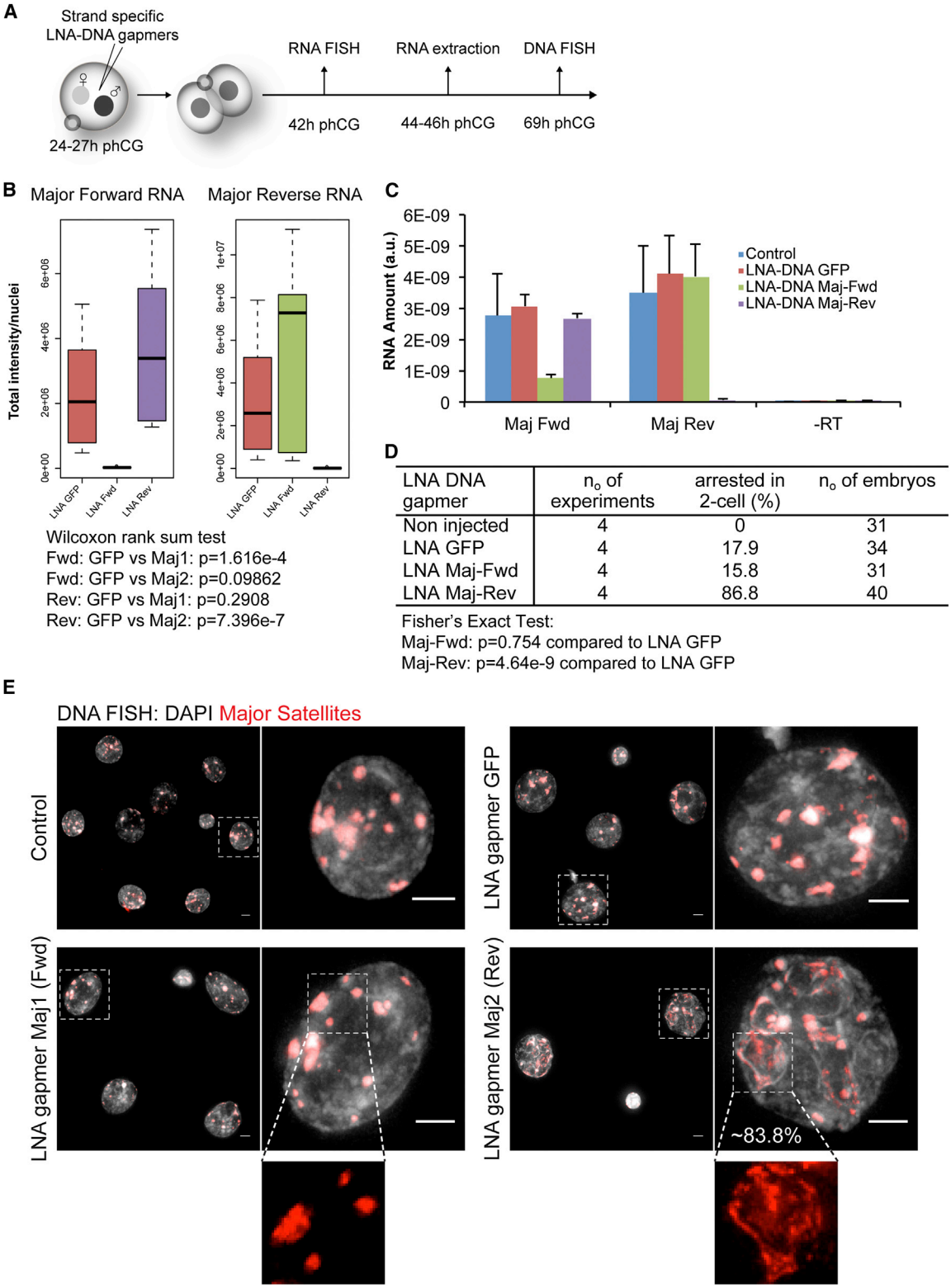


Figure 4. Parthenotes Require Major Satellite Transcripts for Developmental Progression and Proper Heterochromatin Consolidation

(A) Oocytes were collected at 16 hr phCG and activated for ~6 hr in $SrCl_2$ in the presence of cytochalasin B to generate diploid parthenotes. Parthenotes were injected with LNA gapmers targeting either GFP or forward and reverse major satellite transcripts and processed for DNA FISH at 42 hr postactivation (pa).

(B) Table representing the developmental phenotype of parthenotes microinjected with LNA-DNA gapmers.

(C) Noninjected and microinjected parthenotes were processed for DNA FISH with probes revealing major satellites (red). DNA was counterstained with DAPI (gray). Scale bar represents 5 μm. The percentage of parthenotes with ring-like major satellites after knockdown of major satellite transcripts is indicated.



LNA DNA gapmer	n_o of experiments	arrested in 2-cell (%)	n_o of embryos
Non injected	4	0	31
LNA GFP	4	17.9	34
LNA Maj-Fwd	4	15.8	31
LNA Maj-Rev	4	86.8	40

Figure 5. Knockdown of Reverse Major Satellite Transcripts Is Sufficient to Prevent Developmental Progression Past the Two-Cell Stage

(A) Zygotes were injected between 24 and 27 hr phCG with LNA-DNA gapmers directed against GFP or targeting either forward or reverse major satellite transcripts. Embryos were collected for RNA FISH at 42 hr phCG, for RNA extraction at ~44–46 hr phCG, and for immuno-DNA FISH at ~69 hr phCG.

(legend continued on next page)

forward-strand expression. Injecting activated oocytes under experimental conditions identical to those described above (Figure 6A) led to the same developmental phenotype (Figure 6B). Likewise, when parthenotes were injected with strand-specific LNA-DNA gapmers, only parthenotes with reduced levels of reverse major transcripts failed to reorganize part of their major satellites in chromocenters (Figure 6C). Although our results do not exclude other roles for the forward transcripts, we conclude that the reverse transcripts derived from major satellites play a major role in both the clustering of PHC and developmental progression.

DISCUSSION

Our study reveals three important features of PHC during preimplantation development: DNA replication is dispensable for promoting PHC clustering during the two-cell stage, pericentric transcripts are required for PHC reorganization regardless of the chromatin marks associated with distinct parental PHC domains, and pericentric transcripts derived only from the reverse strand are necessary for both nuclear reorganization and developmental progression beyond the two-cell stage. The latter finding reveals that only one of the two complementary major satellite transcripts contributes to the large-scale nuclear reorganization of heterochromatin, a role that was previously unsuspected.

Replication and Transcription Affect Heterochromatin Organization Differently

In somatic cells, where the transcription levels of major satellites are relatively low compared with the early embryo, PHC domains that are organized in chromocenters replicate from mid to late S phase (Guenatri et al., 2004; Quivy et al., 2004). This typical behavior of a heterochromatic state contrasts with the early replication of transcriptionally active euchromatin (Mazzotti et al., 1990; Nakayasu and Berezney, 1989; O'Keefe et al., 1992). In two-cell embryos, prior to chromocenter formation and despite the strong transcriptional activity of major satellites, a replication pattern similar to that of somatic cells is already established. Thus, the global replication timing of PHC domains is imposed by rules independently of their nuclear organization. The earlier initiation of replication of paternal PHC parallels the earlier zygotic genomic activation of the male pronucleus (Aoki et al., 1997; Bouniol-Baly et al., 1997) and the higher levels of forward major satellite transcripts produced from the paternal genome at the two-cell stage (Probst et al., 2010). This bias in the timing of both replication and transcription at the paternal genome may arise from the lack of most somatic repressive histone modifications and reduced binding of HP1 proteins (Santos et al., 2005). It would thus be interesting to challenge factors that are responsible for the setting of histone marks during early mouse development, such as ESET/Setdb1 (Cho et al., 2012), LSD1/KDM1 (Ciccone et al., 2009; Macfarlan et al., 2012), and KAP1 (Quenneville et al., 2011), to determine the impact on the observed parental bias in replication and transcription. These differences may explain why the rearrangement of paternal PHC is more sensitive to interference with major satellite transcripts. Although it is less sensitive, maternal PHC equally requires major satellite RNAs to reorganize appropriately, as shown by our data from parthenogenetic embryos. Thus, major satellite transcripts are critical for both PHC domains to cluster into chromocenters, irrespective of the parental chromatin organization and their associated marks.

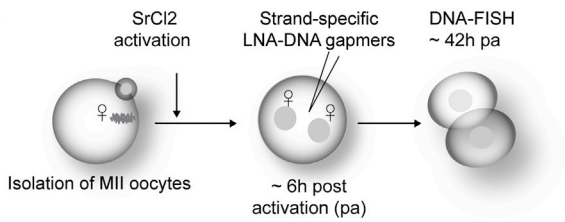
Our comparison between embryos blocked with aphidicolin and embryos microinjected with LNA-DNA gapmers targeting major satellite transcripts emphasizes the importance of transcription over replication for heterochromatin reorganization. This suggests that large-scale nuclear reorganization can take place even when replication is inhibited, as observed in terminally differentiated nuclei transplanted into *Xenopus* enucleated oocytes that do not replicate (Byrne et al., 2003). We thus propose that in the early embryo, although replication may contribute to large-scale nuclear reorganization of PHC in time, it is the appropriate transcription of major satellites that is the dominant driving force in promoting the reorganization of PHC domains and their release from NPBs.

The Reverse Major Satellite Transcripts Are Essential for PHC Organization

During the two-cell stage, the expression of forward and reverse strands of major satellites is temporally and spatially regulated (Probst et al., 2010). The increasing number of long ncRNAs revealed in a recent study (Djebali et al., 2012) led us to reconsider major RNAs as potential single-stranded, long ncRNAs. Indeed, our data suggest that the regulation of the transcriptional dynamics of each strand is likely independent, as depleting one transcript does not affect the levels of the complementary strand. In this respect, the recent identification of several transcription-factor-binding sites in PHC and their importance in regulating expression of forward and reverse transcripts in somatic cells (Bulut-Karslioglu et al., 2012) is particularly interesting. It will be important to further explore how these

(B) Quantification of the total intensity of the RNA FISH signals per nucleus for forward and reverse major satellite transcripts in two-cell embryos microinjected with LNA-DNA gapmers.
(C) qRT-PCR of major satellite transcripts after strand-specific reverse transcription. Mean of the expression levels of transcripts ± SD in two-cell embryos injected with LNA-DNA gapmers targeting either the forward or reverse major satellite RNAs compared with LNA-DNA gapmers targeting GFP and noninjected embryos from three independent experiments.
(D) Table representing the developmental phenotype of embryos microinjected with strand-specific LNA-DNA gapmers.
(E) Control and microinjected embryos were collected at ~69 hr phCG and processed for DNA FISH. Panels with respective enlargements show DNA FISH for major satellites (red) of representative embryos for each set of LNA-DNA gapmers injected. DNA was counterstained with DAPI (gray). Close-ups illustrate major satellite organization in different groups of embryos. Scale bar represent 5 μm. The percentage of embryos with ring-like major satellites after knockdown of reverse major satellite RNA is indicated.
See also Figure S5.

A



B

LNA DNA gapmer	n_o of experiments	arrested in 2-cell (%)	n_o of parthenotes
Non injected	3	3.1	65
LNA GFP	3	43.9	57
LNA Maj-Fwd	3	50	58
LNA Maj-Rev	3	96.4	56

Fisher's Exact Test:
Maj-Fwd: p=0.577 compared to LNA GFP
Maj-Rev: p=2.71e-10 compared to LNA GFP

C

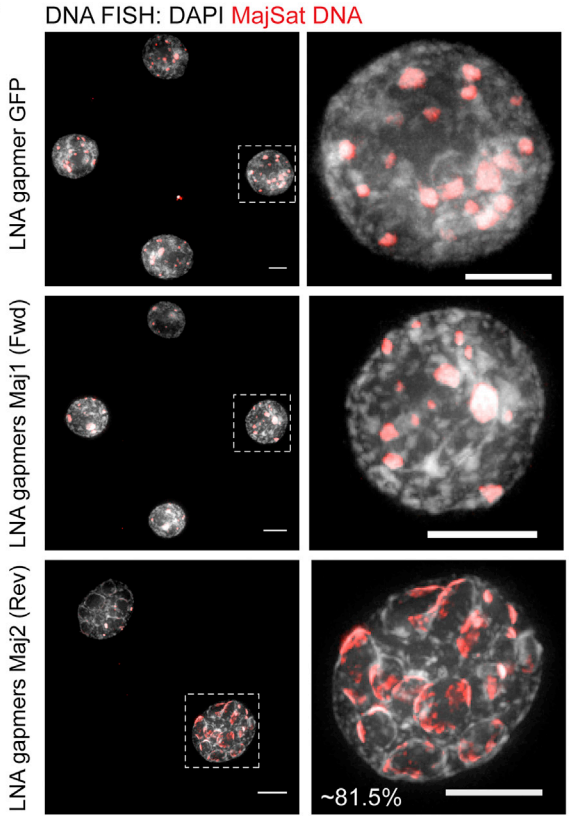


Figure 6. Parthenotes Require Reverse Major Satellite Transcripts for Proper Developmental Progression and Heterochromatin Organization at the Two-Cell Stage

(A) Oocytes were collected at 16 hr phCG and activated for ~6 hr in $SrCl_2$ in the presence of cytochalasin B to generate diploid parthenotes. After activation, parthenotes were injected with strand-specific LNA-DNA gapmers targeting either GFP or forward or reverse major satellite transcripts. At 42 hr pa, the parthenogenetic embryos were processed for DNA FISH.

(B) Table representing the developmental phenotype of parthenotes microinjected with strand-specific LNA-DNA gapmers.

(C) Parthenotes microinjected with LNA-DNA gapmers targeting GFP or forward or reverse major satellite transcripts were processed for DNA FISH with probes targeting major satellites (red). DNA was counterstained with DAPI (gray). Scale bar represents 10 μm. The percentage of parthenotes with ring-like major satellites after the strand-specific knockdown of major satellite transcripts is indicated.

transcription factors contribute to the regulation of the distinct and independent transcriptional dynamics of the two strands in two-cell embryos.

In this study, we demonstrate that only the reverse strand of major satellite transcripts is required for PHC reorganization and developmental progression at the two-cell stage. We propose that the different classes of transcripts arising from major satellites play distinct roles in PHC organization. Given the prominent peak of forward major satellite RNAs from the paternal genome during the two-cell stage (Probst et al., 2010) and their specific interaction, in the context of heterochromatin formation, with sumoylated-HP1 proteins (Maison et al., 2011), we propose that these transcripts have other, as yet unrevealed, functions during development. While we have focused on early events during embryonic development, a broader role for both forward and reverse transcripts should be evaluated in different cellular contexts in later developmental stages, such as primordial germ cells, somatic and stem cells undergoing major cellular reprogramming, and tissues under cellular stress (e.g., DNA damage). The pressing importance of these issues is emphasized by reports of several human and mouse cancers in which heterochromatin is globally altered and silenced repeats become aberrantly expressed (Eymery et al., 2009; Ting et al., 2011; Zhu et al., 2011).

Initially, the functional nature of major satellite transcripts was connected with double-stranded RNA and the small interfering RNA machinery based on work in *Schizosaccharomyces pombe* (White and Allshire, 2008) and more recent work in mouse embryos (Santenard et al., 2010). Although we cannot exclude the possibility that hybrid species exist in a transient manner, they do not seem to be critical for the clustering of PHC. Based on our data, we hypothesize that the appropriate expression of reverse RNA (at the G2-M stage) is critically needed to ensure the proper reorganization of PHC of both parental genomes into chromocenters and allow entry into mitosis. Among the emerging roles for long ncRNAs in genomic regulation (Rinn and Chang, 2012), reverse major satellite transcripts could represent a class of ncRNAs with a structural role in PHC organization. It is tempting to consider that reverse transcripts may help to set up a scaffold/platform that acts as a recruitment/stabilization hub for different RNAs or proteins, such as Polycomb repressive complex 1 (PRC1) complexes that accumulate at the paternal pericentric domains in the zygote (Puschendorf et al., 2008) or HP1 proteins that are enriched on the maternal PHC (Santos et al., 2005). The recent finding that the destabilization of Xist RNA with LNA gapmers led to its quick release from the inactive X chromosome and consequent displacement of the PRC2 complex (Sarma et al., 2010) is in line with this view. Future work focusing on both the structure of major satellite transcripts and the molecular characterization of ribonucleoprotein complexes associated with forward and reverse transcripts will help elucidate the mode of action of pericentric ncRNAs.

EXPERIMENTAL PROCEDURES

Collection and Culture of Mouse Oocytes and Embryos

Animals were used in accordance with the International Guiding Principles for Biomedical Research Involving Animals, as promulgated by the Society for the Study of Reproduction and the European Convention on Animal Experimentation. Female B6D2F1 mice (6–10 weeks old; Charles River) were superovulated by administration of 7.5 IU of pregnant mare's serum gonadotropin (PMSG), followed 46–48 hr later by injection of 7.5 IU hCG. Zygotes were collected from the ampullae of oviducts ~17 hr after injection of hCG. Embryos were further cultivated in microdrops of M16 medium (Sigma) under mineral oil (Sigma) at 37°C under 5% CO_2. To block replication, we supplemented M16 medium with 2.5 μg/mL aphidicolin (Sigma). To block transcription, we supplemented M16 medium with 10 μg/mL α-amanitin (Sigma). For the generation of diploid parthenotes, we isolated MII oocytes 16 hr phCG and activated them in M16 containing 2 mM EGTA supplemented with 5 mM $SrCl_2$ and 5 μg/mL cytochalasin B for 6 hr.

Microinjection of Mouse Embryos

We injected ~10 pl of LNA-DNA gapmers (5, 10, and 20 μM) diluted in 6 mM HEPES pH 7.5, 60 mM KCl, 0.2 mM $MgCl_2$ into the cytoplasm of mouse zygotes between 20 and 24 hr phCG using an Eppendorf Micromanipulator on a Nikon inverted microscope. We monitored the development of the embryos at regular intervals.

Antibodies and LNA Oligonucleotide Probes

For immunofluorescence staining, we used anti-H3K9me3 (1:200; Upstate and Active Motif) and anti-Ring1B (1:200; MBL) antibodies in combination with highly cross-absorbed Alexa 488-, 594-, or 647-coupled secondary antibodies (Molecular Probes). We obtained fluorescently labeled LNA oligonucleotide probes and LNA-DNA gapmers from Exiqon.

Immunofluorescence Staining, RNA FISH, DNA FISH, and EdU Labeling

We prepared embryos for immunofluorescence, RNA FISH, and DNA FISH as previously described (Okamoto et al., 2004; Probst et al., 2010). For the S phase profiling of two-cell embryos, we started collecting embryos at ~30 hr phCG (when the majority of embryos proceeded through the first mitosis) and at regular 1 hr intervals until completion of S phase. Each group of embryos was placed into M16 medium containing 50 μM EdU (Invitrogen). After 1 hr, EdU detection was performed with a Click-iT Imaging Kit (Invitrogen) according to the instructions of the manufacturer except that detection was prolonged to 1 hr. When combined with EdU labeling, DNA FISH and immunofluorescence were carried out before and after EdU revelation, respectively.

Microscope Analysis and Image Processing

We acquired bright-field images of embryos with a Leica MZ FLIII stereomicroscope, and fluorescent images with a Deltavision RT microscope (Applied Precision; 40×/1.35 NA, 63×/1.4 NA, and 100×/1.4 NA objectives). A z series of ~60–70 slices (0.2 μm z step) were acquired and deconvolved using SoftWorx software (enhanced ratio, 20 iterations; Applied Precision). ImageJ was used for further image processing. The total fluorescence of forward and reverse RNA was quantified with the use of the 3D Object Counter (Bolte and Cordelières, 2006). Images were corrected for chromatic shift in z (minus two planes) and the same threshold was used to quantify the difference in intensity levels among the different groups of microinjected embryos. For quantification of the EdU intensity ratio in nuclei, we used the 3D-FIED macro (Cantaloube et al., 2012). Unless stated otherwise, maximum-intensity projections are shown.

RNA Preparation and RT-PCR Analysis

We used Trizol (Invitrogen) to extract RNA from embryos. Briefly, we collected RNA from at least 10 embryos by resuspending them in Trizol (Invitrogen) in the presence of 0.5 pg of an exogenous standard. The extracted RNA was extensively DNase treated with Turbo DNase (Ambion) and reverse transcribed (Superscript III; Invitrogen) with strand-specific primers (Lehnertz et al., 2003). We measured the levels of major satellite transcripts by real-time PCR using Power SYBR Green (Applied Biosystems) in a 7500 Fast Real-Time PCR system (Applied Biosystems). qPCR was based on a previous report (Terranova et al., 2005).

Sequence of Probes, LNA-DNA Gapmers, and Primers

The sequences of the probes, gapmers, and primers used in this work are provided in Table S1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.08.015.

ACKNOWLEDGMENTS

We are grateful to S. Boissel and I. Grandjean from the Institut Curie Animal Facility; I. Okamoto and K. Ancelin for help with embryo work; S. Cantaloube for the 3D-FIED macro; I. Brito and A. Woolfe for help with statistical analysis; A.V. Gendrel, K. Ancelin, E. Heard, and M. Wossidlo for critical readings of the manuscript; and members of UMR218 for helpful discussions. The group of G.A. is supported by the DEEP Labex, and the Institut Curie is a member of Paris Sciences and Letters (PSL) Research University. This work was supported by La Ligue Nationale Contre le Cancer (Équipe Labellisée Ligue 2010), PIC Programs, the European Commission Network of Excellence EpiGeneSys (HEALTH-F4-2010-257082), and ERC Advanced Grant 2009-AdG_20090506 "Eccentric." M.C. was supported by the ERC "Eccentric" and the Institut de France (Cino et Simone del Duca Fondation Special Scientific Prize attributed to G.A.). M.P. was supported by an EMBO Short Term Fellowship, and A.V.P. was supported by ANR JCJC Dynam'Het.

Received: January 3, 2013
Revised: July 2, 2013
Accepted: August 8, 2013
Published: September 19, 2013

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