

Partitioning of Transposon-Rich Maternal DNA is a Prerequisite for Embryonic Gene Activation

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Abstract

The maternal-to-zygotic transition (MZT) marks a fundamental shift in embryonic control from maternally supplied factors to zygotic gene activation, a process critical for vertebrate development. Contrary to the long-standing view that gene promoters are the earliest transcriptional targets, we demonstrate that maternally inherited transposon-rich repetitive DNA regions in zebrafish embryos are initially transcriptionally active and bound by RNA Polymerase II (Pol II). Following fertilization, these repetitive regions undergo progressive chromatin compaction, directed by the histone methyltransferase G9a, which releases Pol II to relocate from intergenic loci to gene promoters, enabling robust activation of developmental genes. Loss of G9a disrupts this process, causing persistent Pol II binding at repetitive elements and delaying activation of embryonic genes. Our findings redefine vast genomic repetitive DNA regions as a collective regulatory sponge that is essential for timely gene activation and reveal a novel mechanism by which maternal chromatin partitioning governs embryonic developmental progression.

Introduction

After fertilization, genomes from sperm and egg come together to generate the zygotic genome. This union establishes a single diploid genome that must be extensively reprogrammed to support totipotency and subsequent stages of embryonic development. A wave of chromatin reprogramming then ensues, permitting zygotic genome activation (ZGA) (1–3), a developmental stage at which transcription initiates from the embryonic genome rather than relying on maternally deposited transcripts. Control of cellular programming switches from maternal, by way of deposited mRNAs and proteins, to zygotic, via activation of genic transcription (1–3). This transition represents a fundamental handoff in regulatory control that is conserved across metazoans. Robust gene activation during MZT is critical to pluripotent stem cell formation and embryonic development (1–3).

Our previous work in zebrafish found that Placeholder nucleosomes, characterized by histone variant H2A.Z and specific post-translational modifications, occupy developmentally regulated promoters and enhancers to prevent the accumulation of repressive chromatin and permit future activation of genes (4, 5). These nucleosomes serve as molecular placeholders that maintain regulatory potential prior to the onset of transcriptional activation. While the paternal zebrafish genome arrives in fertilized embryos in a chromatin state primed for genic activation, the hypomethylated maternal genome must undergo extensive epigenetic reprogramming (6, 7). Placeholder nucleosomes are critical to preserve locations that will undergo activation (promoters or enhancers), but one key aspect remains unresolved – *how does the remaining portion of the genome remain silent during ZGA?* Given the intrinsic capacity of Pol II to promiscuously engage at cryptic transcription start sites (8–17), some chromatin partitioning mechanism(s) must restrict Pol II access to non-genic regions and ensure faithful Pol II occupancy at genes during ZGA. Recognized repressive chromatin marks – such as H3K9me3 and H3K27me3 – are absent or extremely low in zebrafish prior to ZGA (5, 18–30), indicating that canonical heterochromatin-based silencing mechanisms are not yet established and an alternative silencing mechanism must be involved.

We set out to determine how the maternal genome in zebrafish embryos becomes partitioned into active and repressed domains, shedding light on how Pol II is directed toward genes while being excluded from the remainder of the genome. We identified two distinct yet concurrent chromatin reprogramming processes occurring during MZT. Gene promoters became more open and accessible for Pol II loading, while thousands of intergenic regions underwent progressive compaction. Intergenic regions which became more closed-off were comprised largely of **repetitive DNA elements (REs)**, including transposon sequences and satellite DNAs. Unexpectedly, these regions were bound by Pol II before robust genic activation took place. As chromatin compaction progressed, Pol II was redistributed to genes, coinciding with their expression. This transition was dependent on the histone methyl-transferase G9a (also known as Ehmt2) (31, 32). G9a is known to catalyze the deposition of the repressive histone mark H3K9me2. In **G9a-deficient embryos**, Pol II remained bound at REs longer during MZT, and failed to accumulate at genes, leading to diminished transcriptional activity. These outcomes are indicative of causal relationship between chromatin compaction of intergenic repetitive domains and productive gene activation.

Results

We first assessed chromatin accessibility changes during MZT, by reanalyzing published data generated with the Assay for Transposase-Accessible Chromatin Sequencing (ATAC-Seq) at early MZT (256-cell) and late MZT (Sphere) stages, which occurs before and after ZGA onset (33). ATAC-Seq uses a transposase to insert sequencing adapters into regions of open (accessible) chromatin, commonly serving as a proxy for transcription factor binding and gene activation. Consistent with previous studies (33–36), gene promoters became significantly more accessible during MZT (as compared with background regions) ($p\text{-value} = 10^{-10}$, **Fig. 1A and 1B**). Analysis of nascent Click-iT RNA-Seq data (37) further demonstrated that the most highly accessible promoters also exhibited the highest level of nascent transcription during late MZT (**Fig. S1A**).

We next employed an unbiased 10-kb-tiling approach, dividing the entire zebrafish genome into equal 10-kb genomic regions, to assess genome-wide chromatin dynamics (**Fig. S1B**). This strategy allows for a systematic and unsupervised genome-wide survey of changes in accessibility, independent of DNA context. Consistent with our gene-specific analyses (**Fig. 1A and 1B**), regions which became more accessible, hereafter referred to as “zygotically established” accessible regions, were comprised primarily of promoters, exons, and introns (**Fig. 1D**). Unexpectedly, thousands of genomic regions exhibited the opposite pattern, with initially high accessibility and subsequent compaction during MZT. We refer to these as “initially accessible” regions. Intriguingly, initially accessible regions were enriched for intergenic sequences including REs, especially Long Terminal Repeats (LTRs), Satellite DNAs (Sat DNAs), and low complexity repeats, when compared to randomized background regions (**Fig. 1D & 1E**). In contrast to genes, these RE-rich regions were more accessible during early MZT, a stage when the zebrafish genome is thought to be transcriptionally silenced.

Maternally inherited repetitive DNA is accessible in early embryos

To investigate whether the initially accessible regions were inherited maternally or paternally, we performed *in vitro* fertilization of isolated gametes to generate reciprocal crosses of hybrid zebrafish/medaka embryos (**Fig. S1B**), which undergo MZT normally (38, 39). The large evolutionary distance of 115-200 million years separating zebrafish (*D. rerio*) from medaka (*O. latipes*) (40) enables bioinformatic separation of sequencing reads corresponding to each discrete genome, and by assessing hybrid embryos from reciprocal crosses, we were able to track the zebrafish genome within either the maternal or paternal-specific chromatin landscapes. Unlike the paternal genome, the maternal genome undergoes dynamic epigenetic reprogramming during MZT (6, 7). Accordingly, we found that initially accessible regions were significantly more accessible on the maternal than paternal genome in early MZT embryos (p-value = 10^{-16} , **Fig. 2A**). This was also the case for gene promoters, which were inaccessible on the paternal genome prior to ZGA and then gained accessibility as MZT progressed – unlike randomized background control regions (**Fig. S1C**). We next performed RE-specific analysis of allele-specific accessibility, focusing on uniquely named RE subclasses (**Fig. S1D**). Interestingly, specific LTRs (such as Helitron, DIRS, Crypton/LRS, Copia and Gypsy), Sat DNAs (such as Sat-1, Sat-2 and Sat-5), and LINEs (such as L1 and L2) were found to be enriched within initially accessible regions and were more accessible on the maternal genome than the paternal genome (**Fig. 2B-D and S2A, S2B**). We hereafter referred to these REs as “initially accessible REs” (**Fig. 2E**).

Re-analysis of published Click-iT nascent RNA-Seq data from mid-MZT embryos (1000-cell stage, before ZGA onset) (37) revealed that initially accessible REs were more highly transcribed than protein-coding regions (p-value = 2.76×10^{-13} , **Fig. S2C**). Moreover, the total number of reads from nascent transcripts (Click-iT-Seq) mapping to initially accessible regions was substantially higher than reads mapping to either zygotically established accessible regions, or to gene promoters (**Fig. S2D**), and nascent transcript levels from REs was positively correlated with chromatin accessibility levels (Pearson correlation = 0.46) (**Fig. S2E**). Additionally, RE nascent transcription levels were correlated with total RE transcript levels in oocytes and early MZT embryos (Pearson correlation = 0.94 and 0.92, respectively, **Fig. S2E**). In late MZT embryos, these same REs became progressively less accessible, and corresponding transcripts (re-analysis of total RNA-Seq data (41) and small RNA transcripts (42)) decreased significantly (p-value = 0.03, **Fig. S2F and S2G**). Collectively, these results indicate that maternally inherited REs are initially accessible and nascently transcribed before the major wave of ZGA – representing an early, non-genic phase of transcription that occurs independently of promoter-driven expression – during a developmental period when genic transcription is markedly absent.

RNA Polymerase II binding transitions from maternally accessible repetitive regions to genes during MZT

We next asked whether changes in Pol II engagement occurred in accordance with chromatin accessibility dynamics during MZT. We anticipated that Pol II would accumulate over gene promoters during late MZT. Using CUT&RUN (43, 44) (**Fig. S3A-D**), we found this to be precisely the case (**Fig. 3A**). CUT&RUN provides a high-resolution mapping of protein-DNA interactions with low background, allowing for precise detection of Pol II occupancy at discrete locations. In contrast to gene promoters, Pol II localized to initially accessible REs before ZGA in early MZT embryos, paralleling our nascent RNA-Seq

results (**Fig. 3B-C**). Initially accessible REs exhibited significantly higher Pol II binding in early MZT embryos compared with late MZT embryos (p-value = 0.02, **Fig. 3B**) or with either all REs or control regions in early MZT embryos (p-value = 10^{-8} and 10^{-16} , respectively **Fig. 3B**). Pol II-occupied REs were significantly more accessible in early MZT embryos compared with all REs (**Fig. S3E**), and changes in accessibility were correlated with changes in Pol II engagement (Pearson correlation = 0.54, **Fig. 3D**). Pol II-occupied REs included several types of LTRs, Satellite DNAs and LINEs (p-value = 0.02, **Fig. 3E-F, S3F, S3G**), and they tended to be evolutionarily younger than REs not bound by Pol II (**Fig. S3H**).

Interestingly, HOMER Motifs enrichment analysis identified several DNA sequence features enriched within Pol II-bound regions during MZT. In the late MZT embryos, enriched sequences corresponded with transcription factor (TF) motifs for SOX, FOX, TBX, and POU TF families – TFs known to promote genic transcription at later developmental stages (**Fig. S3I**) (45–55). In early MZT however, Pol II-occupied regions are not enriched for any specific TF motifs, and instead possessed degenerate TATA nucleotide combinations, consistent with promiscuous Pol II engagement at “promoter-like” intergenic loci. (**Fig. S3I**). These results support the possibility that early Pol II binding at REs is somewhat permissive and promiscuous rather than TF-directed.

We also performed immunofluorescence and found Pol II diffusely localized within nuclei in early MZT stage embryos (**Fig. 3G**), while in late MZT Pol II occurred within discrete puncta, consistent with prior reports (56, 57) (**Fig. 3G**). Altogether, our findings indicate that Pol II is diffusely distributed and promiscuously bound to intergenic initially accessible REs in early in MZT embryos, but at later stages it becomes localized toward genes, coincident with their transcriptional activation.

H3K9me2 accumulation correlates with repetitive element repression during MZT.

We and others previously demonstrated that certain repressive chromatin marks are lacking before ZGA, including H3K27me3, and H3K9me3 (5, 18, 19, 23) and that DNA methylation levels are low over the maternally inherited genome (5–7). Thus, existing literature does not provide a mechanism for how during this early developmental period RE-rich intergenic regions undergo chromatin compaction.

Studies of mouse oocytes and embryos indicate that histone methyltransferase G9a may be involved in RE repression in early development (32, 58, 59). G9a deposits H3K9me2, a repressive mark (25–27, 31), and a preprint manuscript has reported low-to-moderate levels of H3K9me2 starting at the one-cell stage in zebrafish (60). We therefore speculated that G9a (via H3K9me2 deposition) may be involved in RE silencing and chromatin partition during zebrafish MZT. To investigate this possibility, we performed CUT&Tag (**Fig. S4A & S4B**) (43, 61) and immunofluorescence staining for H3K9me2 in early zebrafish embryos (**Fig. 4A-D**). Indeed, abundant H3K9me2 was detectable during early and late stages of MZT, with significantly higher enrichment over initially accessible REs compared with all REs, gene promoters or randomized background regions of the genome (p-value = 10^{-5} , **Fig. 4A**). Although levels of H3K9me2 was not significantly different for initially accessible REs when we compared overall enrichment in early and late MZT stages, (as measured by CUT&Tag), increases were observed for several RE subtypes, such

as BRSAT and Helitron, and Gypsy28 elements (**Fig. 4B & 4D, Fig. S4C**). Moreover, REs harboring high H3K9me2 levels in early MZT embryos tended to acquire elevated H3K9me3 levels following ZGA (**Fig. S4D**), indicating that such elements became stably silenced as MZT progressed. Total nuclear H3K9me2 level (as quantified by immunofluorescence) also significantly increased during MZT ($p\text{-value} = 1.9 \times 10^{-29}$, **Fig. 4C**). Together, these results indicate that H3K9me2 is present over initially accessible REs before ZGA, and total H3K9me2 accumulates further as these REs undergo silencing, potentially serving as a precursor to full silencing and stable heterochromatin formation.

Maternally provided G9a is necessary for proper Pol II redistribution from repetitive intergenic regions to gene promoters during MZT

In vertebrates, G9a is considered the primary histone methyltransferase that catalyzes the establishment of H3K9me2 repressive chromatin (31, 62). We therefore hypothesized that RE repression during MZT may occur by maternally provided G9a, ultimately facilitating the release of RNA Pol II from REs in later stage MZT embryos. To investigate this, we generated a CRISPR-based knockout of G9a (also known as Ehmt2) (63, 64), allowing us to disrupt H3K9me2-dependent chromatin silencing of repetitive regions, and potentially prolong the initial phase of RE-centric Pol II occupancy during MZT. Animals homozygous for the knockout allele survived to adulthood. Embryos derived from G9a-deficient female adults, maternal-deficient G9a (MdG9a), underwent the MZT normally, without obvious morphological defects (**Fig. S5A and S5B**). However, they exhibited significantly reduced levels of H3K9me2 compared with wildtype embryos during early and late MZT stages ($p\text{-value} = 10^{-10}$ and 4.3×10^{-86} , respectively, **Fig. 5A and 5B**). Despite several attempts as well as success in WT embryos, CUT&Tag or CUT&RUN strategies consistently failed to detect H3K9me2 enrichment in MdG9a embryos, consistent with the overall reduction in total H3K9me2 observed from fluorescence levels.

The MdG9a embryos thus allowed us to test whether failure to establish repressive H3K9me2 leads to failed RE suppression, as well as prolonged enrichment of Pol II at REs. We therefore assessed Pol II enrichment via CUT&RUN in these embryos (**Fig. S5C & S5D**). Unlike WT embryos, in which REs' Pol II binding was initially high and then decreased during MZT ($p\text{-value} = 0.009$, **Fig. 5D**), for MdG9a embryos, Pol II enrichment remained high over initially accessible REs, even in late MZT ($p\text{-value} < 10^{-16}$, **Fig. 5D**). Impacted REs included numerous Gypsy-class elements, DIRS, and Sat-5 elements (**Fig. 5C, 5E and S5E**). We also employed our unbiased genome-wide 10-kb-tiling approach, in which the zebrafish genome was divided into 10-kb regions. Comparing MdG9a to WT embryos, we identified sites exhibiting either an overall significant increase (5,107 regions) or decrease (3,403 regions) in Pol II binding. In particular, regions which lost Pol II throughout MZT progression in WT, tended to retain aberrantly high levels in MdG9a mutants at later stages of MZT, and regions which normally gain RNA Pol II exhibited significantly reduced levels of Pol II engagement (**Fig. 5F-G**). Regions retaining aberrantly high levels of Pol II were comprised primarily of intergenic and repetitive DNA sequences, whereas the regions that fail to acquire Pol II contained a relatively large number of genic elements, including promoters, introns, and exon sequences (**Fig. S5F**). Much like the Pol II binding sites we identified in WT early MZT embryos, genomic regions exhibiting ectopic accumulation of Pol II in MdG9a mutants during late MZT were not

enriched for any specific TF motifs and possessed degenerate TATA nucleotide combinations (**Fig. S5G**) – consistent with persistent promiscuous Pol II engagement. Taken together, these data indicate that MdG9a embryos maintain abnormally high levels of Pol II occupancy at intergenic and repetitive regions, and these embryos also failed to establish appropriate levels of Pol II binding at genes and their promoters during late MZT. These outcomes suggest that G9a-dependent H3K9me2 deposition is required to shift Pol II occupancy from non-genic repetitive regions (during early-MZT) to gene promoters (at later MZT stages).

Failure to acquire Pol II occupancy at genes is associated with disrupted genic activation

Focusing our subsequent analyses on genes, we next found that the majority of Pol II-bound genes in WT embryos failed to acquire sufficient Pol II occupancy in MdG9a embryos even at late MZT stages (p-value < 10^{-16} , **Fig. 6A and 6B**), exemplified by several individual genes that are known to become transcriptionally active from the zygotic genome during MZT, such as *nanog*, *cldne*, and *sox19b* (**Fig. 6C**). Immunofluorescence analysis was consistent with these observations: at late MZT after ZGA, WT embryos exhibited Pol II puncta within the nucleus, but significantly fewer puncta formed in MdG9a embryos (p-value = 1.7×10^{-87} , **Fig. 5A and 5B**). We next performed total RNA-Seq analyses to investigate impacts on transcription (**Fig. S6**). To specifically assess zygotic gene activity, we created a comprehensive list of embryonically activated genes during wild-type MZT. This was done by analyzing published Click-iT nascent RNA-Seq from late MZT embryos (37) and defining low relative read-count-thresholds (see Methods). Remarkably, we found that the majority of differentially expressed embryonic genes (523 out of 565 genes) failed to become transcriptionally active in MdG9a embryos (**Fig. 6D**). This outcome was exacerbated further when we examined zebrafish embryos deficient for both maternal and zygotic contribution of G9a (MZG9a). Among 1,482 differentially expressed genes in MZG9a mutants, 1,384 (94%) were downregulated compared to WT (**Fig. 6E**). Overall, our Pol II CUT&RUN, immunofluorescence and RNA-Seq data support the hypothesis that repression of intergenic and repetitive regions by G9a ensures transcriptional fidelity and permits robust levels of genic expression during zebrafish MZT.

Discussion

From decades of research, the zebrafish community has concluded that the major wave of ZGA commences approximately 3.5 hours after fertilization, with a minor wave occurring slightly earlier (2hpf) for a hand-full of genes (1, 2, 50, 56, 65). This view has taken shape largely based on studies of transcriptional activators, leading to a framework in which discrete developmental and molecular switches guide gene activation events (1–3, 37, 48, 50, 52, 66). Our study challenges this long-standing model by demonstrating that transcription initiation of intergenic repetitive loci begins substantially earlier than previously understood – before robust genic activation occurs. We there propose an alternative model, in which genome-wide chromatin partitioning is required – subdividing the genome into an accessible or inaccessible regions – for proper genic transcription to occur.

We show that the unpartitioned maternally inherited genome possesses intergenic repetitive regions which recruit RNA Pol II through promiscuous binding. This pervasive Pol II occupancy constitutes an early, non-genic form of transcriptional initiation that precedes promoter-driven gene expression. Thus, while embryonic genes remain mostly silent until later stages of MZT, transcription from REs is already underway. As a result, the onset of RE-centric transcription is not a consequence of ordered TF-driven recruitment mechanisms, which occurs for genes, and instead, transcriptional initial begins as an unguided process in which Pol II takes the “path of least resistance”, and chromatin partitioning is necessary to carve the appropriate path forward.

In early MZT, we find that Pol II does not preferentially associate with promoters and is instead distributed broadly across the genome landscape. Repetitive elements – due to their abundance and accessibility – become the first genomic regions to engage Pol II. Our results position this early, repetitive-element-centric Pol II binding phase of MZT as having a mechanistically critical function: to prevent premature transcription of zygotic genes while epigenetic reprogramming of the parental genomes is occurring. Because Pol II is inherently promiscuous, gene promoters must compete with the remaining portion of the genome for engagement, which includes a vast number of accessible transposons and repeats. During early MZT, before proper partitioning of REs into repressive chromatin occurs, competition for Pol II is dominated by accessible REs, resulting in sequestration away from genic promoters. Accordingly, protein-coding genes remain Pol II-depleted and transcriptionally inactive in early MZT embryos. In this way, early spurious transcription at repetitive domains in early MZT imposes the repression of zygotic gene expression which has been previously observed but lacked a comprehensive explanation (1–3, 67). Our model is also consistent with prior studies in which decreasing nuclear concentrations of core histones and increasing levels of transcription factors and/or nuclear-cytoplasmic ratios help to overcome silencing (by better competing against REs) to facilitate genic transcription during ZGA (68–76).

We also identify maternally provided G9a as required for re-localization of Pol II from REs to genes. We find that maternally provided G9a deposits H3K9me2 across vast regions of intergenic repetitive-element-rich DNA, leading to the establishment of semi-repressive chromatin domains notably before genic robust activation occurs. This G9a-dependent chromatin partitioning is therefore required for genes to exhibit robust Pol II loading and transcriptional initiation. Genome partitioning through maternal H3K9me2 ensures that transcription is directed toward gene promoters rather than intergenic repetitive elements, a critical step in ensuring developmental fidelity. Our study suggests that what has been traditionally described as the “onset of ZGA” is, in fact, a *secondary* phase of Pol II engagement devoted primarily to genic promoters – the remaining portion of the genome that is not subject to G9a-dependent silencing.

Our revised model is consistent with other observations across metazoans. In *Drosophila*, mice, and humans, repetitive elements are among the first transcripts detected, yet the functional significance of this early activation has remained only partially understood. Our findings provide a mechanistic explanation: early repetitive element transcription emerges naturally from accessible maternal chromatin

landscape, which functions to suppress premature zygotic gene activation. Only after these non-genic regions are compacted and partitioned away can promoter-driven robust genes activation occur.

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Figures

Fig 1.

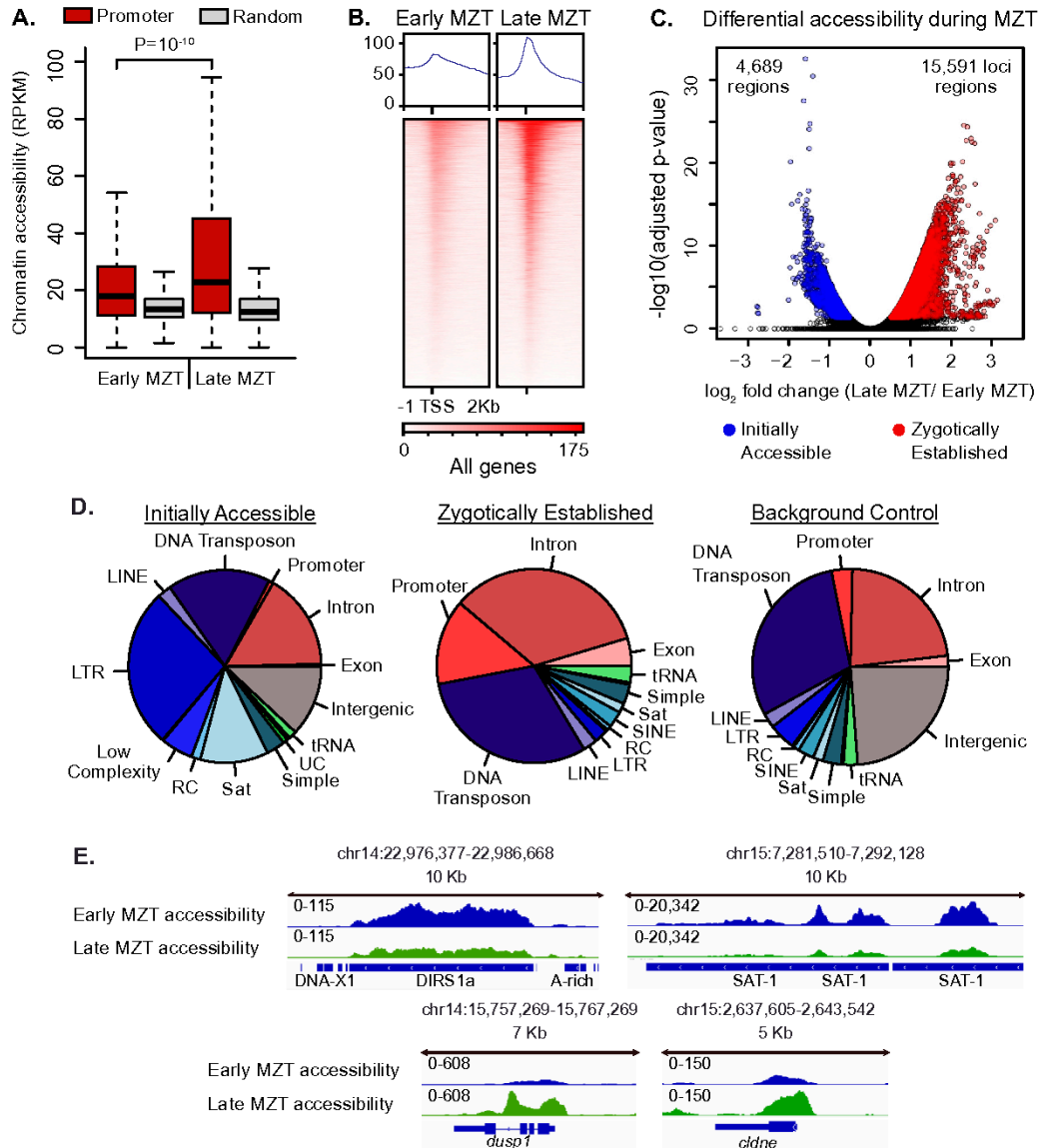


Figure 1

Chromatin accessibility establishment at genes and compaction at repetitive elements during the maternal-to-zygotic transition (MZT).

A. Chromatin accessibility (RPKM) at all zebrafish gene promoters ($n = 38,134$) and random background regions ($n = 50,000$) during early MZT (2–2.5 hpf) and late MZT (4 hpf). Pairwise Wilcoxon rank-sum test, $p\text{-value} = 1.5 \times 10^{-10}$.

B. Heatmap of chromatin accessibility (RPKM) at transcription start sites (TSSs) of all genes during early and late MZT.

C. Genome-wide changes in chromatin accessibility across 10-kb genomic bins when comparing late MZT/early MZT embryos. Initially accessible regions that lose accessibility (adjusted p-value < 0.05 and $\log_2FC < -0.5$, total 4,689 regions) are shown in blue and zygotically established regions (adjusted p-value < 0.05 and $\log_2FC > 0.5$, total 15,591 regions) that gain accessibility are shown in red.

D. Genomic annotation of initially accessible regions, zygotically established regions, and background control regions. Promoter (TSS ± 1 kb), introns, exons, intergenic regions (annotated with neither genic or RE features), transfer RNA (tRNA), unclassified repeats (UC), simple repeats, satellite DNA (Sat), short interspersed nuclear elements (SINE), rolling-circle transposons (RC), low complexity repeats, long terminal repeats (LTR), long interspersed nuclear elements (LINE), and DNA transposons are indicated.

E. IGV browser views of chromatin accessibility (RPKM) at initially accessible REs during early (blue) and late (green) MZT in the top row, and zygotically activated gene promoters in the bottom row.

Fig 2.

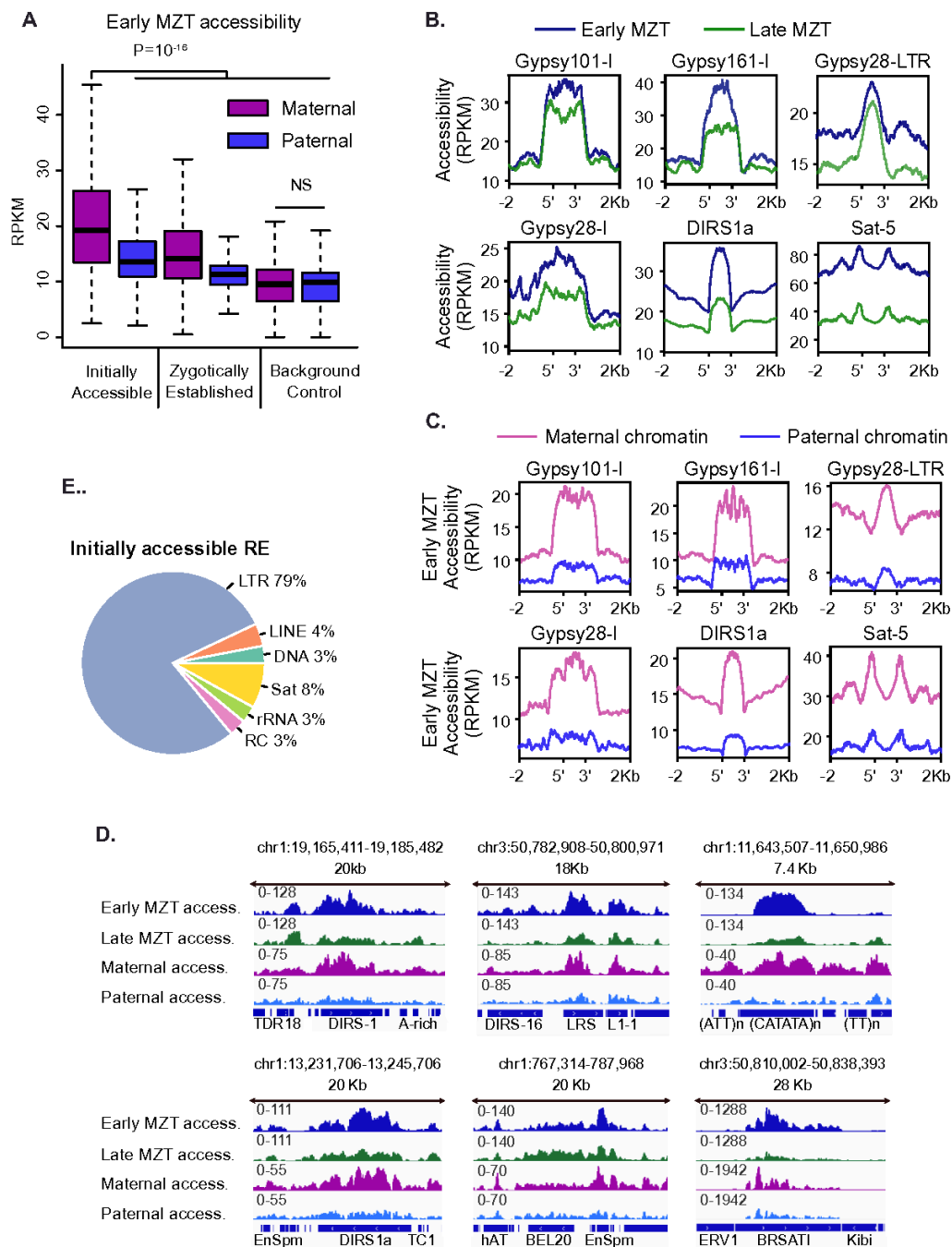


Figure 2

Initially accessible REs reside within the maternally inherited genome

A. Parental-specific chromatin accessibility (RPKM) at initially accessible regions, zygotically established regions, and background regions during early MZT. Wilcoxon rank-sum tests show significantly higher maternal accessibility at initially accessible regions (p -value = 10^{-16}).

B, C. Examples of chromatin accessibility (RPKM) at initially accessible REs during early and late MZT (**B**) and parental-specific accessibility (RPKM) during early MZT (**C**). Accessibility was averaged across all RE genomic copies and their ± 2 -kb flanking regions.

D. IGV browser views of chromatin accessibility (RPKM) at initially accessible REs during early (blue) and late (green) MZT, and parental-specific accessibility during early MZT (maternal and paternal accessibility highlighted in purple and light blue, respectively).

E. Proportion of RE classes comprising initially accessible REs are displayed as a pie chart.

Fig 3.

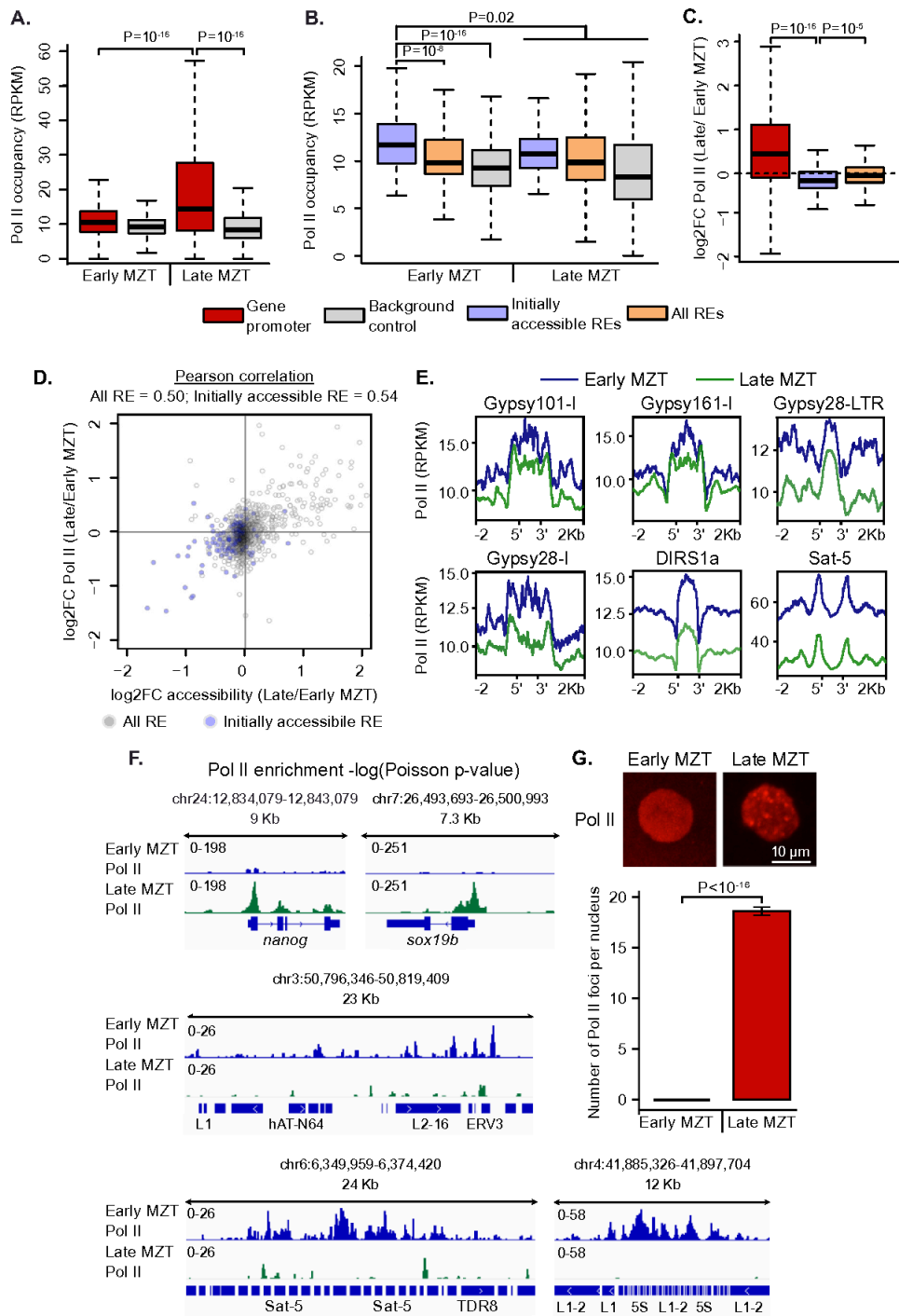


Figure 3

Transcriptionally competent RNA polymerase II occupies repetitive elements prior to genic activation.

A. Boxplot of Pol II occupancy (RPKM) at gene promoters during early and late MZT. Wilcoxon rank-sum tests compare late versus early MZT and randomized background regions (p-value = 10^{-16} and 10^{-16}).

B. Pol II occupancy at initially accessible REs decreases during MZT. Initially accessible REs are

significantly enriched for Pol II occupancy compared with all REs and randomized background regions in early MZT (p-value = 10^{-8} and 10^{-16}) and show reduced occupancy in late MZT (p-value = 0.02). Pairwise Wilcoxon rank-sum tests.

C. Log₂ fold change in Pol II occupancy (late MZT/early MZT embryos) at gene promoters, initially accessible REs, and all REs. Pol II occupancy change during MZT at initially accessible REs is significantly different from that at all genes and all REs (p-value = 10^{-16} and 10^{-5} , pairwise Wilcoxon rank-sum test).

D. Pearson correlation between changes in chromatin accessibility and Pol II occupancy during MZT. All REs are in dark grey (R = 0.50) and initially accessible REs are highlighted in blue (R = 0.54).

E. Examples showing reduced Pol II occupancy (RPKM) at initially accessible REs during MZT.

F. IGV views of Pol II establishment at genes (e.g., *nanog*, *sox19b*) and Pol II loss at initially accessible REs.

G. Representative Pol II immunostaining during early and late MZT and quantification of Pol II foci per nucleus (p-value < 10^{-16} , two-tailed Student's *t*-test).

Fig 4.

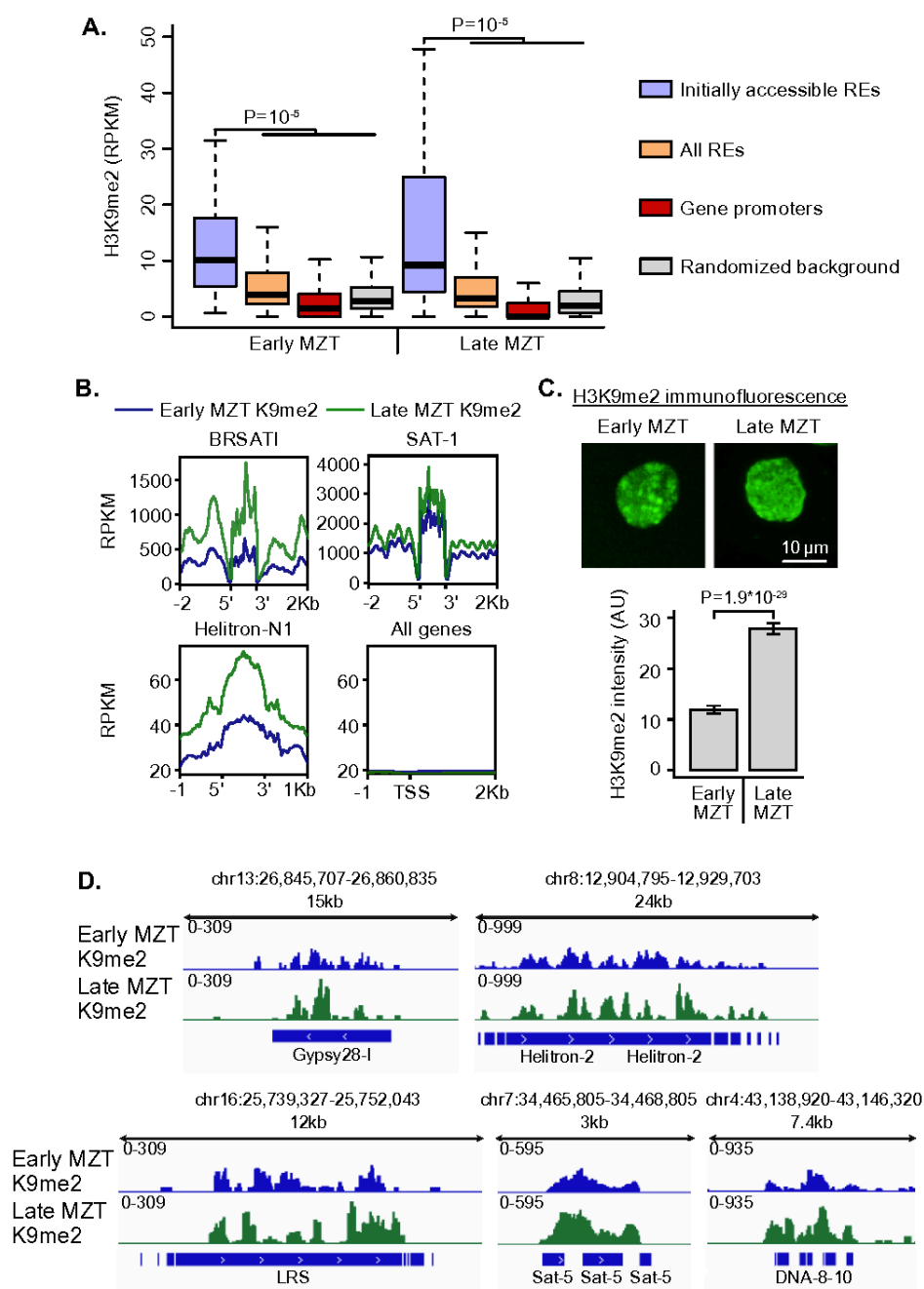


Figure 4

H3K9me2 marks repetitive elements destined to be stably repressed in late MZT.

A. Boxplots of H3K9me2 levels (RPKM) across genomic features during early and late MZT. H3K9me2 is significantly enriched at initially accessible REs (p -value = 10^{-5} , pairwise Wilcoxon rank-sum test).

B. Average H3K9me2 levels at select examples of initially accessible REs, displayed as profile plots,

- compared with all genes.
- C. H3K9me2 immunostaining and quantification during early and late MZT (p-value = 10^{-29} , two-tailed Student's *t*-test).
- D. IGV view showing maintenance of H3K9me2 at initially accessible REs during MZT.

Fig 5.

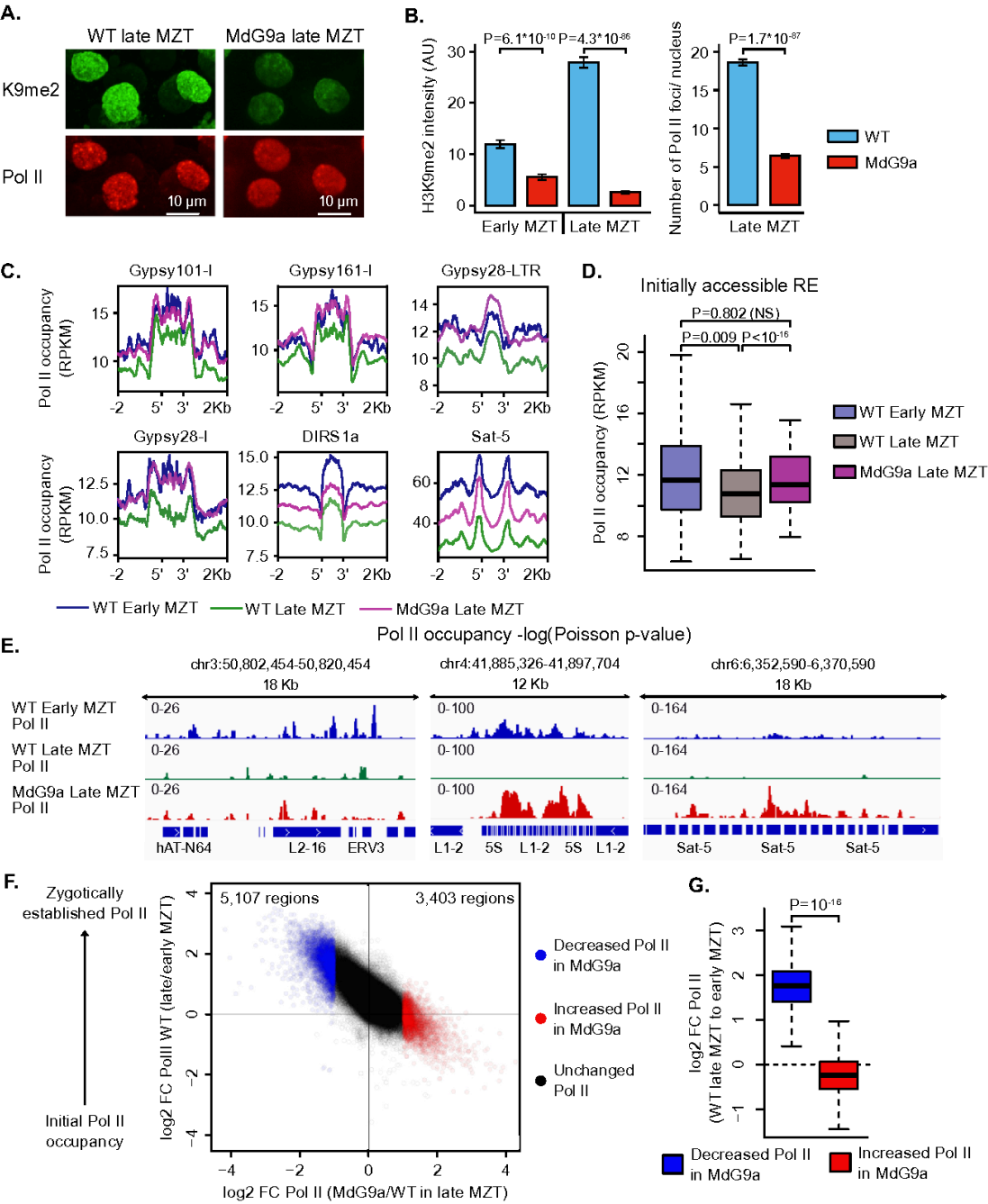


Figure 5

Maternal loss of G9a causes Pol II retention at repetitive elements and impaired genic localization.

- A.** Immunofluorescence staining of H3K9me2 (K9me2) and Pol II in WT and MdG9a embryos at late MZT.
- B.** Quantification of H3K9me2 intensity and Pol II foci per nucleus. Two-tailed Student's *t*-tests show significant H3K9me2 depletion and reduced Pol II foci in MdG9a embryos.
- C.** Pol II occupancy at selected initially accessible REs in WT and MdG9a embryos, displayed as profile plots.
- D.** Quantification of Pol II occupancy at initially accessible REs, displayed as boxplots. Pairwise Wilcoxon rank-sum tests indicate Pol II retention in MdG9a embryos.
- E.** Genome Browser view of Pol II enrichment from CUT&Run, $-\log(\text{Poisson } p\text{-value compared with negative control})$.
- F.** Scatterplot comparing Pol II changes during WT development with changes in MdG9a embryos.
- G.** Boxplot comparing Pol II occupancy changes (WT late MZT/early MZT embryos) of genomic regions with altered Pol II occupancy in MdG9a embryos ($p\text{-value} = 10^{-16}$, pairwise Wilcoxon rank-sum test).

Fig 6.

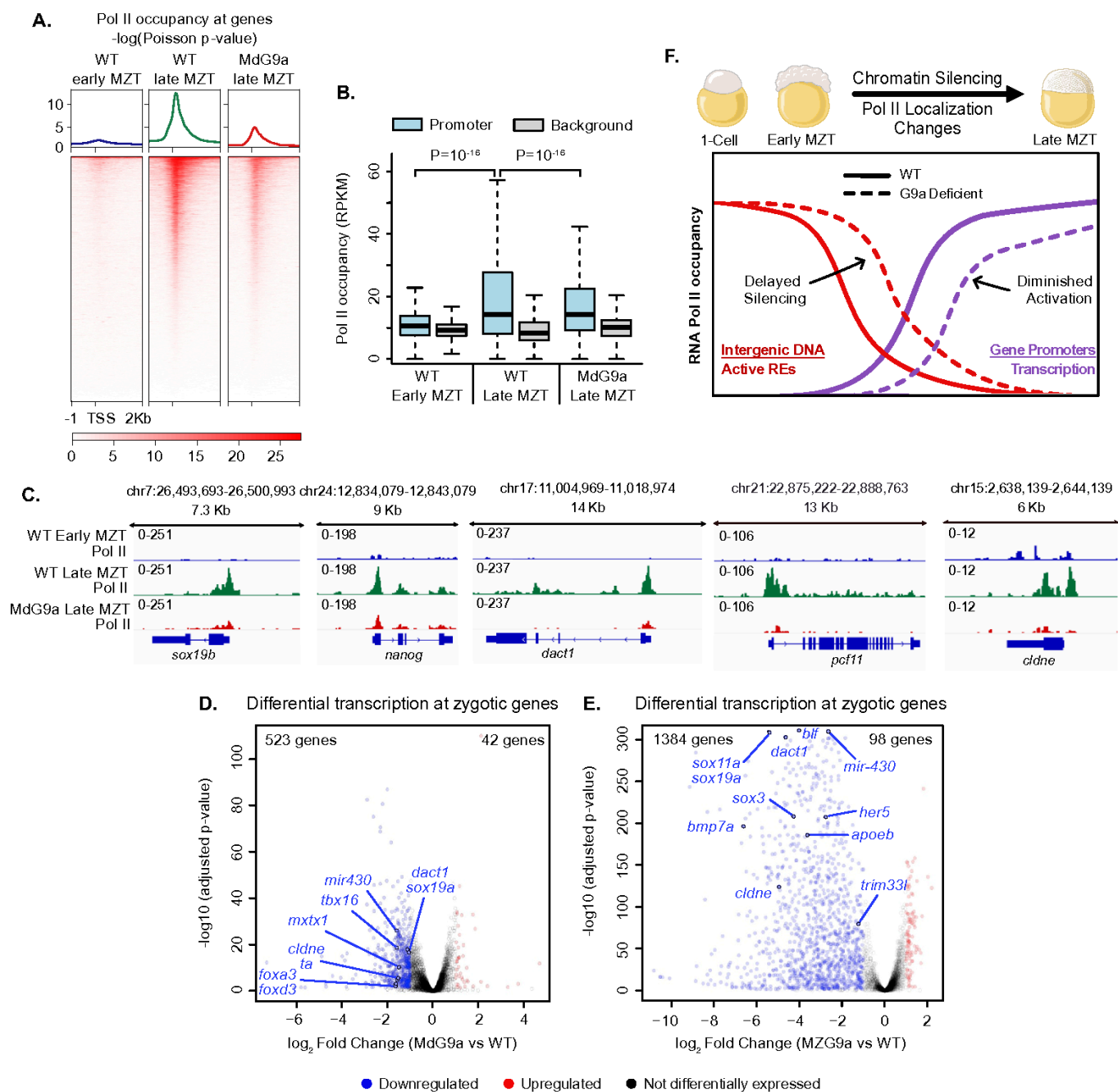


Figure 6

Failed Pol II localization to genes is associated with reduced zygotic transcription in G9a mutants.

A. Heatmaps depict Pol II occupancy measured as $-\log_{10}(\text{Poisson p-value})$ at all genes in WT early MZT, WT late MZT, and MdG9a late MZT embryos.

B. Boxplot comparisons of Pol II occupancy (RPKM) at gene promoters (TSS $\pm$ 1 kb) and randomized

background regions in WT early MZT, WT late MZT, and MdG9a late MZT embryos. Pairwise Wilcoxon rank-sum tests compare WT late MZT with WT early MZT and MdG9a late MZT (p-value = 10^{-16} for both).

C. IGV genome browser views of Pol II occupancy ($-\log_{10}(\text{Poisson p-value})$) at representative zygotically transcribed genes in WT early MZT, WT late MZT, and MdG9a late MZT embryos.

D. Differential expression of zygotically transcribed genes in MdG9a versus WT late MZT embryos.

E. Differential expression of zygotically transcribed genes in MZG9a versus WT late MZT embryos. Downregulated genes are shown in blue and upregulated genes in red.

F. Model for repetitive-element-mediated regulation of Pol II binding transition. Pol II initially localizes to accessible repetitive elements in eggs and early MZT embryos. G9a-mediated H3K9me2 deposition silences initially accessible REs, enabling Pol II redistribution to genes for proper zygotic transcription during late MZT. Loss of G9a results in Pol II retention at derepressed REs and reduced genic transcription.

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