

Single-cell TimeLapse-seq reveals MTA is an essential retrotransposon orchestrating mouse zygotic genome activation

Corresponding Author: Professor Shaorong Gao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Wu et al have optimized a low input version of the previously published TimeLapse-seq method. They have utilized this method to elucidate temporal pattern of zygotic genome activation in mice. Wu et al have analysed expression dynamics of transposable elements (TE), focusing on the LTRs, which have previously shown to be expressed in early mouse development and provide cis regulatory elements.

Authors determine three zygotic genome activation waves based on the gene expression dynamics, investigate maternal and paternal transcript expression and then focus on TE expression dynamics and regulation.

We acknowledge the novelty in addressing the roles of MaLRs in mouse ZGA, as well as the role of Sall4 as a potential MTA element regulator. Authors present interesting findings that bring relevant insights to the field, however, major improvements are needed prior to publishing this manuscript.

Major comments

1. Concerning the manuscript structure, we suggest that the Figure 6 results would be moved from the Discussion to the Results section.
2. The clarity of the manuscript text should be improved because at times it is slightly challenging to follow and this may undermine the significance of these data. We have specifically drawn attention to some lines below.
3. Authors state that ASO treatment also affected other MT subfamilies. How were the MTA targeting ASO sequences designed and how their specificity was evaluated?
4. Regarding the MTA anti-sense oligo-treatment, it appears that the treated embryos were collected at late 2-cell stage, while the embryos that resulted from MII oocyte injections, were arrested already at 1-cell stage. Authors should clarify at which stage they measured the knock-down efficiency for each experiment and at which stage they performed actual analyses of these embryos.
5. In case the MTB-derived Nfya, we suggest that authors write about predicted isoform, or experimentally validate the alternative TSS, for instance by cloning the alternative transcript. Authors could also utilize the published Polymerase II data to investigate whether Pol II is accumulated at the predicted TSS.
6. Could MTB-derived Nfya code for protein? We suggest that authors discuss this.
7. Regarding the MTB Nfya knockdown embryos, authors should clarify what they mean by "partially arrested" embryos? Do they mean that all embryos are delayed? This should be clarified and taken into the context of canonical Nfya perhaps being able to compensate the missing MTB Nfya.
8. We suggest that authors discuss the role of MTB-derived Nfya. Why is the expression of the isoform required in early mouse development? What is the function of the MTB-derived Nfya?
9. Authors should expand Discussion to better relate their findings to published literature. We feel that several relevant papers have been cited, however, they have not been discussed in the context of the findings made in this manuscript.
10. Authors have used Sall4 knockout data and show that MTAs are downregulated. However, downregulation is quite modest and therefore, authors should discuss whether Sall4 might, for example, act redundantly with other factors.
11. Results regarding the transcript transfer to cytoplasm are interesting. Authors should also plot TEs to elucidate whether they are also transported to cytoplasm?
12. Authors should discuss potential mechanisms related to MTA silencing and resulting genome accessibility?
13. Authors show (Figure 6) that the motifs are enriched at the internal part and not the LTR of the MTA. Authors could

discuss whether these regulators only activate complete MTA elements and not the solo LTRs which lack the internal parts.
14. Lines 322-324: We think that this statement is slightly bold and should be tuned down.

Minor comments

1. Lines 32-34 (Abstract): Please clarify the phrasing.
2. Lines 184-187: Please clarify the phrasing.
3. Lines 254-258: Please clarify the phrasing.
4. Line 488 (Methods): "unless indicated". Should this be "unless otherwise indicated"?
5. Lines 566-570: Please provide the ASO sequences.
6. Please provide a reference to Kimura two-parameter model.
7. Lines 844-845: Please provide a web address to the Genome Sequence Archive.
8. Code Availability: Please indicate whether original code was produced and where it will be made available (for example Github or Zenodo).
9. Line 289: Please clarify the phrasing (by removing "respectively").
10. Figure 3f: Are these sub families enriched in this analysis? Please show the subfamily TE copy number in the genome.
11. Figure 4c: Which samples are in question?

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)

Understating the transcriptional dynamics during very early development is an exciting and challenging field. In their manuscript, "Sequential Activation of Retrotransposons Regulate Zygotic Genome Activation and Embryo Development," Wu, Shi, Yan and colleagues describe systematic measurements of changes in transcription across ZGA, focusing on the role of retrotransposons. They find interesting expression of TEs from the maternal genome and characterize a specific type of chimeric transcript. Overall, this is a very study, but needs substantial work to better contextualize both the new methodology described here as well as the novel biology.

Major points

- 1) The novelty of the biology is not described with sufficient scholarship. The authors' title and abstract read as if this is the first description of the sequential waves of temporally controlled expression of retroelements, yet this has been known for over a decade. For example, it is known that terminal repeats derived from endogenous retroviruses are temporally controlled (see for example, from 2012, DOI: 10.1038/nature11244), and many related findings have been reported in the intervening years. Similarly chimeric transcripts of the type described here are already described (reviewed in DOI: 10.1016/j.tig.2023.10.010). Findings related to these points have already been extended to human embryos (DOI: 10.1126/science.adv5257). The authors are clearly aware of this, as their text in some places is nuanced and emphasizes the authors new findings using allelic analyses of early transcripts and specific TEs/regulatory factors. The authors need to re-write their manuscript (especially their title and abstract) to consistently and clearly indicate what novel claims they are making. Relatedly, the authors tend to overstate their findings, for example by declaring their results to be "comprehensive," when it is more appropriate to describe them as broad or systematic as there are transcripts and timepoints that have not been fully analyzed.
- 2) The novelty of the methodology also needs to be better contextualized. The authors do a nice job describing their approach to adapt TimeLapse-seq to single-cell analyses, and importantly the authors put considerable effort into the validation of their protocol. Nonetheless, the authors need to provide context for readers who are not aware that 4-thiouridine-based approaches have been previously adapted for single-cell measurements. The authors' contributions to methodology here are significant but need more context.
- 3) The manuscript needs substantial editing. While many of the issues are merely poorly worded sentences (I'm not sure how a "description" can "lack high-sensitivity," for example, see p3 line 63), there are larger issues too. Important claims are garbled, including the final sentence of their abstract, which reads as if the authors are claiming that their study is what inspired retrotransposons to have an essential role in pre-implantation development.

Minor points

- 1) The authors refer to their scTLS-seq measurements as reporting on "nascent transcripts" throughout most of the manuscript except for the first section in the results, where the authors more accurately describe these transcripts as "newly-synthesized" transcripts. With experiments that last 4 hours and beyond, very few of the transcripts are nascent, which is a term reserved for RNA that is still being transcribed.
- 2) The authors refer to their time-course study as "real time" (p5, line 138) which it is not.
- 3) Why is scTimeLapse-seq abbreviated scTLS-seq as opposed to scTLS or scTL-seq?
- 4) The experiment described in Fig. 2d-e and related supplementary figures is confusing as is the authors' interpretation. Shouldn't new transcription in the whole embryo stop after including the inhibitors?
- 5) The steady increase shown in Fig. S3c could be due to an increase in transcription as the authors conclude, but it also could be due to an increase in the T-to-C mutation rate due to changes in the 4SUTP pool.

6) The figures also need editing and increased clarification. For example, there is a typo in Fig 1a “existed RNA.” What units are shown on the y-axis of Fig. 1c? Why is it on a different scale from Fig 1.e?

Reviewer #4

(Remarks to the Author)

Retrotransposon Elements (RTEs), once regarded as “junk” DNA, has recently become a major research focus, with accumulating evidence revealing their importance in development, disease and cancer. After a long period of transcriptional silence during meiosis, early embryos undergo extensive chromatin remodeling and initiate Zygotic Genome Activation (ZGA), ultimately completing the maternal-to-zygotic transition. However, the transcriptional regulation of RTEs during ZGA and early embryogenesis has remained poorly understood, largely due to the lack of low-input, high-efficiency methods for detecting nascent transcript.

In this submission, the authors developed single-cell TimeLapse-sequencing (scTLS-seq), a technology that enables sensitive detection of nascent RNA from trace material. Using scTLS-seq, the authors reveal the temporal activation dynamics of RTEs during ZGA and uncover their regulatory roles in ZGA. These findings are interesting and contribute valuable insights to both the RTEs and the early embryo development fields.

In my view, several aspects of the study would benefit from further clarification or additional experimental support to strengthen the conclusions. I have outlined a series of comments and concerns below, careful revisions are required for improving the quality of this manuscript.

Major comments:

1. Line 115-116: “about 1300 genes detected in IP samples but not in 115 scTLS-seq were low-expressed, likely background noise through IP method. (Supplementary information, Fig. S1g)”. As the total RNA input for scTLS-seq is extremely low, I think the 1,300 undetected low-expressed gene are more likely due to limited sensitivity rather than background noise through IP method. Authors should give more evidence to support that these low-intensity genes are not real nascent genes. Besides I wonder what is the lowest RNA abundance that scTLS-seq is actually capable of detecting? In addition, in Fig. S1g, for genes within the same RPKM range, are the sets of genes detected by 4sU-seq and scTLS-seq the same? How high is the overlap between them?
2. Line 123-124: “Testing different concentrations led us to determine that a 2mM 4sU treatment for 4h is optimal for nascent RNA labeling in early embryos...” The 4sU concentration used (2mM) is relatively high, and the treatment duration (4 hours) is long. Therefore it is important to assess whether such conditions might disturb the homeostasis of oocytes and early embryos. The authors should provide the IVM and developmental rates under 2mM 4sU treatment. Furthermore, for the 4sU concentration gradient, 2mM is 20-fold higher than 100μM, it would be valuable to know whether the effects of 1mM 4sU exerts effects comparable to 2mM.
3. Line 146 and Figure 1g. It appears as though there is roughly double the amount of nascent transcription occurring at GV compared to PN3. Is this accurate? It suggest that the GV oocyte has detectable and relatively (to PN3 and PN5) higher translation. How does this compare to previous methods? I’m under the assumption that that the level was too low to detect, or at least lower than Minor ZGA. Could this mean in PN stages, transcripts are made and quickly degraded? Also, GV-stage oocytes can be classified into Surrounding Nucleus (SN) and Non-Surrounding Nucleus (NSN) based on chromatin condensation. Meiosis in SN GV oocytes is expected to be transcriptionally silent. It is unclear whether the authors separated SN and NSN oocytes, and whether all sequenced oocytes were NSN. Furthermore, in Fig. S2a, the T > C mutation ratio in 0μM group appears higher than in the 100 μM group, raising the concern that the treatment itself may introduce mutations. Therefore, an MII group treated with 2mM 4sU would help to further validate the robustness of scTLS-seq.
4. Line 217: Does the PWK reference genome have a sufficient assembly (T2T?) for this analysis? Could this factor into why Maternal (B6) reads are more abundant? The detection sensitivity can be influenced by the number of informative SNPs present in the paternal and maternal genomes. The authors should clarify whether the numbers of detectable SNPs from the two parental genomes are comparable, and the reciprocal cross would clarify this.
5. Line 220 to 229: The mapping of TEs is significantly more difficult using this approach. The combination of 4sU along with B6vsPWK is not trivial. Much more information on how this analysis was done is needed. Can you further clarify in lines 676–686?
6. Line 262: Could you go into more detail about how unique mapping was done in the context of T to C mutation as the output of scTLS-Seq? As I understand it, mapping of TEs is initially difficult to achieve with traditional methods, so I would imagine applying this additional analysis was a challenge. How was it handled? the unique mapping procedure in line 647 using the parameter ‘--unique-only’. The methods do not go into sufficient detail.
7. Line 269 to 282: The interpretation of these TEs being cis regulatory units might be premature. The null hypothesis here would be they are simply inheriting the local epigenetic and transcriptional landscape of the nearby gene. The next few sections showing positive correlation could also support the null hypothesis.
8. As clarified by the author and shown in Fig S7c and S7e, the ASO used in this study also target other MT family members because of the sequential similarity. Therefore, the current data are not sufficient to support the conclusion that Transcription of MTA is essential for ZGA. To strengthen this, I recommend performing an MTA rescue experiment to determine whether resupply MTA can rescue early embryonic development.
9. Line 292 and Figure 4C: I am not sure the figures shows this. The KD just shows the MTA_Mm-int and MTA_Mm in the main figure while S7a-c just shows MT-INT goes down (can you also show MTB and MTC LTRs? This would be consistent with the upcoming data on the Nfya section. Or does just the MTB driving Nfya decrease expression (S7e)?

10. Line 323: This result is somewhat unexpected. KD of MTA leads to an arrest, which could suggest other TEs would also be arrested or stalled, but instead you observe premature activation of subsequent TEs, suggesting they take on the TE transcriptional profile of later stage embryos, presumably 4C based on the ERVK levels. Can you speculate as to "how"? The DNASE I-TUNEL assay is not clear to me. I understand DNASE I sensitivity suggests open chromatin and TUNEL should label the newly formed breaks, but how does just TUNEL staining look in both conditions without DNase? The H2AX in S7I has a somewhat downward trend in the MTA-ASO but is N.S. ZGA is associated with opening of male and female pronuclei, which is a fairly quick process after fertilization, with the male pronuclei opening quicker. Might we be seeing the timing of this process altered due to the ASO interference? In the representative image in S7I, the female pronuclei appears "smaller" in the MTA-ASO vs NC-ASO. Can you go back and measure these ratios on the Zygotes used to quantify in S7M?

11. Line 355 and figure 5B: This is a little confusing. MTB appears to be an Oocyte (GV) family, which is supported by the Chimera's expression (Fig5C). The previous results suggested that after MTA KD, an accelerated TE profile was seen (we see 2C and 4C TEs prematurely). I am a little confused how MTB was now being focused on. Was the link meant to be from Chromatin accessibility (from the previous section)? The Chimera expression seems to vanish prior to ZGA. Does the protein persist into 2C (where the chromatin role was reported in line 354)? How do we know this is the Chimera or Canonical action? They appear to be equal to each other in terms of expression (5B and 5C).

Minor comments:

1. In general, there is a lack of telling the number of samples in many experiments, especially in embryo survival graphs (percentages).
2. Abstract:
 - Line 34: Sounds too similar to "RNAi".
 - Line 36: TEs could also be coding. See MERV1-GAG and HERV1-GAG in mouse and human embryos. There are likely many others (RTs, for example).
 - Line 42: ERVK? Are there representative families like muERV1? IAP?
 - Line 48: TE Reprogramming. Is this the standard term? It makes sense to me but I haven't seen it written this way before.
3. Introduction:
 - Line 72: The sentence should have "is": " , which is transiently highly expressed"...
 - Line 73: This is a major motivation for the paper. The LINE and SINE examples should be explained a little bit more.
4. In Fig. 1H/I/J/L/M/N, the GV groups are missing but should be included.
5. Fig. S2 and S3c, this study primarily focuses on ZGA, therefore it is unclear why the figures present the nascent ratio in morula. Furthermore, the morula data are not utilized elsewhere in the manuscript, even not mentioned in the schematic diagram in Fig. S3A. This dataset appears to be redundant, and it should be replaced with data from other stages.
6. Line 126-127: "...we used a T to C / T to A > 3 cut off for nascent gene and TE definition..." I am wondering if the high concentration of 4sU can perturb the T > A mutation ratio that is attributed to transcription. Considering this potential confounding effect, it may be more appropriate to use alternative mutation types, such as G > C, as the basis for the cutoff?
7. In Fig. S3b, the variation in the mutation ratio across developmental stages is not clearly presented. The authors should provide detailed values or fold changes for the T>C groups relative to the non-T>C groups.
8. Line 202: The text begins to jump between 1C and PN3/5 without much explanation for when/how the cell is referred to either. Some clarification or sticking to one type of designation is helpful.
9. Line 227: The Cdx2 hit is interesting and unexpected. This is associated with specification of the trophoblast lineages. Is this the earliest report of Cdx2 expression?
10. Line 243: It is not clear what is meant by "excluding MaLR in this paper".
11. Line 264: Somewhat hard to read, please revise.
12. In Fig 4h, the nascent genes of 36 hpf MTA-ASO embryos seem be closer to the M2C stage along PC1. If so, why these embryos arrest at the 1-cell stage rather than progressing to E2C stage?
13. In Fig 4h, the nascent gene profiles of 36 hpf NC embryos are closer to the L2C stage, whereas in Fig 4l, the nascent TE profiles of the same samples are closer to the M2C stage. This suggests that TE activation lags behind gene activation. The authors should clarify whether this result is biologically reasonable.
14. Line 319: Typo, please revise.
15. Line 349 and Figure 5A: How does the analysis define "exon"? I understand a TSS originating from an intron (cryptic) or intergenic (upstream) like in the examples shown in 5B, but I cannot distinguish how "exon" can be a TSS. Can you elaborate or show examples? Most of this is explained in S8A, but I am not sure I see "exon".
16. In Fig. 5C, How are the junction reads determined? Directly above it in 5B is an example showing 217 vs 198 reads. In Panel 5C at PN5, we are shown 44 vs 49.
17. Line 364 and figure 5E: I might be interpreting this wrong, but the "common" exon would indicate to me that after siRNA KD, only a very tiny amount of "total transcripts" went down, meaning the Canonical generates the majority of all Nfya? If this is correct, then the minor reduction in 5F and 5G might be explained. What are the N-Values for each experiment here (5F and 5G)? This is missing from legend. "Crucial" might be too strong of a term. (Line 369), as the blastocyst rate at 108hpf are relatively similar. Setd2 shows more of a dynamic range.
18. Line 379 and Figure 5H: How was this done? The 5H schematic looks like it targets this locus 3 ways: Upstream, Downstream and Internal. I'd imagine the internal would have many off targets. Is that what is shown in 5I and 5J when "TE" is mentioned on the X Axis? As in: a large portion of MT2 and ORR1B1 are reduced in this experiment, with these chimeras (and canonical as well?) being a part of that global downregulation? According to 5B, roughly half of the expression should be coming from the canonical Setd2 expression. Does the canonical compensate? (Primers can be designed against just the canonical isoform transcript to confirm, similar to what is shown in S8C and S8D). The details of this strategy are not clear to me in the methods.

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have significantly improved their analyses, and the presentation of their work has improved. We have the following additional comments that should be addressed.

1. We have requested the authors to bring their findings in the context of published findings in the discussion, however, only few published papers have been referred to. Thus, we encourage the authors to work on the discussion.
2. Regarding the requested ASO validations it is unclear whether the other MT family members are expressed in this context, making it difficult to interpret the absence of downregulation as evidence of ASO specificity.
3. Lines 323–325 / Fig. S7g: Please clarify what the z-score represents in this analysis.
4. The new analysis distinguishing solo from full-length MTA elements is interesting and useful for the community. However, the methods lack detail on how these classes were derived from RepeatMasker annotations, which do not directly define full-length elements, but provide separate annotations for LTR and internal regions. The authors should describe the analyses in detail.
5. Lines 311–312: The conclusion that “transcriptional activation of MTA is essential” is an overstatement based on this experiment. ASOs primarily degrade RNA and do not directly inhibit transcription, so the data do not distinguish between a requirement for the endogenous MTA RNA itself and transcriptional activation from the endogenous MTA loci.
6. Lines 452–456 / Fig. 6a: Please clarify whether motif enrichment was performed using all proteins detected in the embryonic proteomics dataset or a filtered subset, and if the latter, describe the selection criteria.

Reviewer #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

I appreciate the authors' detailed and constructive responses to my comments. Overall, I found the rebuttal to be thoughtful and responsive, and I think the manuscript has been substantially strengthened. In particular, the authors have added useful controls and clarifications regarding the sensitivity of scTL-seq, the effects of 4sU treatment, allele-specific and TE-mapping strategies, MTA-ASO specificity, MTA rescue, and the validation of TE-derived chimeric transcripts. I remain generally positive about the study. My main view is that the paper is now much improved, but that some of the mechanistic conclusions, especially regarding MTA function, should still be phrased conservatively in the revised manuscript.

The additional validation of the scTL-seq method addresses many of my concerns. I appreciate that the authors revised the interpretation of the 4sU-IP-specific genes and no longer describe them simply as background noise. The added PRO-seq comparison, the discussion of detection thresholds, and the overlap analysis between 4sU-IP and scTL-seq are helpful. I also appreciate the added developmental assays showing that 2 mM 4sU treatment for 4 hours does not have an obvious detrimental effect on oocyte maturation or blastocyst development. These data make the methodological foundation of the study more convincing.

The authors also responded directly to my concerns about MTA-ASO specificity and the need for a rescue experiment. The rescue experiment is informative, even though exogenous full-length MTA RNA did not rescue the developmental phenotype. In my view, this result argues against a simple model in which freely supplied MTA RNA is sufficient, and instead supports a more nuanced interpretation in which MTA function may depend on genomic context, local transcription, or cis-regulatory activity. I think this is an important point, and I would encourage the authors to make sure that the revised manuscript does not overstate the conclusion that MTA RNA itself acts directly in trans unless this is clearly presented as a hypothesis.

The MTB-Nfya section is also improved. The RT-PCR/Sanger validation of the chimeric transcript, together with the protein-size and localization experiments, provides stronger evidence that MTB-Nfya is a genuine transcript that encodes a protein indistinguishable from canonical NFYA. I also appreciate that the authors softened the developmental phenotype from “partial arrest” to a developmental delay and discussed the possibility of compensation from canonical Nfya. This makes the interpretation much more balanced.

I also appreciate the additional explanation of the TE and allele-specific mapping strategy. The authors now provide a clearer description of whole-genome alignment, unique mapping, HISAT-3N usage, SNP masking, base-quality filtering, read-end filtering, paired-read filtering, and RepeatMasker-based TE assignment. This clarification is helpful. At the same time, because unique mapping necessarily captures only a subset of TE-derived reads, I think it would be useful for the revised manuscript to explicitly acknowledge this limitation and the possibility that some TE copies or families may be underrepresented.

MINOR:

There are a few remaining points that I think should be addressed in the revised manuscript, mostly by ensuring that the relevant data and caveats are actually included rather than only described in the rebuttal. First, some useful validation data, including the EU-labeling and MII robustness controls, appear to be discussed in the response but may not be incorporated into the manuscript or supplement. Since these data help address important concerns, I think the key controls should be included. Second, the proposed mechanism by which MTA suppresses other TEs remains speculative, particularly because the ChIRP-seq data are preliminary, from ESCs, and not included in the manuscript. Therefore, any model in which MTA RNA directly represses other retrotransposons should be clearly framed as a possible mechanism rather than a demonstrated conclusion. Third, although I understand the authors' explanation for why a reciprocal cross was not feasible, the strong asymmetry in informative SNP numbers and the lack of reciprocal cross still leave some room for allele-assignment bias. I do not think a reciprocal cross is necessary at this stage, but I would ask that this limitation be stated clearly. Finally, for the DNaseI-TUNEL chromatin accessibility assay, the authors clarified the rationale, but it would be helpful to know whether a no-DNase TUNEL control was performed or to include a brief statement addressing this point.

Overall, I think the authors have taken the comments seriously and have made substantial improvements. I remain supportive of the manuscript, provided that the revised version includes the promised controls and maintains appropriately cautious language around the mechanistic interpretation of MTA function. I would support publication after VERY minor revision.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Wu et al have optimized a low input version of the previously published TimeLapse-seq method. They have utilized this method to elucidate temporal pattern of zygotic genome activation in mice. Wu et al have analysed expression dynamics of transposable elements (TE), focusing on the LTRs, which have previously shown to be expressed in early mouse development and provide cis regulatory elements.

Authors determine three zygotic genome activation waves based on the gene expression dynamics, investigate maternal and paternal transcript expression and then focus on TE expression dynamics and regulation.

We acknowledge the novelty in addressing the roles of MaLRs in mouse ZGA, as well as the role of Sall4 as a potential MTA element regulator. Authors present interesting findings that bring relevant insights to the field, however, major improvements are needed prior to publishing this manuscript.

We thank the reviewer#1 for the constructive and thorough comments, which have greatly helped us improve our manuscript. Below we summarize the key changes we have made in response to the reviewer's suggestions.

First of all, we validated the specificity of our ASO pool and showed that it mainly targets MTA_Mm-int and MTA_Mm, with negligible knockdown of majority of MT family members. Besides, we analyzed the activation pattern of MT family members and found that the predominantly activated form in early embryos is the full-length MTA.

Secondly, we re-examined the motif enrichment using the JASPAR database instead of HOCOMOCO after comparison of two motif database, mainly because the SALL4 motif in HOCOMOCO was inconsistent with published reports. After this update, we identified NOBOX, an oocyte-specific transcription factor, as a strong candidate regulator of full-length MTA activation. The motif for NOBOX is significantly enriched in nascent full-length MTA elements, and analyses of Nobox knockout data confirmed its requirement for MTA expression (Figure 6).

Thirdly, we experimentally validated the existence of the MTB-Nfya chimeric transcript by RT-PCR. ORF prediction and in vitro expression of the cloned transcript demonstrated that the encoded protein is identical in size and subcellular localization to canonical NFYA. Thus, MTB may serve as an alternative promoter to ensure sufficient Nfya expression before ZGA, without altering the protein product.

Finally, we have rewritten the abstract to clearly highlight our novel findings while avoiding overstatements. The overall structure of the manuscript has also been optimized, with the regulators of MTA moved to the Results section and the Discussion expanded to better contextualize our findings within the existing literature.

We have addressed all of the reviewer's comments point-by-point in blue font, and the corresponding figures and their locations in the manuscript have been highlighted in the point-to-point response. We hope these revisions adequately address the reviewer's concerns. Should there be any further suggestions, we are happy to make additional modifications.

Major comments

1. Concerning the manuscript structure, we suggest that the Figure 6 results would be moved from the Discussion to the Results section.

We thank the reviewer for this constructive suggestion. Accordingly, we have revised the manuscript by moving the results originally presented in Figure 6 from the Discussion to the Results section, and have added a new subsection entitled “**Potential regulators of full-length MTA elements**” to present these findings. In the updated Figure 6, we redefined the motif of MTA and its potential transcription factor binding, ultimately identifying and validating NOBOX as a potential activator of MTA. We believe that the reviewer's suggestions have greatly improved the structure and readability of the manuscript, as well as strengthened the significance of our work.

2. The clarity of the manuscript text should be improved because at times it is slightly challenging to follow and this may undermine the significance of these data. We have specifically drawn attention to some lines below.

We thank the reviewer for this valuable suggestion. In response, we have rewritten the abstract and carefully revised the manuscript by supplementing and adjusting the data and content according to the reviewer's comments. We mainly reorganized the content of the MTA knockdown section (Figure 4) and the MTA motif and regulator section (Figure 6). The overall clarity and readability of the text have also been substantially improved. The revised content has been highlighted in yellow in the manuscript. If the reviewer has any further suggestions regarding the manuscript text, we are glad to further address them.

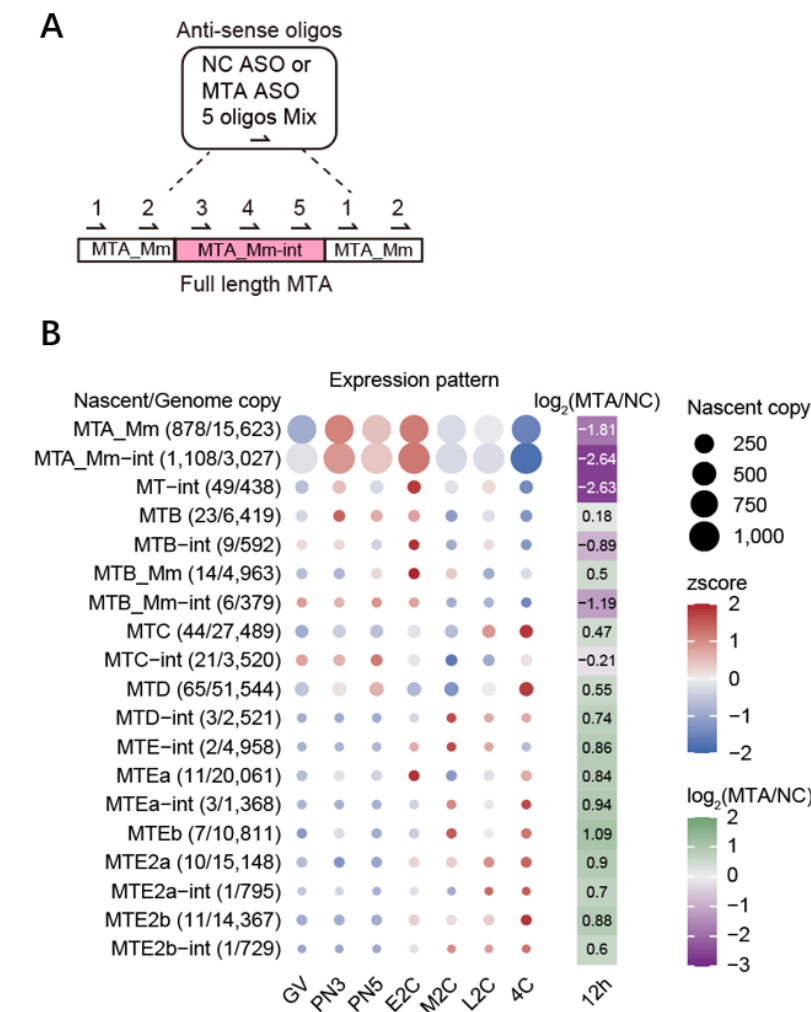
3. Authors state that ASO treatment also affected other MT subfamilies. How were the MTA targeting ASO sequences designed and how their specificity was evaluated?

We thank the reviewer for this important question that ASO specificity need to be fully evaluated.

First, the ASOs targeting MTA were designed as follows: we designed three ASOs against the most actively expressed MTA_Mm-int in wave 1 (W1) and two ASOs against its LTR element, MTA_Mm (**Response Figure 1A**).

To evaluate the specificity of the ASO sequences, we examined the expression changes of all MT family members at 12 hpf after ASO injection into MII oocytes. The most pronounced downregulation was detected for MTA_Mm-int and MTA_Mm, followed by MT-int, while other MT families showed no significant changes (MTB-int and MTB_Mm-int trended downward). In addition, genomic and transcribed copy number analyses further revealed that, aside from MTA, other MT family members (including MT-int, MTB-int and MTB_Mm-int) have substantially fewer active copies, indicating the impact on these family members is limited (**Response Figure 1B**).

In summary, we consider that our set of five ASOs primarily knocks down MTA_Mm-int and its LTR element MTA_Mm, with limited effect on other MT families. The corresponding results have been revised in the manuscript (Fig4c, Supplementary Fig. S7g).



Response Figure 1 : Specificity of MTA-ASO

(A) Schematic diagram of ASO-mediated MTA knockdown.

(B) Expression patterns and knockdown levels of MT family members. Left numbers indicate nascent and genomic copy numbers. Bubble plot shows expression changes at different developmental stages (bubble size represents nascent copy number). Right heatmap shows RNA fold-change at 12 hpf after ASO injection in MII oocytes.

4. Regarding the MTA anti-sense oligo-treatment, it appears that the treated embryos were collected at late 2-cell stage, while the embryos that resulted from MII oocyte injections, were arrested already at 1-cell stage. Authors should clarify at which stage they measured the knock-down efficiency for each experiment and at which stage they performed actual analyses of these embryos.

We apologize for the lack of clarity regarding the experimental timing and methods. In the revised manuscript, we have now clarified these points and updated the experimental schematic accordingly.

Knockdown efficiency was assessed by RT-qPCR and RNA-seq. For RT-qPCR, ASOs were injected into zygotes, and embryos were collected at the 2-cell stage (24 hours post-injection) to evaluate the knockdown efficiency of MTA-int and MTA_Mm (Fig. 4d). Zygotic injection results in impaired development but not arrest at 1-cell stage.

For RNA-seq, ASOs were injected into MII oocytes, and embryos were collected at two time points: 12 hours post-fertilization (hpf) and 36 hpf. Since MTA knockdown following MII injection resulted in nearly complete arrest at the 1-cell stage, the L2C time point was collected in parallel with the control group (NC) based on the developmental timing (36 hpf) of the controls, even though the knockdown embryos did not divide to 2 cells. All RNA-seq-based analyses shown in Figure 4 and Supplementary information Fig.S8 were performed on samples collected at these two time points (12 hpf or 36 hpf) after MII injection, with the collection times indicated in the figure panels. The DNaseI-TUNEL assay, pronuclear size measurements, and γ H2AX staining were all performed on 1-cell embryos collected at 12 hpf following MII injection.

To avoid confusion, we have simplified the schematic in Fig. 4c and added detailed descriptions of the collection time points in the Results section.

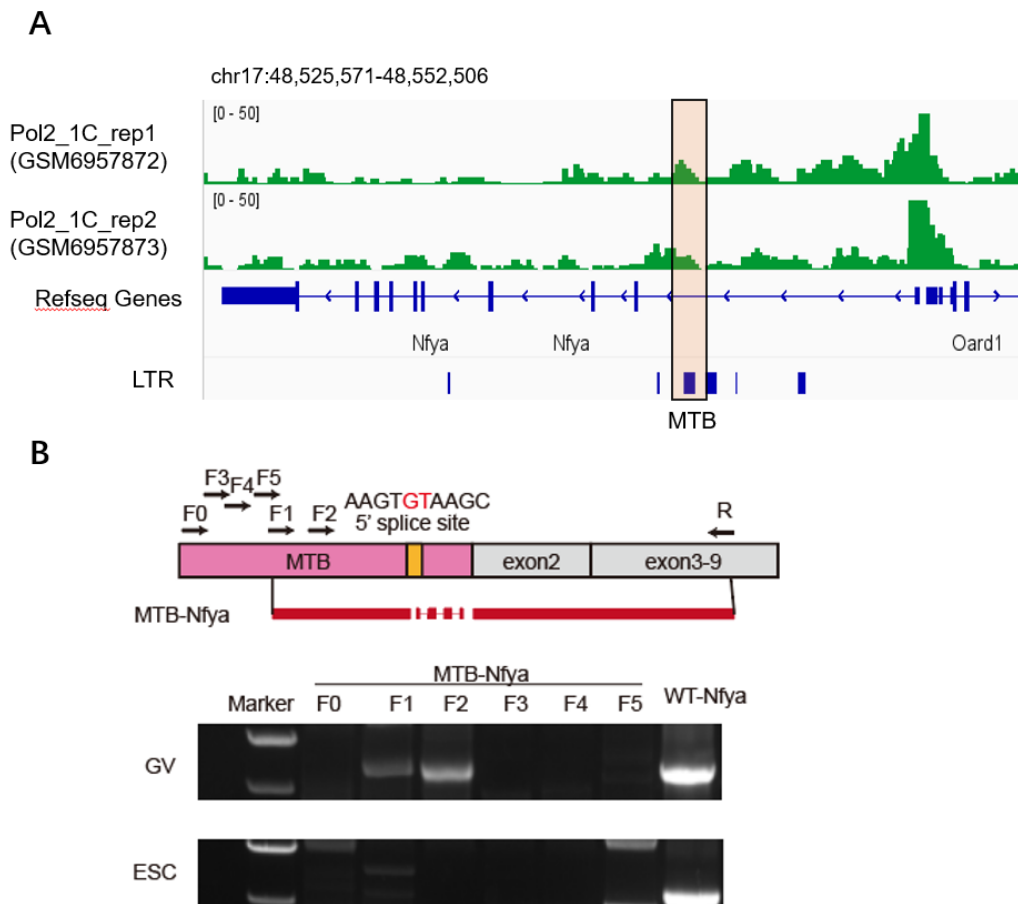
5. In case the MTB-derived Nfya, we suggest that authors write about predicted isoform, or experimentally validate the alternative TSS, for instance by cloning the alternative transcript. Authors could also utilize the published Polymerase II data to investigate whether Pol II is accumulated at the predicted TSS.

We thank the reviewer for this valuable suggestion. We agree that it is essential to clarify whether MTB-Nfya represents a genuine alternative isoform and whether an alternative transcription start site (TSS) truly exists.

First, we examined published Pol II Stacc-seq data from 1-cell embryos (GSM6957872 and GSM6957873). We observed modest Pol II binding signals at the MTB locus, which indicates a possibility of Pol II occupancy on this region (**Response Figure 2A**).

Additionally, following the reviewer's recommendation, we performed experimental validation by RT-PCR and Sanger sequencing. Based on scLT-seq data, we designed multiple forward primers targeting different regions of MTB and a reverse primer located at the 3' end of Nfya to clone the full-length MTB-Nfya. RT-PCR results showed that the combination of F1 (and its downstream primers F2) with the reverse primer successfully amplified the MTB-Nfya from GV cDNA. Sanger Sequencing results showed fusion sequence between MTB and Nfya, with splicing occurring at the 5' splice site of MTB and exon 2 of Nfya, which matched the chimeric transcript identified in our scTL-seq data (**Response Figure 2B**).

Taken together, these results confirm the existence of the MTB-Nfya chimeric transcript. The revised manuscript now includes RT-PCR experimental validation (**Supplementary Fig. S9e**). If the reviewer requests inclusion of the Pol II Stacc-seq data, we can readily add them to the manuscript.



Response Figure 2. Identification of the MTB-Nfya chimeric transcript.

(A) Pol II binding signals at the 1-cell stage. Data were obtained from GSM6957872 and GSM6957873.

(B) RT-PCR products obtained with different primer sets targeting MTB-Nfya, and the schematic alignment of Sanger sequencing results confirming the splice junction.

6. Could MTB-derived Nfya code for protein? We suggest that authors discuss this.

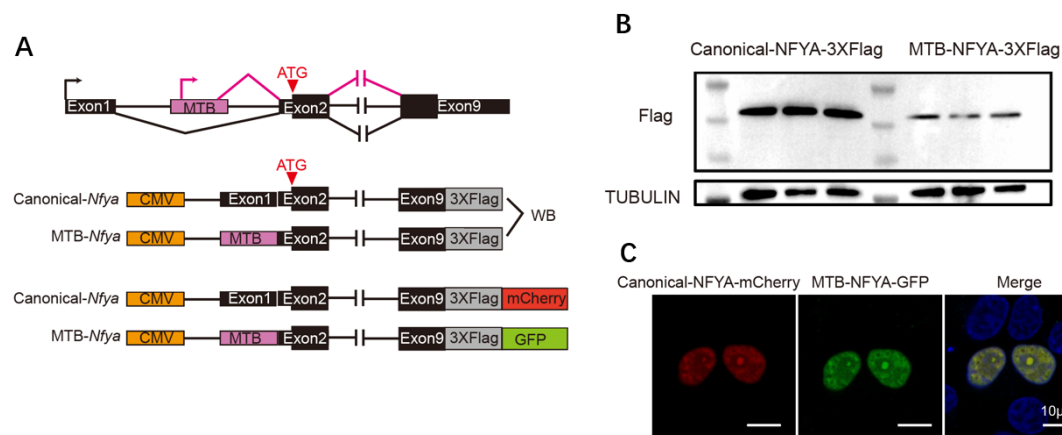
We thank the reviewer for raising this important point. We have investigated whether the MTB-derived Nfya transcript can produce a protein.

First, we predicted the open reading frame (ORF) of the full-length MTB-Nfya chimeric transcript. The predicted start codon (ATG) was found to be located in exon 2, at the same position as that of the canonical Nfya ORF. This suggests that MTB-Nfya does not encode a novel chimeric protein; rather, the MTB element serves as an alternative transcription start site that alters the 5' UTR sequence without affecting the protein-coding sequence (CDS) (**Response Figure 3A**).

To experimentally validate this prediction, we cloned both the canonical Nfya (5' UTR-CDS) and the full-length MTB-Nfya sequence into a 3×Flag expression vector. Western blot analysis showed that the two constructs produced proteins of identical size (**Response Figure 3B**). Furthermore,

fluorescence imaging revealed that both proteins exhibited the same nuclear localization pattern (**Response Figure 3C**). These results validate that the protein encoded by MTB-Nfya is indistinguishable from that of the canonical Nfya transcript.

The revised manuscript now includes these data (**Supplementary Fig. S9f–h**).



Response Figure 3. Validation of protein from MTB-Nfya chimeric transcript.

- (A) Schematic diagram of the splicing patterns and predicted transcription start sites (red arrow, ATG) of canonical Nfya and MTB-Nfya, along with the expression vector design.
- (B) Western blot showing the protein sizes produced by the canonical Nfya and MTB-Nfya expression constructs in human 293T cells.
- (C) Fluorescence reporter assays showing that both canonical NFYA and MTB-NFYA exhibit the same nuclear localization pattern in 293T cells.

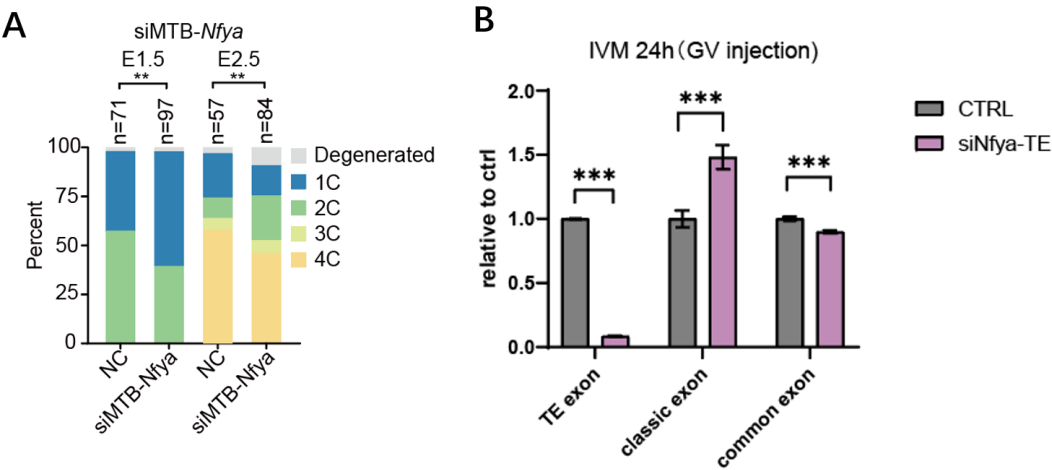
7. Regarding the MTB Nfya knockdown embryos, authors should clarify what they mean by “partially arrested” embryos? Do they mean that all embryos are delayed? This should be clarified and taken into the context of canonical Nfya perhaps being able to compensate the missing MTB Nfya.

We thank the reviewer for this question. We agree with the reviewer that the phenotype of MTB-Nfya knockdown embryos should be described as developmental delay rather than “partial arrest”.

As shown in Figure 5f (and **Response Figure 4A**, which now includes the total embryo numbers each group), the knockdown group exhibited a developmental delay compared to the control, particularly in the timing of entry into the 2-cell and 4-cell stages. At 24 h post-IVF, 56% of control embryos had reached the 2-cell stage, whereas only 40% of knockdown embryos had done so. However, the proportion of embryos arrested at the 1-cell stage at 48 h did not differ substantially between the control (22%) and knockdown (15%) groups. Thus, we concluded that knockdown of MTB-Nfya does not cause developmental arrest, but rather leads to a noticeable delay.

This partial phenotype may indeed be explained by incomplete knockdown of maternal proteins by siRNA and compensatory upregulation of canonical Nfya transcripts. When we injected siRNAs into GV oocytes and collected them after 24 h of in vitro maturation, we observed a significant upregulation of canonical Nfya levels (**Response Figure 4B**).

Notably, a recent study using a dTAG mouse model for NFYA reported that rapid degradation of NFYA protein causes early embryonic arrest at the 2-cell stage (doi: <https://doi.org/10.64898/2026.03.30.715371>). The stronger phenotype observed in that study is likely due to the complete loss of NFYA protein, whereas in our knockdown experiment, the overall reduction of Nfya transcript levels was only modest. Consequently, we observed only a developmental delay. We have revised the conclusion accordingly to more accurately reflect these observation as: *“However, a noticeable delay in progression to the 2C stage was evident, indicating that MTB-initiated Nfya transcripts promotes the ZGA process. The relatively mild phenotype is likely explained by partial compensation from the upregulated canonical Nfya transcripts.”*



Response Figure 4. Compensation of canonical Nfya upon MTB-Nfya knockdown.

(A) Developmental ratio at E1.5 and E2.5 post-IVF after NC and siMTB-Nfya injection in GV and IVM.

(B) RT-qPCR showing expression levels of the indicated transcripts relative to control (non-targeting siRNA, NC) in GV oocytes after 24 h of in vitro maturation following siRNA injection.

8. We suggest that authors discuss the role of MTB-derived Nfya. Why is the expression of the isoform required in early mouse development? What is the function of the MTB-derived Nfya?

We thank the reviewer for this insightful suggestion. We have now expanded the Discussion section to address the potential function of MTB-derived Nfya in early mouse development.

Although the two transcripts (MTB-Nfya and canonical Nfya) encode identical NFYA proteins and differ only in their promoter usage and 5' UTR sequences, we propose that this dual-promoter configuration serves as a “two-engine system” during the maternal-to-zygotic transition. The MTB

retrotransposon provides an additional promoter that ensures increased expression of NFYA prior to ZGA, thereby meeting the protein demand during this critical window of epigenetic reprogramming. It remains to be determined whether the two different 5' UTRs lead to differences in RNA stability and translational efficiency, which requires further investigation.

9. Authors should expand Discussion to better relate their findings to published literature. We feel that several relevant papers have been cited, however, they have not been discussed in the context of the findings made in this manuscript.

We thank the reviewer for this constructive suggestion. In the revised manuscript, we have substantially restructured and expanded the Discussion section. Specifically: First, the content regarding the regulators of MTA has been moved to the last subsection of the Results section. Second, we have added a discussion on the potential mechanisms by which MTA regulates other transposable elements. Besides, we have also expanded the discussion on the function, protein-coding potential, and possible regulatory mechanisms of TE-derived chimeric transcripts.

We believe these revisions now appropriately place our findings in the context of previously published work.

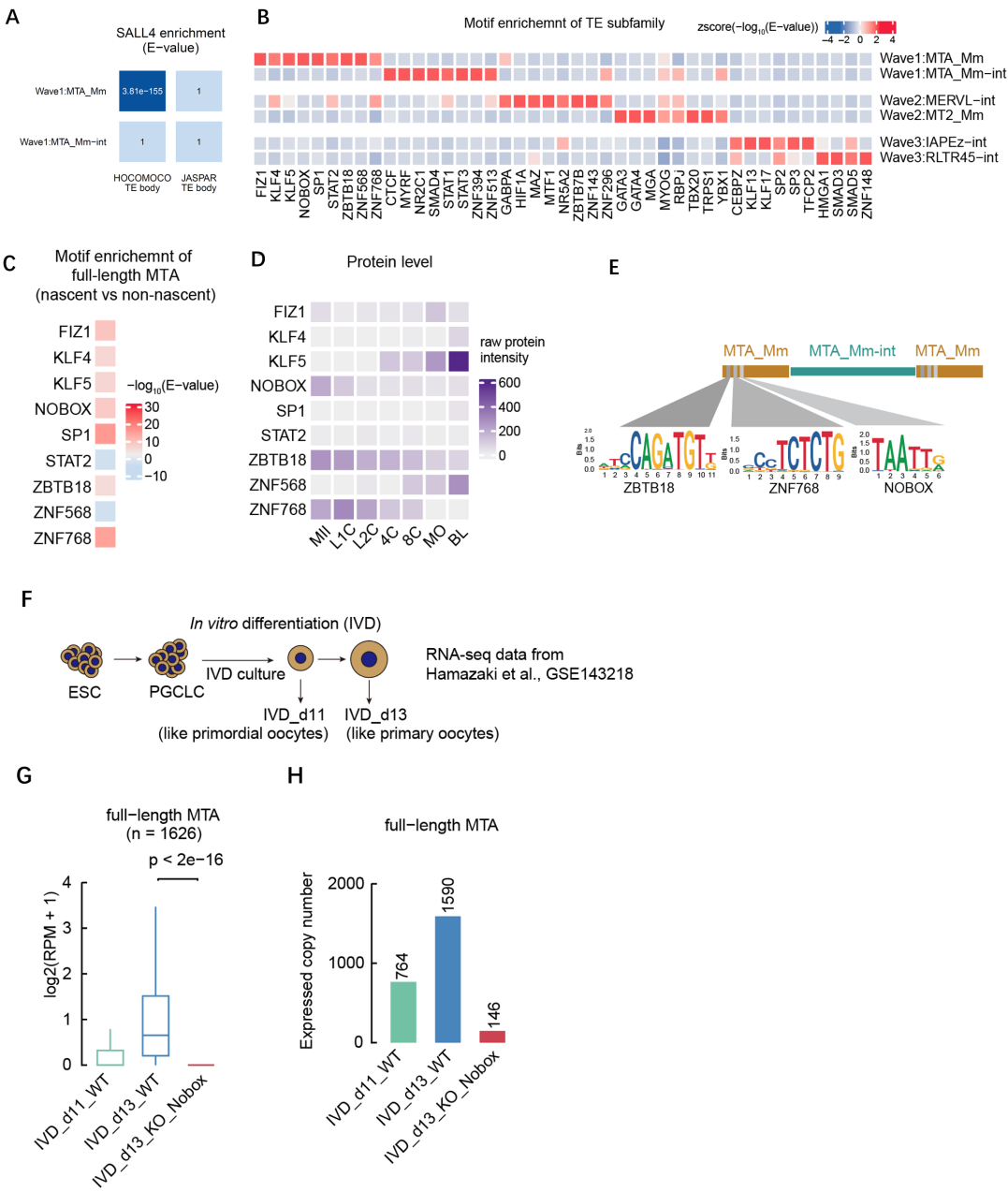
10. Authors have used Sall4 knockout data and show that MTAs are downregulated. However, downregulation is quite modest and therefore, authors should discuss whether Sall4 might, for example, act redundantly with other factors.

To further investigate the regulatory landscape of MTA elements, we re-examined the TF motif analysis and updated the motif annotation database from HOCOMOCO to JASPAR, which provides manually curated and experimentally supported TF binding profiles that are widely used in mouse regulatory genomics studies. In addition, the SALL4 motif in JASPAR closely matched the canonical AT-rich SALL4 binding sequence reported in previous studies (e.g., PMID: 36257403, 33406418), whereas the corresponding HOCOMOCO motif showed lower similarity to the experimentally reported motif. Following re-analysis with the updated motif database, the SALL4 motif was no longer significantly enriched in MTA elements (**Response Figure 5A**). The enrichments of KLF4/5, SP1, STAT2, and ZNF568 remained robust, and several additional TFs became enriched in MTA_Mm elements, including the oocyte-specific transcription factor NOBOX and ZBTB18 (**Response Figure 5B**). Among these, NOBOX, ZBTB18, and ZNF768 were significantly enriched in active full-length MTA elements compared to non-nascent MTA elements, and all three have detectable protein abundance in oocytes (**Response Figure 5C, D**). Their binding motifs are predominantly located at the 5' end of MTA_Mm (**Response Figure 5E**).

NOBOX is known as an oocyte-specific transcription factor essential for oocyte development; Nobox knockout mice exhibit oocyte loss and arrested folliculogenesis (PMID: 15326356). In an ESC-to-oocyte differentiation system (PMID: 33328630), Nobox knockout caused oogonial arrest at day 13 (equivalent to primary oocytes at p6–p7 stages), and transcriptomic data showed reduced MTA expression. Using this dataset, we examined the expression of full-length MTA elements

during differentiation. In wild-type cells, full-length MTA expression and the number of active copies were significantly increased at day 13 (primary oocyte-like) compared to day 11 (primordial oocyte-like). However, in Nobox knockout cells, both expression levels and active copy numbers remained dramatically reduced, even below the day 11 wild-type levels, indicating that Nobox is required for MTA activation (**Response Figure 5F–H**).

Taken together, our revised analysis indicates that SALL4 is unlikely to be a key regulator of MTA. Instead, our data point to **NOBOX** as a critical transcription factor that activates full-length MTA elements in mouse oocytes. The relatively modest downregulation of MTA in the Sall4 knockout data (mentioned by the reviewer) is consistent with this revised conclusion. We have updated the corresponding results and discussion in the revised manuscript accordingly (**Figure 6**).



Response Figure 5: predictive MTA regulator

(A) SALL4 motif enrichment (E-value) on MTA_Mm and MTA_Mm-int from HOCOMOCO (previously used) and JASPAR database (now updated).

(B) Motif enrichment of different TE subfamilies. The transcription factors listed are proteins detected in proteomics data of mouse early embryos.

(C) Motif enrichment comparison between nascent full-length MTA elements and non-expressed full-length MTA elements.

(D) Raw protein intensity of potential transcription factors binding to MTA_Mm in mouse early embryos.

(E) Schematic illustration of the motif sequences of ZBTB18, ZNF768, and NOBOX, and their predicted binding sites within full-length MTA elements.

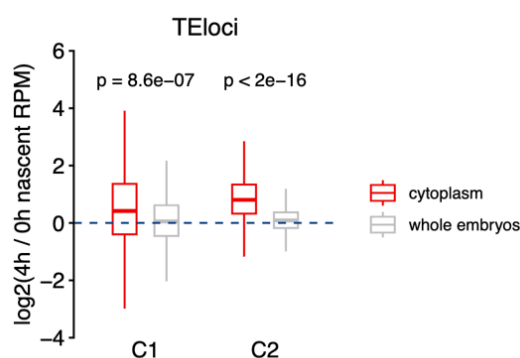
(F) Experimental scheme of in vitro differentiation (IVD) toward oocytes. RNA-seq data from IVD day 11, IVD day 13, and Nobox knockout at IVD day 13 were obtained from GSE143218 and used for panels (G) and (H).

(G) Expression levels of all expressed full-length MTA elements at IVD day 11 and day 13. “N” indicates the total number of detected copies. P-values (Wilcoxon rank-sum test) indicate significance between WT day 13 and Nobox KO day 13.

(H) Genomic copy numbers of all expressed full-length MTA elements in each group.

11. Results regarding the transcript transfer to cytoplasm are interesting. Authors should also plot TEs to elucidate whether they are also transported to cytoplasm?

We appreciate the reviewer’s suggestion. We have now quantified the cytoplasmic fraction of nascent transcripts for TEs in the C1 and C2 categories. As shown in the figure below, TE-derived nascent RNAs are also transported to the cytoplasm, analogous to the behavior of coding transcripts. We have not included this result in the current manuscript because TEs are introduced later in the Results section than this experimental result. If the reviewer considers it important, we can further incorporate it into the TE section.



Response Figure 6: Cytoplasmic enrichment of nascent transcripts from C1 and C2 TEs.

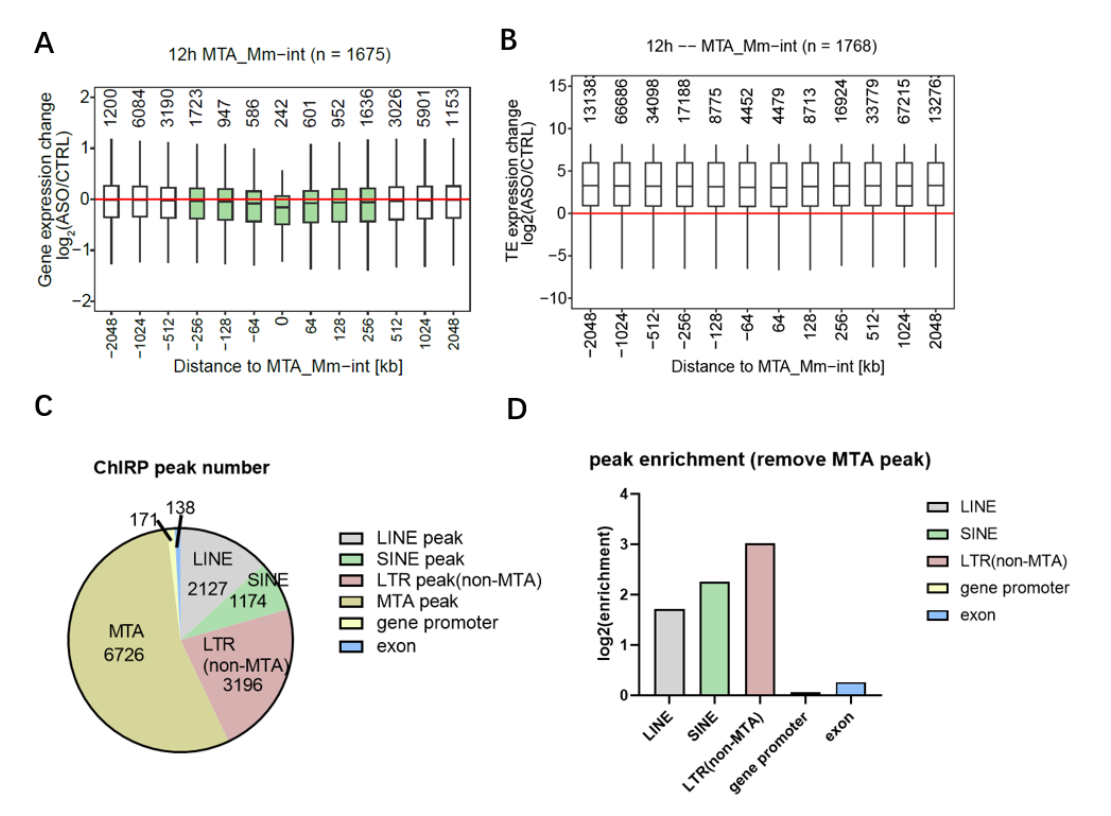
12. Authors should discuss potential mechanisms related to MTA silencing and resulting genome accessibility?

We thank the reviewer for raising this important question, which is also of great interest to us.

We currently propose that the increased chromatin accessibility upon MTA knockdown is primarily due to the loss of MTA RNA, which relieves the suppression of other TEs, leading to their widespread activation and subsequent chromatin opening.

Further analysis of the MTA knockdown data revealed that MTA knockdown leads to downregulation of neighboring genes at 12 hpf (**Response Figure 7A**), which may occur through a cis-regulatory mechanism. However, the upregulation of 392 TE families at 12 hpf was not correlated with genomic distance (**Response Figure 7B**). Preliminary data from Chromatin Isolation by RNA Purification sequencing (ChIRP-seq) experiments in embryonic stem cells (ESCs) suggest that MTA RNA directly binds to approximately thousands of retrotransposon loci with high enrichment (**Response Figure 7C,D**), potentially suppressing their transcription and modulating their chromatin state.

Gene and TE expression change results are supplement in [Fig.4j](#) and [Supplementary Fig.S8k](#). Since these ChIRP-seq results from ESCs are unpublished, we have not included them in the current manuscript. Nevertheless, we have added a discussion of this hypothesis in the revised Discussion section.



Response Figure 7: MTA silencing and chromatin accessibility.

A. Boxplot showing expression change for the genes within indicated distances to the downregulated MTA_Mm-int upon MTA ASO at 12h (hpf) after MII injection. The number of expressed genes (RPKM >1 in Ctrl or ASO samples) in each distance category is annotated above the boxes.

B. Boxplot showing expression change for the TEs (MT families excluded) within indicated

distances to the downregulated MTA_Mm-int upon MTA ASO at 12hpf after MII injection. The number of expressed TEs (RPKM >0.3 in Ctrl or ASO samples) in each distance category is annotated above the boxes.

C. Peak number and peak ratio of MTA ChIRP-seq in ESCs.

D. Peak enrichment (remove MTA peaks) of MTA ChIRP-seq in ESCs.

13. Authors show (Figure 6) that the motifs are enriched at the internal part and not the LTR of the MTA. Authors could discuss whether these regulators only activate complete MTA elements and not the solo LTRs which lack the internal parts.

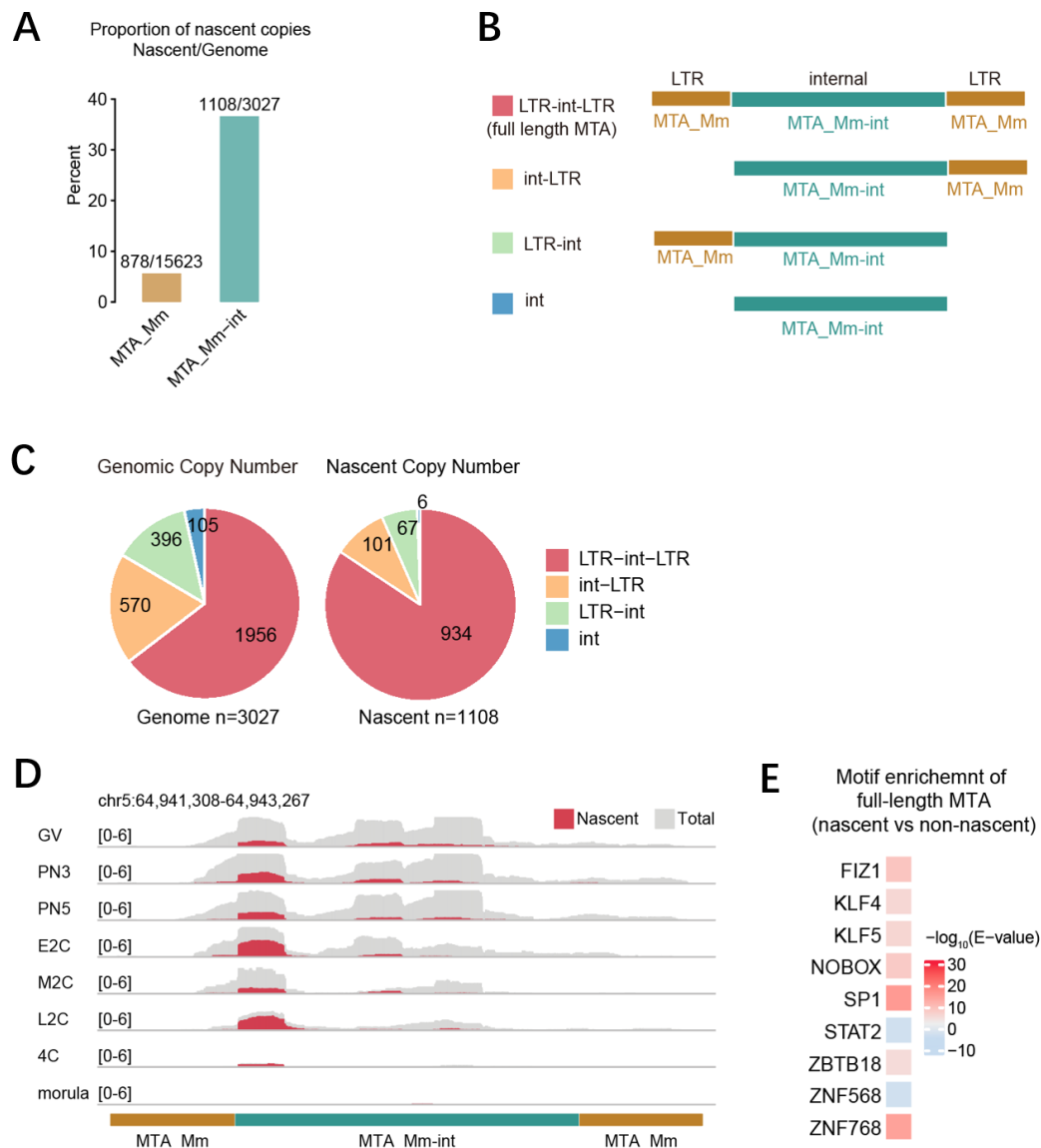
We thank the reviewer for this insightful question. We have now clarified the issue regarding motif enrichment and the differential activation of solo LTRs versus full-length MTA elements.

In our original analysis, we calculated motif enrichment in the promoter region of each TE (from 1 kb upstream to 100 bp downstream of the TSS). For MTA_Mm-int, this region included its upstream LTR (MTA_Mm). Thus, the observed enrichment “in the internal part” actually reflects enrichment in the adjacent LTR sequence. The apparent lack of motif enrichment in the MTA_Mm promoter region is likely due to poor sequence conservation upstream of MTA_Mm. To avoid this misunderstanding, we re-analyzed motif enrichment within the TE body region after switching to the JASPAR database. The results showed that TFs previously enriched in the promoter of MTA_Mm-int (e.g., KLF4/5, SP1, STAT2, ZNF568) now showed enrichment within the MTA_Mm sequence itself (**Response Figure 5**), indicating that the MTA_Mm element carries conserved motifs and may act as a promoter to drive MTA_Mm-int expression.

To answer whether these regulators only activate complete MTA elements and not the solo LTRs which lack the internal parts. We firstly analyzed activation levels of solo MTA LTRs versus internal elements. Approximately 40% of MTA_Mm-int copies exhibit nascent expression, whereas only about 5% of solo MTA_Mm copies are transcriptionally active, indicating that solo MTA_Mm elements are not broadly activated (**Response Figure 8A**). Among the expressed MTA_Mm-int copies, the vast majority (934 out of 1,008) are flanked by MTA_Mm LTRs on both sides, a configuration we term full-length MTA (LTR-int-LTR) (**Response Figure 8B-D**). These data demonstrate that the primary transcriptionally active entities in early embryos are full-length MTA elements, not solo LTRs.

Furthermore, we compared motif enrichment between nascent and non-nascent full-length MTA elements. The motif for SP1, NOBOX, ZNF768 et al., was significantly enriched in nascent full-length MTA elements compared to non-nascent ones (**Response Figure 8E**). Therefore, the more conserved motif features of full-length MTA elements likely account for their transcriptional activation at the 1-cell stage.

We have incorporated these clarifications and additional analyses into the revised manuscript (Fig.4b, Fig.6 and Supplementary Fig. S7a-c.)



Response Figure 8. Genomic configuration and activation status of MTA_Mm and MTA_Mm-int elements.

- (A) Nascent copy numbers versus genomic copy numbers of MTA_Mm and MTA_Mm-int.
- (B) Schematic diagram illustrating the association of MTA_Mm LTRs flanking MTA_Mm-int in the genome.
- (C) Genomic and nascent copy numbers for each configuration type shown in (B).
- (D) IGV snapshot showing an example of a transcriptionally active full-length MTA element (LTR-int-LTR).
- (E) Motif enrichment comparison between nascent full-length MTA elements and non-expressed full-length MTA elements.

14. Lines 322-324: We think that this statement is slightly bold and should be tuned down.

Thank you for the suggestion. We have made appropriate revisions in the text, as detailed in: **“In summary, our findings reveal that MTA knockdown caused a burst of hundreds of transposable elements, including ERVs and L1s, followed by failure of ZGA gene activation.”**

Minor comments

1. Lines 32-34 (Abstract): Please clarify the phrasing.

We thank the reviewer for this suggestion. We have revised the abstract accordingly and clarified the phrasing as recommended.

2. Lines 184-187: Please clarify the phrasing.

Thank you for the suggestion. We have made appropriate revisions in the text, as detailed in:

“Compared with other clusters, M2C-active C4 genes have fewer exons and shorter lengths, consistent with the preferential activation of short or intron-less genes during early ZGA as reported in other species.”

3. Lines 254-258: Please clarify the phrasing.

We have made appropriate revisions in the text, as detailed in: **“Sequence divergence analysis revealed that the MaLR copies active in W1 are evolutionarily younger than those expressed at other stages; similarly, ERVL copies active in W2 are relatively young, and ERVK copies expressed in GV and W3 are also younger (Supplementary information, Fig. S6c). These findings suggest a link between the sequential activation of LTR retrotransposons and their evolutionary age, with preferentially younger elements being activated at each stage.”**

4. Line 488 (Methods): “unless indicated”. Should this be “unless otherwise indicated”?

Thank you for the suggestion; we have revised the text accordingly.

5. Lines 566-570: Please provide the ASO sequences.

ASO sequences was provided in Supplementary Table 4.

6. Please provide a reference to Kimura two-parameter model.

Thank you for the suggestion. We apologize for the ambiguity in our original description. In this study, we did not explicitly apply the Kimura two-parameter model. Instead, we directly used the milliDiv values from RepeatMasker annotations as measures of sequence divergence from the consensus sequence. To avoid misunderstanding, the Method section has been revised as follows: **“To estimate the evolutionary age of TEs, the milliDiv values reported by RepeatMasker were used as measures of sequence divergence from the consensus sequence. The milliDiv values were divided by 10 to obtain percentage divergence values (div) for comparison.”**

7. Lines 844-845: Please provide a web address to the Genome Sequence Archive.

Thank you for the comment. We have added the web addresses for the Genome Sequence Archive entries in the revised manuscript. The corresponding links are provided below:

human: <https://ngdc.cncb.ac.cn/gsa-human/s/I4Ibvt5B>

mouse: <https://ngdc.cncb.ac.cn/gsa/s/JQ0J6hje>

8. Code Availability: Please indicate whether original code was produced and where it will be made available (for example Github or Zenodo).

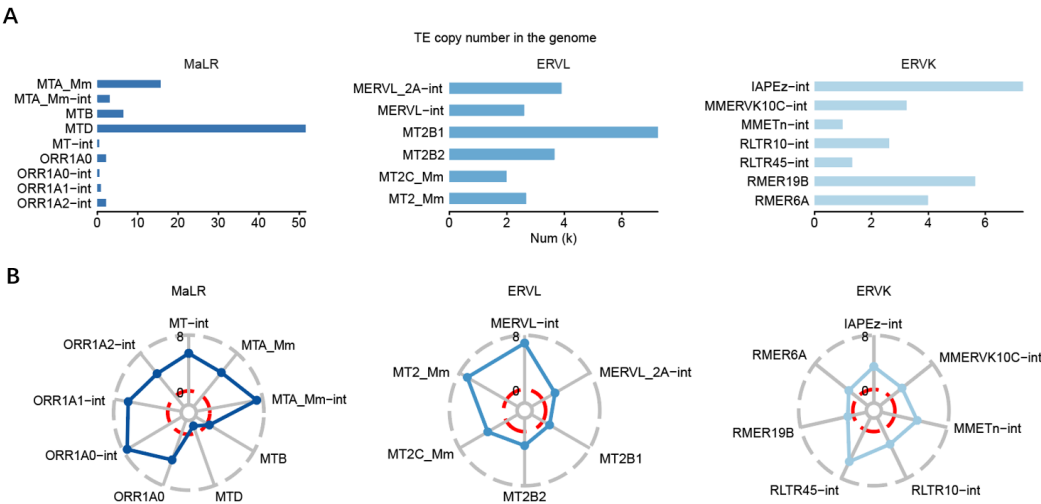
Thank you for the suggestion. We confirm that all original scripts and analysis code used in this study have been deposited in a public GitHub repository (<https://github.com/S2111266/Single-cell-TimeLapse-seq>) and are freely accessible.

9. Line 289: Please clarify the phrasing (by removing “respectively”).

Thank you for the suggestion; we have revised the text accordingly. **“To investigate the role of full-length MTA, we designed anti-sense oligos (ASO) targeting both MTA_Mm and MTA_Mm-int, and injected them into MII oocytes and zygotes (Fig. 4c).”**

10. Figure 3f: Are these sub families enriched in this analysis? Please show the subfamily TE copy number in the genome.

We thank the reviewer for this suggestion. In response, we have supplemented the radar plot of enrichment analysis and also calculated the genomic copy numbers of these TE subfamilies (**Response Figure 9A,B**). These results have now been incorporated into the respective figures in the manuscript (**Supplementary Fig. S6d, e**).



Response Figure 9. Genomic copy numbers and enrichment levels of LTR retrotransposon subfamilies.

(A) Genomic copy numbers of LTR elements that account for a high proportion of expressed (nascent) copies.

(B) Radar plot showing the enrichment levels of LTR subfamilies with high expressed copy number proportions.

11. Figure 4c: Which samples are in question?

We thank the reviewer for pointing this out. Figure 4c shows RT-qPCR analysis of MTA-int and MTA_Mm knockdown efficiency in zygote-injected embryos collected at the 2-cell stage (24 h post-injection). The Results section has been revised to clarify this.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3 (Remarks to the Author):

Understating the transcriptional dynamics during very early development is an exciting and challenging field. In their manuscript, “Sequential Activation of Retrotransposons Regulate Zygotic Genome Activation and Embryo Development,” Wu, Shi, Yan and colleagues describe systematic measurements of changes in transcription across ZGA, focusing on the role of retrotransposons. They find interesting expression of TEs from the maternal genome and characterize a specific type of chimeric transcript. Overall, this is a very study, but needs substantial work to better contextualize both the new methodology described here as well as the novel biology.

We thank the reviewer for the insightful and constructive comments, which have helped us substantially improve the manuscript. Below we summarize the key revisions we have made.

We have rewritten the title and abstract to clearly state our novel findings and avoid overstatements, and we have significantly improved the clarity and language throughout the manuscript. The advances of our methodology are optimization of chemical conversion for low-input and single-embryo samples, with validation that 4sU incorporation for 4 h does not impair oocyte maturation or blastocyst development. We also provided a computational pipeline that identifies transcriptionally active TE copies at unique genomic loci via stringent mapping and mutation calling, enabling locus-specific resolution essential for our functional discoveries.

We have also added several key experimental results to improve the novelty of the manuscript. First, we performed MTA rescue experiments and found that exogenous full-length MTA RNA could not rescue the knockdown phenotype, suggesting that MTA may function likely involve cis-regulatory mechanisms. Second, we validated that the MTB-Nfya chimeric transcript encodes a protein identical to canonical NFYA, supporting a “dual-engine” promoter model. Finally, we identified NOBOX as a potential oocyte-specific transcriptional activator of full-length MTA elements.

We have addressed all of the reviewer’s comments point-by-point in blue font, and the corresponding figures and their locations in the manuscript have been highlighted in the point-to-point response. We hope these revisions fully address the reviewer’s concerns.

Major points

1) The novelty of the biology is not described with sufficient scholarship. The authors’ title and abstract read as if this is the first description of the sequential waves of temporally controlled expression of retroelements, yet this has been known for over a decade. For example, it is known

that terminal repeats derived from endogenous retroviruses are temporally controlled (see for example, from 2012, DOI: 10.1038/nature11244), and many related findings have been reported in the intervening years. Similarly chimeric transcripts of the type described here are already described (reviewed in DOI: 10.1016/j.tig.2023.10.010). Findings related to these points have already been extended to human embryos (DOI: 10.1126/science.adv5257). The authors are clearly aware of this, as their text in some places is nuanced and emphasizes the authors new findings using allelic analyses of early transcripts and specific TEs/regulatory factors. The authors need to re-write their manuscript (especially their title and abstract) to consistently and clearly indicate what novel claims they are making. Relatedly, the authors tend to overstate their findings, for example by declaring their results to be “comprehensive,” when it is more appropriate to describe them as broad or systematic as there are transcripts and timepoints that have not been fully analyzed.

We fully appreciated the reviewer for this very important suggestion regarding the novelty of our work. We fully agree that we are not the first to describe the concept of temporal waves of retroelement expression. However, the precise identities of the transposable element (TE) families activated in each wave during early embryonic development have not been systematically defined. The current definition of TE waves (Andrew J. et al., Cell, 2021; doi.org/10.1016/j.cell.2021.09.021) was largely derived from re-analysis of single-cell RNA-seq data from early mouse embryos (Xue et al., Nature, 2013; doi.org/10.1038/nature12364). In those studies, the definition of TE expression levels suffered from methodological limitations.

In contrast, our scTLS-seq method uses random primers to capture the full transcriptome of nascent RNA, substantially improving the detection of TE transcripts, particularly at the minor ZGA stage. Furthermore, by using unique-mapping reads to assign repetitive sequences to unique genomic positions, we not only obtained the overall expression levels of each TE family but also identified the exact copy number and genomic locations of transcriptionally active TE copies.

Based on these technical advantages, we discovered that the MT family member MTA is actively transcribed at the 1-cell stage (wave 1), and we uncovered its role in silencing other TEs and in supporting early embryonic development. Using our nascent transcriptome data, we redefined more than 600 TE-derived chimeric transcripts. Notably, we identified for the first time a chimeric transcript formed by MTB and Nfya, and characterized its impact on early development.

In addition, in the revised manuscript we have included the identification of Nobox as a potential transcriptional activator of MTA elements (**Figure 6**).

Following the reviewer’s suggestion, we have revised the manuscript, the abstract, and the title to clearly state our novel claims while avoiding overstatements.

2) The novelty of the methodology also needs to be better contextualized. The authors do a nice job describing their approach to adapt TimeLapse-seq to single-cell analyses, and importantly the authors put considerable effort into the validation of their protocol. Nonetheless, the authors need

to provide context for readers who are not aware that 4-thiouridine-based approaches have been previously adapted for single-cell measurements. The authors' contributions to methodology here are significant but need more context.

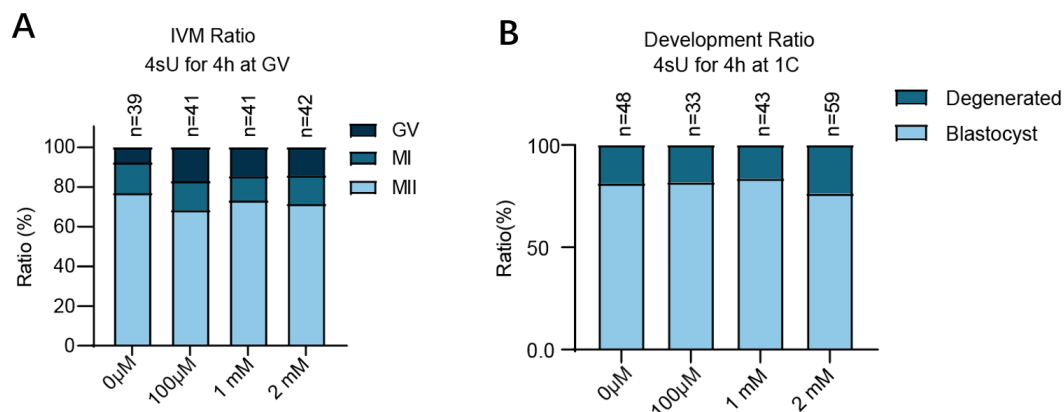
We thank the reviewer for this insightful comment, which helps us better contextualize our methodological contribution.

We fully acknowledge that pioneering single-cell methods based on 4sU metabolic labeling, such as scSLAM-seq (Erhard et al., Nature, 2019) and sciFATE2 (Rory et al., Cell Systems, 2024), have been previously developed. However, these methods were designed to resolve transcriptional differences among individual cells within a population to better characterize cellular heterogeneity, at the cost of low sequencing depth per cell and limited resolution. Moreover, they require a minimum of ten thousand cells as starting material and are therefore not applicable to precious low-input samples such as embryos. In contrast, our work provides a distinct and valuable advance for the field of early embryonic development in two key aspects.

First, our optimization of the chemical conversion chemistry is critical for application to precious embryonic materials. The original TimeLapse-seq protocol was designed for cultured cells with abundant starting material. Our adaptation to low-input and single-embryo settings, validated from 2 µg down to 10 ng or a single embryo, represents a significant technical leap that makes nascent RNA sequencing accessible to research with limited sample availability. In the revised manuscript, we have added data showing the effects of different 4sU concentrations on oocyte maturation and embryo development rates, demonstrating that our labeling conditions do not compromise embryonic development (**Response Figure 10**).

Second, we developed a dedicated computational workflow to resolve the unique complexities of repetitive elements in the genome. While generic nascent RNA methods can quantify total TE expression, our method goes a crucial step further by identifying transcriptionally active TE copies at unique genomic loci. This is achieved through stringent unique mapping and careful mutation calling, allowing us to distinguish the specific TE copies that are transcriptionally active. This locus-specific resolution has been instrumental for our functional discoveries, such as the precise genomic configuration of MTA elements and their transcriptional activation patterns in early embryos.

We have supplement the development rate in **Supplementary Fig.S2d,e** and revised the manuscript to more clearly articulate these methodological innovations and the distinct challenges they address.



Response Figure 10. Effect of 4sU concentration on embryos.

(A) Maturation rates of GV oocytes treated with different 4sU concentrations for 4 hours followed by 24 hours of in vitro maturation (IVM). n: number of oocytes per group.

(B) Blastocyst rates of zygotes treated with different 4sU concentrations for 4 hours and then cultured to the blastocyst stage. n: number of embryos per group.

3) The manuscript needs substantial editing. While many of the issues are merely poorly worded sentences (I'm not sure how a "description" can "lack high-sensitivity," for example, see p3 line 63), there are larger issues too. Important claims are garbled, including the final sentence of their abstract, which reads as if the authors are claiming that their study is what inspired retrotransposons to have an essential role in pre-implantation development.

We thank the reviewer for pointing out these language and clarity issues. We have now thoroughly edited the entire manuscript, corrected poorly worded sentences (e.g., the "description lacks high-sensitivity" phrase), and revised the final sentence of the abstract to avoid any misinterpretation that our work "inspired" retrotransposon functions. The revised sentence clearly states that our study demonstrates the essential roles of TEs. We believe the manuscript is now much clearer and more accurate.

Minor points

1) The authors refer to their scTLS-seq measurements as reporting on "nascent transcripts" throughout most of the manuscript except for the first section in the results, where the authors more accurately describe these transcripts as "newly-synthesized" transcripts. With experiments that last 4 hours and beyond, very few of the transcripts are nascent, which is a term reserved for RNA that is still being transcribed.

We fully agree with the reviewer's semantic distinction. In strict terms, "nascent" refers to RNA chains still engaged with RNA polymerase. However, in the field of 4sU-based transcriptomics, it has become common practice to use "nascent" to operationally denote newly synthesized RNA over a labeling period, typically 10–30 minutes in cultured cells. In early mouse embryos, due to low 4sU uptake efficiency, a longer labeling period (4 h) is required to achieve sufficient signal. To avoid confusion with strictly defined "nascent" RNA, we have added an operational definition in the Results section **clarifying that "nascent" in this study refers to RNA newly synthesized during**

the 4-h labeling window. We believe this balances precision with practical readability.

2) The authors refer to their time-course study as “real time” (p5, line 138) which it is not. We thank the reviewer for pointing this out. We have replaced “real-time” with “temporal” to more accurately reflect the time-course nature of our study. The revision has been made in the manuscript.

3) Why is scTimeLapse-seq abbreviated scTLS-seq as opposed to scTLS or scTL-seq? We thank the reviewer for this suggestion. The abbreviation "scTLS-seq" has been replaced with **scTL-seq** throughout the manuscript.

4) The experiment described in Fig. 2d-e and related supplementary figures is confusing as is the authors' interpretation. Shouldn't new transcription in the whole embryo stop after including the inhibitors?

We apologize for the confusion caused by the description of this experiment. The aim of this experiment was to determine whether transcripts generated at the PN3 stage are processed and exported to the cytoplasm, or whether they represent unprocessed, transient transcripts that are rapidly degraded in the nucleus.

To address this question, we designed the following experimental scheme: First, newly synthesized transcripts were labeled with 4sU for 4 hours at the PN3 stage. After labeling, 4sU was removed and transcription inhibitors were added to block any further RNA synthesis. During the subsequent 4-hour period (in the presence of inhibitors), no new RNA synthesis is expected. We then examined the nucleo-cytoplasmic distribution of the pre-labeled RNAs. If the amount of labeled RNA in the cytoplasm increased over this 4-hour chase period, it would indicate that these transcripts are being actively processed and exported, rather than retained in the nucleus.

In this experiment, “whole embryo” samples served as a control to monitor the total level of labeled RNA, while “cytoplasm” samples specifically reported changes in the cytoplasmic fraction. Our results showed that labeled transcripts from categories C1 and C2 increased in the cytoplasm during the chase period, supporting the conclusion that PN3-generated transcripts are competent for nuclear export.

We have now revised the Results section to include a clearer description of the experimental rationale and details.

5) The steady increase shown in Fig. S3c could be due to an increase in transcription as the authors conclude, but it also could be due to an increase in the T-to-C mutation rate due to changes in the 4SUTP pool.

We appreciate the reviewer's concern. The 4sUTP concentration in the medium and the labeling time (4 h) were identical for all stages tested. Thus, the intracellular 4sUTP pool should not vary systematically across stages. We therefore interpret the progressive increase in T-to-C mutation rate as reflecting genuine increases in transcriptional activity rather than changes in the 4sUTP pool.

6) The figures also need editing and increased clarification. For example, there is a typo in Fig 1a

“existed RNA.” What units are shown on the y-axis of Fig. 1c? Why is it on a different scale from Fig 1.e?

We thank the reviewer for the careful reading and valuable suggestions. The typo in Fig. 1a (“existed RNA”) has been corrected to “existing RNA”. The y-axis label in Fig. 1c has been changed from “Mutation rate” to “Nascent RNA ratio”, defined as the proportion of T-to-C converted reads among all mapped reads, as also revised in [Supplementary Fig. S1c](#). In contrast, Fig. 1e shows the per-base mutation frequency (T to C rate at nucleotide resolution). Therefore, these two panels are based on different levels of quantification (read-level versus base-level), which explains the difference in scale.

Reviewer #4 (Remarks to the Author):

Retrotransposon Elements (RTEs), once regarded as “junk” DNA, has recently become a major research focus, with accumulating evidence revealing their importance in development, disease and cancer. After a long period of transcriptional silence during meiosis, early embryos undergo extensive chromatin remodeling and initiate Zygotic Genome Activation (ZGA), ultimately completing the maternal-to-zygotic transition. However, the transcriptional regulation of RTEs during ZGA and early embryogenesis has remained poorly understood, largely due to the lack of low-input, high-efficiency methods for detecting nascent transcript.

In this submission, the authors developed single-cell TimeLapse-sequencing (scTLS-seq), a technology that enables sensitive detection of nascent RNA from trace material. Using scTLS-seq, the authors reveal the temporal activation dynamics of RTEs during ZGA and uncover their regulatory roles in ZGA. These findings are interesting and contribute valuable insights to both the RTEs and the early embryo development fields.

In my view, several aspects of the study would benefit from further clarification or additional experimental support to strengthen the conclusions. I have outlined a series of comments and concerns below, careful revisions are required for improving the quality of this manuscript.

Summary of responses to Reviewer #4:

We sincerely thank the reviewer for the thorough and insightful comments, which have significantly improved our manuscript.

First of all, we have rewritten the title and abstract to clearly state our novel findings and avoid overstatements, and we have significantly improved the clarity and language throughout the manuscript.

Second, we have provided additional data to define the lower detection limit of our method for nascent RNA, tested the effects of different 4sU concentrations on embryonic development. We have also clarified the SNP calling strategy and allele-assignment pipeline, especially for TE calculation.

Third, we systematically evaluated the specificity of our MTA-targeting ASOs. We performed a rescue experiment by co-injecting full-length MTA mRNA lacking the ASO target sequence. The failure of rescue suggests that MTA's function may depend on its genomic context and transcription activity. Further analysis revealed that MTA knockdown causes downregulation of nearby ZGA genes in a distance-dependent manner, indicating a cis-regulatory role.

Finally, we validated the MTB-Nfya chimeric transcript by RT-PCR and Sanger sequencing, and we showed that it encodes a protein identical to canonical NFYA in size and subcellular localization. Thus, MTB may serve as an alternative promoter ensuring sufficient Nfya expression before ZGA. We also assessed off-target effects of the sgRNAs targeting TE-derived chimeric transcripts (ORR1B1-Setd2 and MT2-Tead4) and found that only the targeted TE families were affected. The canonical transcripts of these genes showed limited compensation, suggesting the essential contribution of the TE-driven isoforms.

We have addressed all of the reviewer's comments point-by-point in blue font, and the corresponding figures and their locations in the manuscript have been highlighted in the point-to-point response. We hope these revisions adequately address the reviewer's concerns. Should there be any further suggestions, we are happy to make additional modifications.

Major comments:

1. Line 115-116: "about 1300 genes detected in IP samples but not in 115 scTLS-seq were low-expressed, likely background noise through IP method. (Supplementary information, Fig. S1g)". As the total RNA input for scTLS-seq is extremely low, I think the 1,300 undetected low-expressed genes are more likely due to limited sensitivity rather than background noise through IP method. Authors should give more evidence to support that these low-intensity genes are not real nascent genes. Besides I wonder what is the lowest RNA abundance that scTLS-seq is actually capable of detecting? In addition, in Fig. S1g, for genes within the same RPKM range, are the sets of genes detected by 4sU-seq and scTLS-seq the same? How high is the overlap between them?

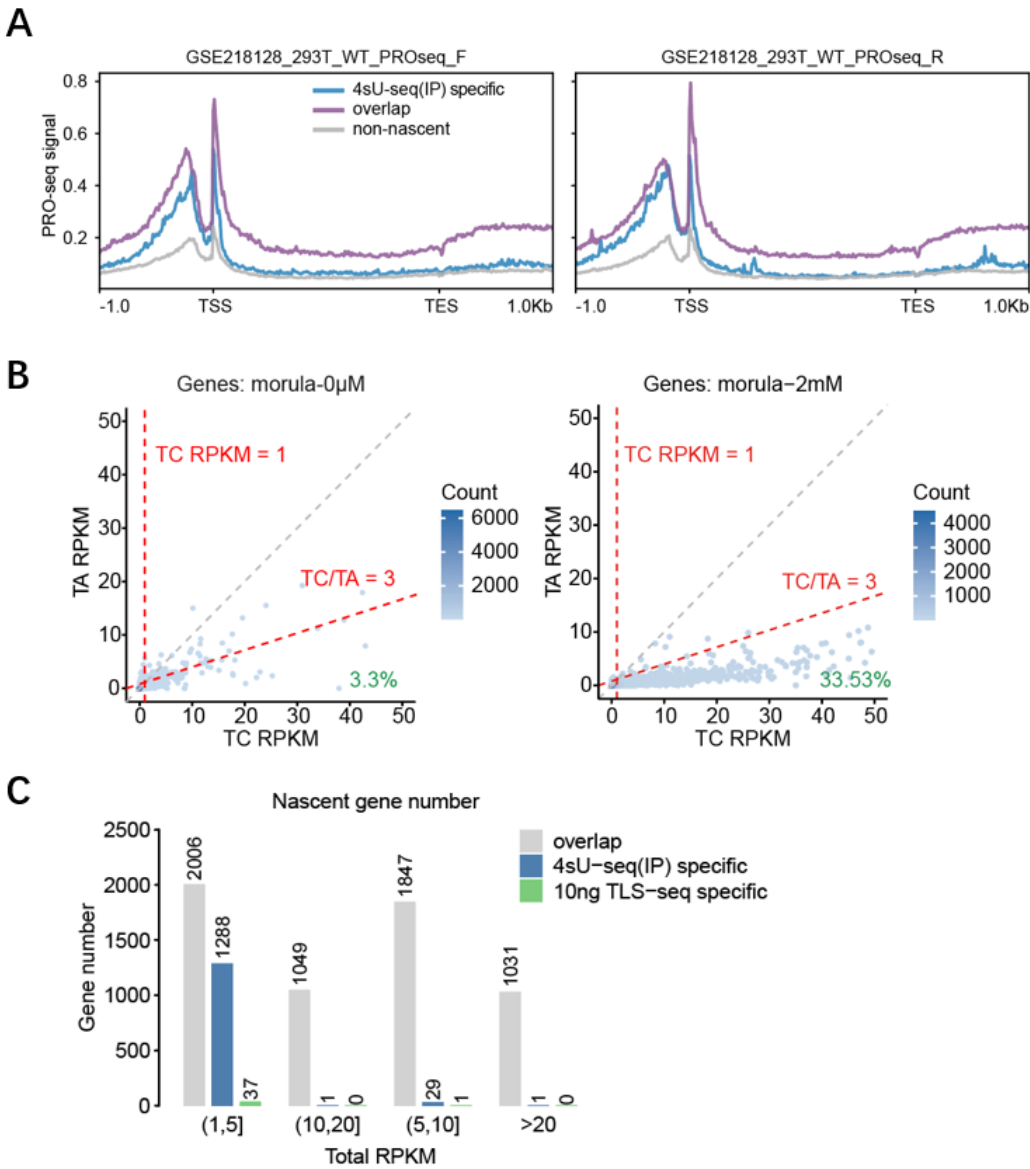
We thank the reviewer for raising these important points regarding sensitivity and detection thresholds of scTL-seq.

First, we agree that our previous description referring to the ~1,300 genes detected by 4sU-seq (IP) but not by scTL-seq as "background noise" was inappropriate. To further evaluate the transcriptional activity of these genes, we examined their signals in PRO-seq data (PMID: 26965480). We found that these IP-only genes (blue lines) exhibited relatively weak transcriptional signals, with profiles across gene bodies and TES regions closely resembling those of non-nascent genes, suggesting low transcriptional activity rather than strong nascent transcription (**Response Figure 11A**). PRO-seq result was also added in **Supplementary Fig. S1i**.

Regarding the lowest RNA abundance detectable by scTL-seq, we evaluated the performance of different thresholds using labeled and unlabeled control samples (**Response Figure 11B**). We observed that a considerable fraction of low-abundance genes (nascent RPKM < 1) could still be detected in labeled samples, indicating that scTL-seq is capable of capturing low-level T-to-C

conversion signals. To ensure robust identification of true nascent transcription while minimizing false positives arising from background mutations, we selected a stringent cutoff based on the distribution of signals in labeled versus unlabeled controls ($T\text{-to-C}/T\text{-to-A} > 3$), which provides an optimal balance between sensitivity and specificity. This threshold-based filtering ensures reliable detection of nascent transcripts while reducing random PCR- or sequencing-derived mutations, and was applied uniformly in downstream analyses.

Regarding the question: in Fig. S1g, for genes within the same RPKM range, are the sets of genes detected by 4sU-seq and scTL-seq the same? We further analyzed the overlap between the two methods across different RPKM ranges. For genes with $RPKM > 5$, the overlap was as high as 99%. For genes in the $(1, 5]$ RPKM range, the overlap was approximately 60%, and the discrepancy was mainly due to 4sU-seq (IP) detecting 1288 more genes (**Response Figure 11 C**). This result was also added in **Supplementary Fig. S1h**.



Response Figure 11. Comparison of nascent gene detection between scTL-seq and 4sU-seq (IP).

(A) Overlap and specific number of nascent genes identified by scTL-seq and 4sU-seq (IP) across different RPKM ranges.

(B) T-to-C(TC) and T-to-A(TA) reads level of each gene (RPKM) in morula samples with 0 μ M 4sU (left) and 2 mM 4sU treatment (right). Grey dashed line represents TC/TA=1. Red dashed line represents TC=1 and TC/TA=3, green number represents gene percentage with TC>1 and TC/TA>3.

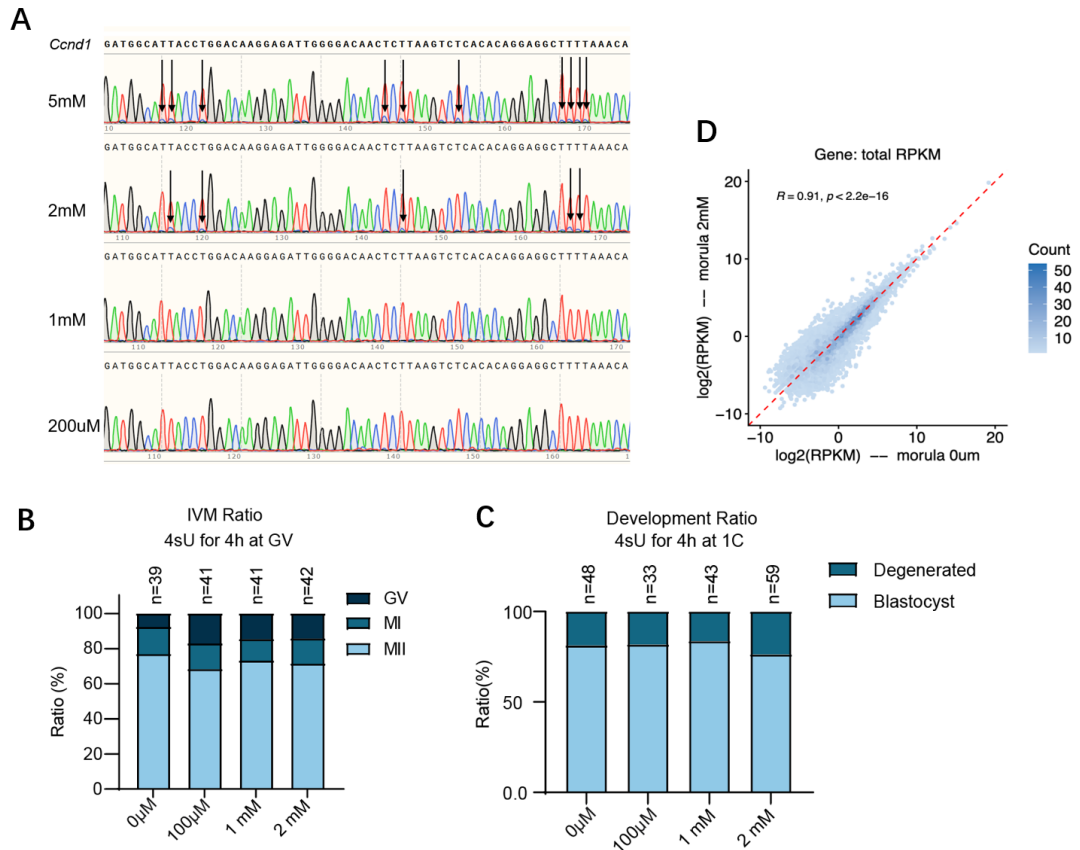
(C) PRO-seq signal intensity profiles (-1 kb of TSS to +1 kb of TES) for overlapping nascent genes, 4sU-IP specific genes, and non-nascent genes in 293T cells (data from GES218128). Left panel: forward sequences PRO-seq. Right panel: reverse sequences of PRO-seq.

2. Line 123-124: “Testing different concentrations led us to determine that a 2mM 4sU treatment for 4h is optimal for nascent RNA labeling in early embryos...” The 4sU concentration used (2mM) is relatively high, and the treatment duration (4 hours) is long. Therefore it is important to assess whether such conditions might disturb the homeostasis of oocytes and early embryos. The authors should provide the IVM and developmental rates under 2mM 4sU treatment. Furthermore, for the 4sU concentration gradient, 2mM is 20-fold higher than 100 μ M, it would be valuable to know whether the effects of 1mM 4sU exerts effects comparable to 2mM.

We thank the reviewer for raising these important points regarding 4sU concentration and treatment duration.

Regarding the 4sU concentration selection, we initially tested different 4sU concentrations in mouse embryonic stem cells (R1) by Sanger sequencing of a highly transcribed gene (*Ccnd1*). We observed clear T-to-C mutation peaks at 2 mM and 5 mM, whereas 1 mM and 200 μ M did not produce obvious T-to-C signals (**Response Figure 12A**). Therefore, we did not test additional intermediate concentrations like 1 mM in embryos.

To address whether 2 mM 4sU treatment for 4 hours perturbs homeostasis of oocytes and early embryos, we performed additional developmental assays using different 4sU concentration. The results showed that 2 mM 4sU treatment for 4 hours did not affect the in vitro maturation (IVM) rate of GV oocytes, nor did it affect the blastocyst rate when embryos were treated at the 1-cell stage for 4 hours (**Response Figure 12B, C**). Furthermore, total RNA levels in morula-stage embryos were comparable between the 2 mM 4sU-treated and untreated groups, with a correlation >0.9 (**Response Figure 12D**). These data indicate that the 2 mM, 4-hour treatment has no obvious detrimental effect on early embryonic development. We have now included these developmental rate results in the revised manuscript (**Supplementary Fig. S2d, e**).



Response Figure 12. Effect of 4sU concentration on embryos.

(A) T-to-C mutation rates in *Ccnd1* transcripts detected by Sanger sequencing after reverse-transcription PCR from ESCs treated with different 4sU concentrations. Arrowheads indicate a C peak beneath the T peak, representing T-to-C conversion.

(B) Maturation rates of GV oocytes treated with different 4sU concentrations for 4 hours followed by 24 hours of in vitro maturation. n: number of oocytes per group.

(C) Blastocyst rates of zygotes treated with different 4sU concentrations for 4 hours and then cultured to the blastocyst stage. n: number of embryos per group.

(D) Gene expression levels and correlation between 2 mM 4sU-treated and unlabeled morula-stage embryos. Color depth represents gene density.

3. Line 146 and Figure 1g. It appears as though there is roughly double the amount of nascent transcription occurring at GV compared to PN3. Is this accurate? It suggest that the GV oocyte has detectable and relatively (to PN3 and PN5) higher translation. How does this compare to previous methods? I'm under the assumption that that the level was too low to detect, or at least lower than Minor ZGA. **Could this mean in PN stages, transcripts are made and quickly degraded?** Also, GV-stage oocytes can be classified into Surrounding Nucleus (SN) and Non-Surrounding Nucleus (NSN) based on chromatin condensation. Meiosis in SN GV oocytes is expected to be transcriptionally silent. It is unclear whether the authors separated SN and NSN oocytes, and whether all sequenced oocytes were NSN.

Furthermore, in Fig. S2a, the T > C mutation ratio in 0uM group appears higher than in the 100 uM group, raising the concern that the treatment itself may introduce mutations. Therefore, an MII group

treated with 2mM 4sU would help to further validate the robustness of scTLS-seq.

We thank the reviewer for the detailed questions and concerns.

Question 1: It appears as though there is roughly double the amount of nascent transcription occurring at GV compared to PN3. Is this accurate? To address the transcriptional activity between GV and PN stages, we performed EU labeling on GV and PN3 embryos. The results showed that EU intensity was higher in GV oocytes than in 1-cell stage embryos (**Response Figure 13A, B**). Together, these data indicate that GV oocytes (mainly the NSN subpopulation) have higher transcriptional activity than 1-cell embryos.

Question 2: Could this mean in PN stages, transcripts are made and quickly degraded? As the reviewer noted, we have tested whether PN3-generated transcripts can be processed and exported to the cytoplasm (**Fig. 2d, e**). Our results demonstrate that a substantial fraction of PN3 nascent RNAs are not rapidly degraded (as the RNA levels in whole embryos did not decrease dramatically after 4 hours) but are instead exported to the cytoplasm.

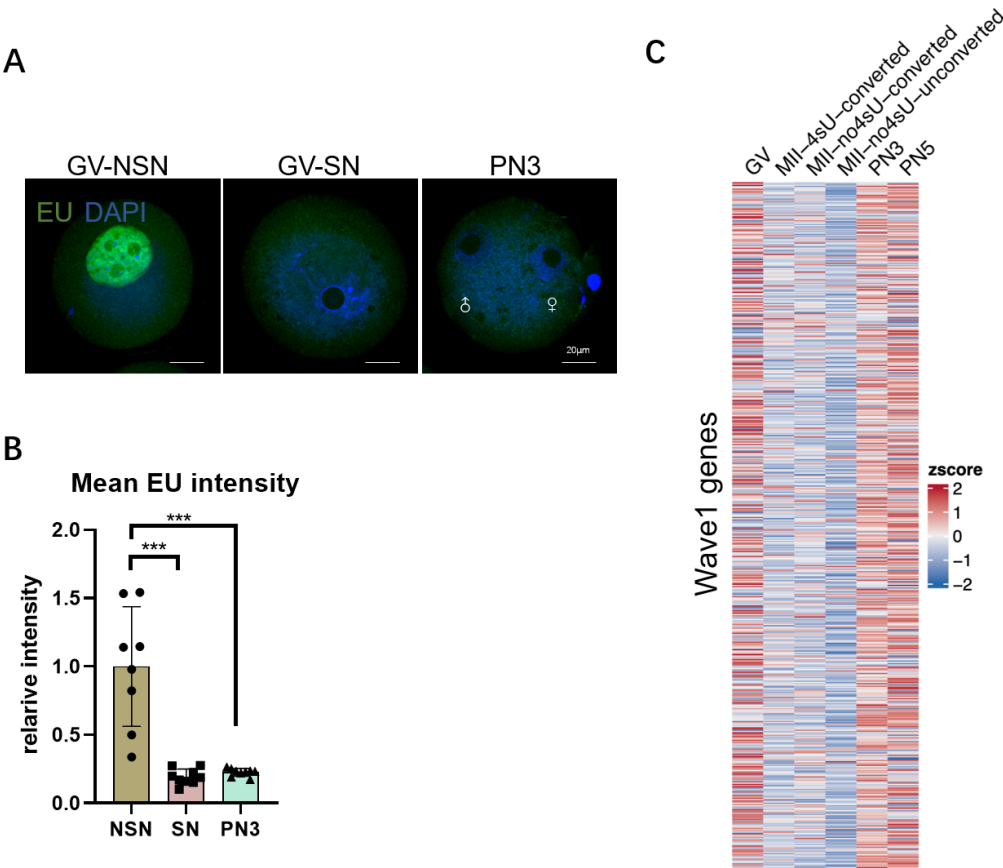
Question 3: It is unclear whether the authors separated SN and NSN oocytes, and whether all sequenced oocytes were NSN? Regarding the transcriptional activity at the GV stage and NSN/SN classification, we did not separate NSN and SN oocytes; instead, we pooled about 20 GV-stage oocytes for a replicate for scTL-seq. Based on our previous experience and published reports (PMID: 37831603), NSN oocytes represent approximately 30–50% of the GV pool, and it is well proved that NSN oocytes are transcriptionally active (PMID: 36306357). To confirm this, we performed EU labeling on sorted NSN and SN oocytes. Consistent with previous reports, NSN oocytes showed strong transcriptional signals, whereas SN oocytes exhibited no detectable EU incorporation (**Response Figure 13A, B**). Therefore, it is not surprising that pooled GV oocytes display detectable transcriptional activity.

Question 4: in Fig. S2a, the T > C mutation ratio in 0μM group appears higher than in the 100 μM group, raising the concern that the treatment itself may introduce mutations. Concerning the high T-to-C mutation ratio in the 0 μM group (Fig. S2a), we acknowledge that the background T-to-C mutation rate appears higher than other mutation types. This is because we intentionally lowered the mapping quality threshold for T-to-C reads to retain as many mutation-carrying reads as possible, given that such mutations can reduce mapping scores. Therefore, the raw T-to-C ratio should not be interpreted as absolute transcription level. To rigorously define nascent transcripts, we applied a strict cutoff: T-to-C / T-to-A ratio > 3 and nascent RPKM > 1 (for TE copy, nascent RPM>0.3). All subsequent clustering and functional analyses (Fig. 2, etc.) were based on these stringently defined nascent genes, not on raw mutation ratios. Thus, the main conclusions are robust against background noise.

Question 5: an MII group treated with 2mM 4sU would help to further validate the robustness of scTLS-seq. To evaluate whether the labeling/conversion process itself introduces mutations, we performed additional sequencing on three MII oocyte groups: (i) no 4sU, no conversion; (ii) no 4sU, with conversion; (iii) 2 mM 4sU labeled, with conversion. A heatmap of wave-1 genes across these MII groups and other developmental stages showed that nascent RPKM in MII samples was consistently lower than in PN stages (**Response Figure 13C**). Moreover, the conversion-only group

showed similar nascent RPKM to the unconverted control, indicating that the chemical conversion step does not artificially introduce mutations. These results validate the robustness of our method.

We have not included these results in the current manuscript. However, if the reviewer wishes us to present these validation data in the Results section, we are glad to further revise the manuscript accordingly.



Response Figure 13. Transcriptional activity in oocytes and 1-cell.

(A) EU incorporation in NSN and SN GV oocytes and PN3 1-cell embryos.

(B) Quantification of EU signal intensity in the samples shown in (A).

(C) Heatmap showing nascent RNA levels (nascent RPKM) of wave-1 genes across MII control groups and other developmental stages.

4. Line 217: Does the PWK reference genome have a sufficient assembly (T2T?) for this analysis? Could this factor into why Maternal (B6) reads are more abundant? The detection sensitivity can be influenced by the number of informative SNPs present in the paternal and maternal genomes. The authors should clarify whether the numbers of detectable SNPs from the two parental genomes are comparable, and the reciprocal cross would clarify this.

We thank the reviewer for raising this important point.

Question 1: Does the PWK reference genome have a sufficient assembly(T2T)?

To our knowledge, a telomere-to-telomere (T2T) assembly for the PWK strain is not yet available.

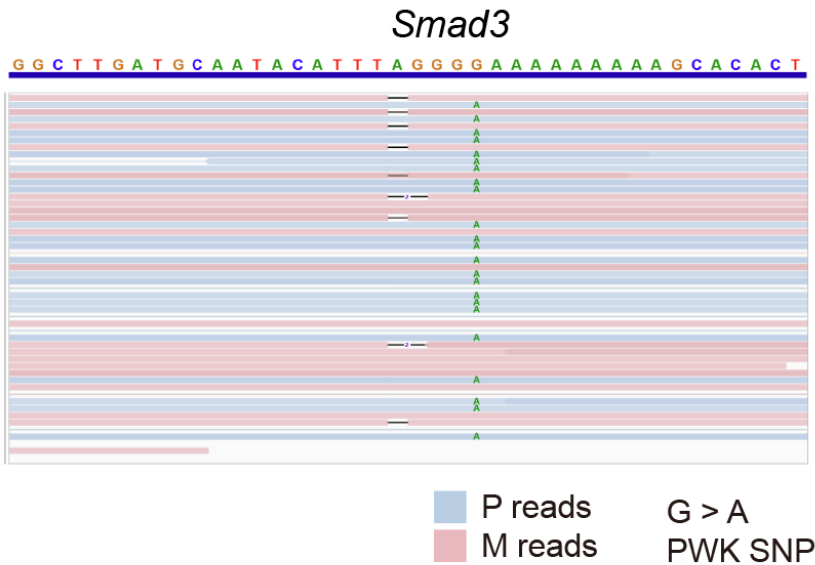
Question 2: Could this factor into why Maternal(B6) reads are more abundant?

The reviewer raised a valid concern about whether the difference in SNP numbers between PWK and B6 might bias the detection of parental-origin transcripts. We used the PWK strain as the paternal line precisely because it provides a larger number of informative SNPs for allele assignment. The current numbers are: **20,308,039 SNPs for PWK and 7,293 for C57**. We retained only strain-specific SNPs and applied stringent quality filters (excluding sites within 5 bp of a read end or with base quality < 30). Potential 4sU-induced mutations were also discarded.

Reads were then classified into three categories: maternal, paternal, or unsplit as follows:

- 1) Reads containing a PWK-specific SNP were assigned as paternal; reads containing a C57-specific SNP were assigned as maternal.
- 2) When multiple SNPs were present in the same read, the parental origin was determined by majority voting.
- 3) Reads lacking SNPs but mapping to regions that were uniquely defined as PWK- or C57-specific based on SNP-containing reads were assigned to the opposite parental origin.**
- 4) Reads mapping to regions lacking strain-specific SNPs or containing equal numbers of maternal and paternal SNPs were classified as unsplit.

Because we also utilize SNP-free reads that fall into allele-informative regions, our method effectively leverages the full set of informative genomic intervals rather than being limited to reads that happen to capture a SNP. This design ensures that the final allelic read counts are not biased by differences in the total number of SNPs between the two parental genomes. The schematic diagram for distinguishing parental-origin transcripts was shown in [Fig.2f](#). A representative example (fragment of *Smad3*) of allele assignment is shown below, where G>A is a PWK SNP.



Question 3: Reciprocal cross:

We agree that a reciprocal cross (PWK♀ × B6♂) would provide additional validation. However, based on our own observations, embryos from this cross suffer from severe developmental failure, likely due to cytoplasmic-nuclear incompatibilities. Such abnormalities would confound any transcriptional analysis, and therefore the reciprocal cross could not be reliably performed in this study.

5. Line 220 to 229: The mapping of TEs is significantly more difficult using this approach. The combination of 4sU along with B6vsPWK is not trivial. Much more information on how this analysis was done is needed. Can you further clarify in lines 676–686?

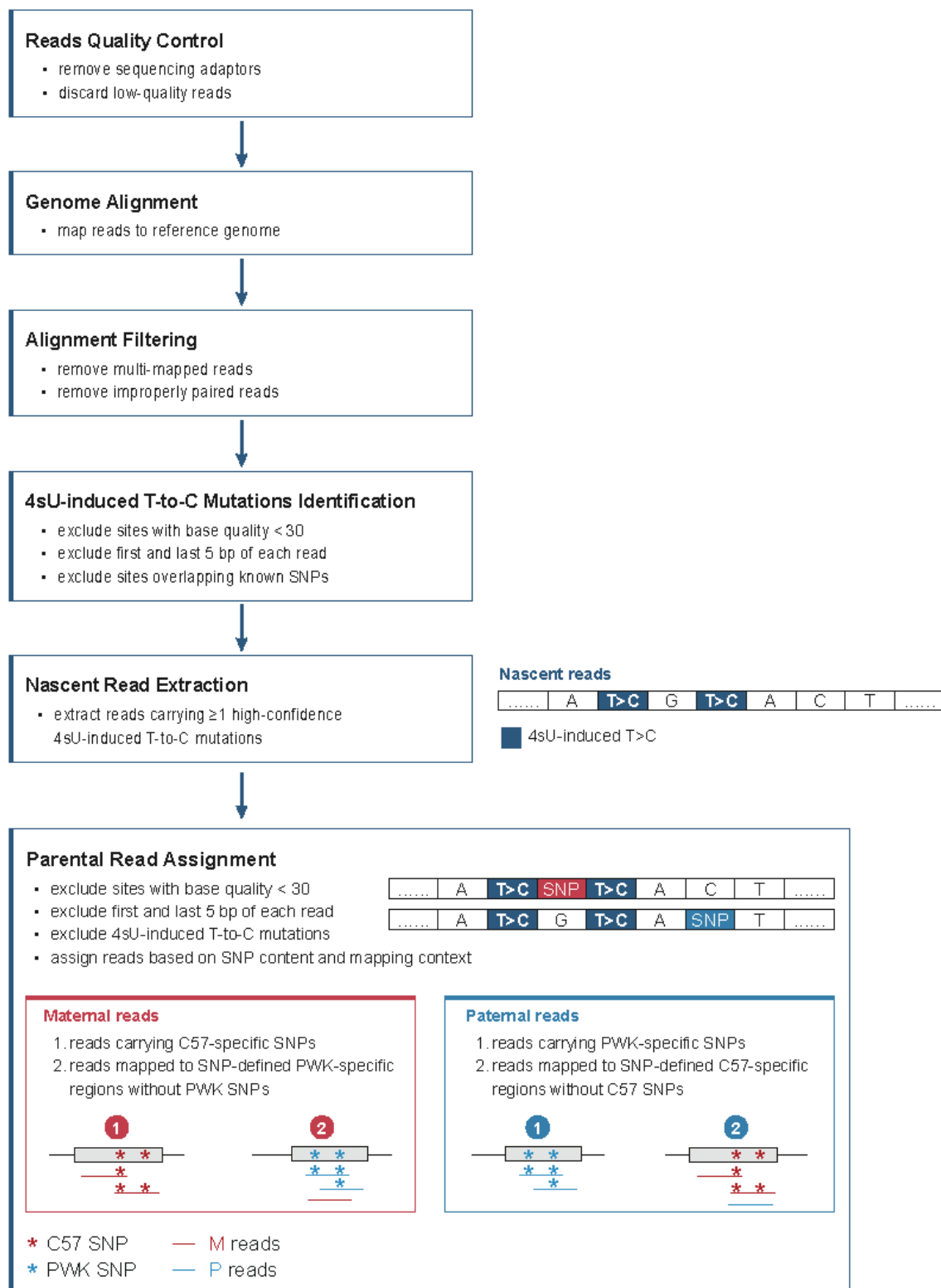
We thank the reviewer for raising this important question.

We agree that analyzing TE expression in scTimeLapse-seq data is technically challenging, as it requires both accurate assignment of reads derived from highly repetitive TE loci and reliable discrimination of true 4sU-induced T-to-C conversions from naturally occurring SNPs in the B6/PWK hybrid background.

In this study, all reads were first aligned to the whole reference genome (GRCm38/mm10), rather than directly mapped to TE consensus sequences. To reduce mapping ambiguity caused by the high sequence similarity among TE copies, only uniquely mapped reads (i.e., reads that could be confidently assigned to a single genomic locus) were retained for downstream analysis and treated as total reads. Subsequently, nascent reads were defined as those containing at least one high-confidence 4sU-induced T-to-C mutation and were assigned to individual TE loci according to RepeatMasker annotations.

To avoid misclassification of strain-specific polymorphisms as nascent transcription signals, several filtering steps were applied. Specifically, strain-specific SNPs for the B6 and PWK genomes were obtained from the Mouse Genomes Project, and all known SNP sites were excluded during mutation calling. In addition, sites with base quality < 30 and mutations located within 5 bp from either end of reads were removed to minimize potential sequencing and alignment artifacts.

The overall workflow was simplified as the below schematic. We have added these clarifications to the revised Methods section.



Response Figure: Schematic of pipeline distinguishing allelic-specific TE reads.

6. Line 262: Could you go into more detail about how unique mapping was done in the context of T to C mutation as the output of scTLS-Seq? As I understand it, mapping of TEs is initially difficult to achieve with traditional methods, so I would imagine applying this additional analysis

was a challenge. How was it handled? the unique mapping procedure in line 647 using the parameter ‘--unique-only’. The methods do not go into sufficient detail.

We thank the reviewer for raising this important question.

In this study, we used the HISAT-3N --unique-only parameter to perform unique mapping. This option retains only reads that have a single best alignment in the reference genome, while reads with multiple equally optimal alignment locations (multi-mapping reads) are discarded, thereby reducing mapping ambiguity caused by the high sequence similarity among transposable element (TE) copies.

Although 4sU-induced T-to-C mutations introduce nucleotide mismatches at the read level, HISAT-3N is designed for nucleotide-conversion sequencing technologies and allows such conversions during alignment; therefore, these mismatches are not expected to substantially affect alignment quality or the unique-mapping criterion.

We further retained only properly paired reads (SAM flags 99, 147, 83, and 163) to improve alignment reliability. Both unique mapping and paired-end filtering steps were performed prior to 4sU-induced T-to-C mutation analysis and TE quantification.

Finally, nascent reads were assigned to individual TE loci based on RepeatMasker annotations for downstream expression quantification.

7. Line 269 to 282: The interpretation of these TEs being cis regulatory units might be premature. The null hypothesis here would be they are simply inheriting the local epigenetic and transcriptional landscape of the nearby gene. The next few sections showing positive correlation could also support the null hypothesis.

We thank the reviewer for this constructive comment and fully agree with the point raised. In the present study, we did not experimentally validate whether each TE with strong co-transcriptional correlation with a nearby gene indeed functions as a cis-regulatory element. Instead, we used genomic positioning and co-transcriptional relationships to infer which LTR families are more likely to possess such regulatory potential. Our data indicate that ERVL and ERVK elements show stronger co-transcriptional associations with adjacent genes. The cis-regulatory function of some of these elements (e.g., MT2 as a promoter for Tcstv3 and Zfp352) has been demonstrated in other systems such as ESCs (Genes & Dev. 2011. 25: 594-607, PMID: 21357675), which supports our proposal that they may act as cis-regulatory units.

We agree with the reviewer that the null hypothesis that these TEs simply inherit the local epigenetic and transcriptional landscape of the neighboring gene, is both conservative and reasonable. Accordingly, we have softened our conclusions in the revised manuscript to better reflect this possibility.

8. As clarified by the author and shown in Fig S7c and S7e, the ASO used in this study also target other MT family members because of the sequential similarity. Therefore, the current data are not sufficient to support the conclusion that Transcription of MTA is essential for ZGA. To strengthen this, I recommend performing an MTA rescue experiment to determine whether resupply MTA can rescue early embryonic development.

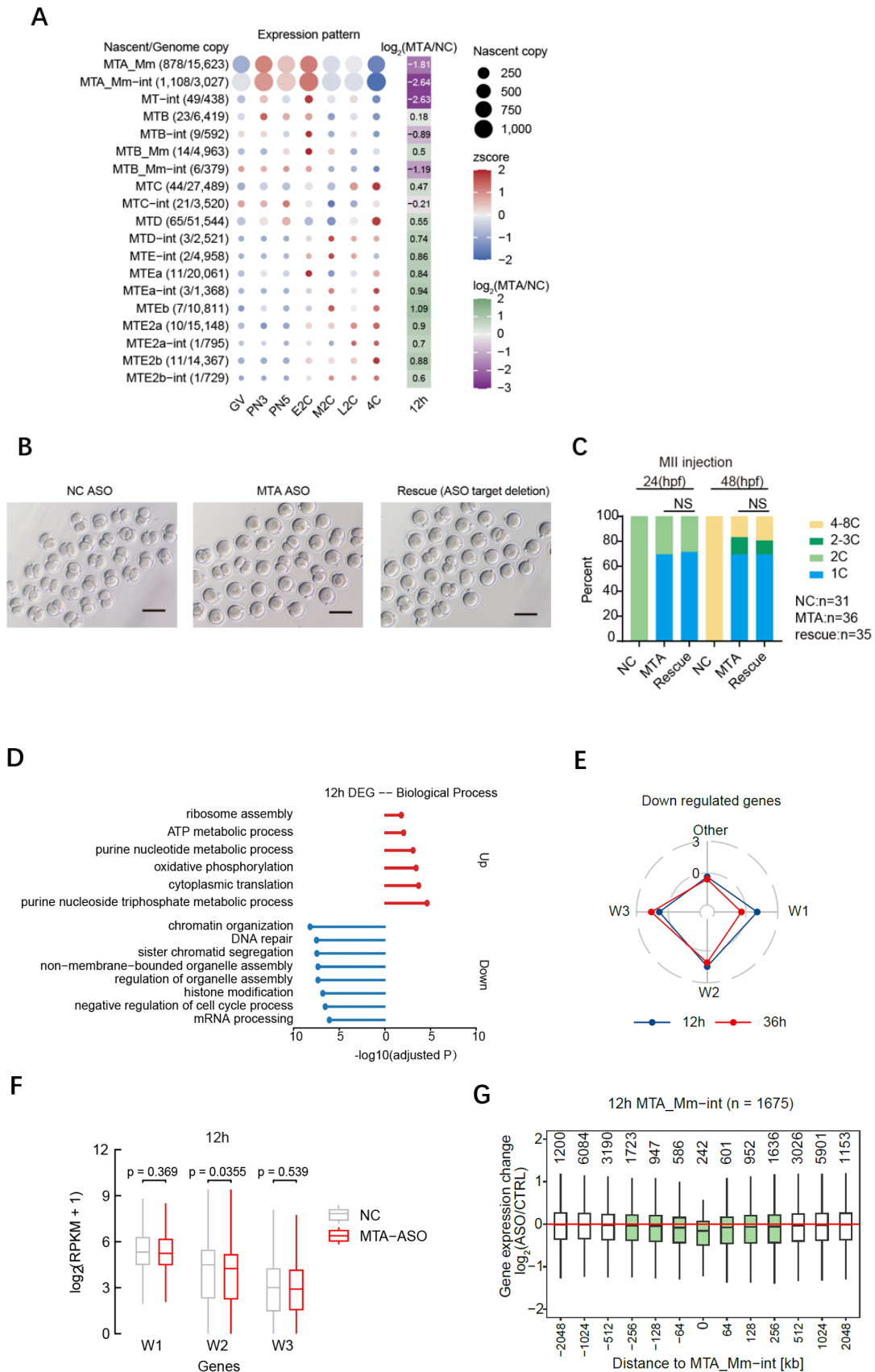
We thank the reviewer for this constructive suggestion.

First, to evaluate the specificity of the ASO sequences, we examined the expression changes of all MT family members at 12 hpf after ASO injection into MII oocytes. The most pronounced downregulation was detected for MTA_Mm-int and MTA_Mm, followed by MT-int, while other MT families showed no significant changes (MTB-int and MTB_Mm-int trended downward). In addition, genomic and transcribed copy number analyses further revealed that, aside from MTA, other MT family members (including MT-int, MTB-int and MTB_Mm-int) have substantially fewer active copies, indicating the impact on these family members is limited (**Response Figure 14A**). The corresponding results have been revised in the manuscript (**Fig4c, Supplementary Fig. S7g**).

To address the concern whether MTA itself is essential for early development, we performed a rescue experiment. We co-injected MII oocytes with MTA-targeting ASOs together with full-length MTA mRNA lacking the ASO target sequence (Δ target) as rescue group. As a result, exogenous full-length MTA RNA failed to rescue the developmental arrest caused by MTA knockdown (**Response Figure 14B,C**). The phenotype was indistinguishable between the knockdown and rescue groups.

To find potential reasons for this lack of rescue, we further analyzed the categories of downregulated genes at 12 hpf after MTA knockdown and their distance to MTA. GO analysis revealed that the 205 downregulated genes at 12 hpf were mainly enriched in chromatin organization, DNA repair, and sister chromatid segregation, correlating with the 1-cell arrest phenotype (**Response Figure 14D**). The downregulated genes at 12 hpf showed relatively broad enrichment across waves 1–3 (**Response Figure 14E**). When comparing the overall expression changes of wave-specific genes, wave 2 genes were significantly downregulated at 12 hpf (**Response Figure 14F**). We then examined whether these aberrantly expressed genes are located close to the knocked-down MTA elements. We calculated the expression changes of genes at various distances from the knocked-down MTA_Mm-int copies. At 12 hpf, genes closer to MTA showed more pronounced downregulation (**Response Figure 14G**). These results suggest that MTA may have a cis-regulatory function in promoting the expression of nearby ZGA genes.

Taken together, we consider that MTA transcription is important and may act as a cis-regulatory element to promote the activation of nearby ZGA genes (mainly wave 2), to ensure developmental progression. The failure of full-length MTA RNA to rescue the phenotype suggests that the lack of MTA RNA is not the key reason of developmental arrest; the relevant reasons are discussed in detail in the Discussion section. All of these results have been added to the Results section (**Fig. 4i,j; Supplementary Fig. S7e–g, S8a,d**).



Response Figure 14. MTA rescue and MTA regulatory function.

A. Expression patterns and knockdown levels of MT family members. Left numbers indicate

nascent and genomic copy numbers. Bubble plot shows expression changes at different developmental stages (bubble size represents nascent copy number). Right heatmap shows RNA fold-change at 12 hpf after ASO injection in MII oocytes.

B. Representative phase-contrast images of embryos at 24 h post-fertilization from NC, MTA-ASO and rescue (Δ ASO target) groups with MII injection. Scale bars, 100 μ m.

C. Developmental rates of NC, MTA-ASO, and rescue groups at 24 h and 48 h post-fertilization. Chi-square test; NS, not significant.

D. Gene ontology of differential expressed genes (DEGs) at 12 hpf after MTA-ASO when MII injection.

E. Rader plot showing down-regulation genes enrichment in W1-3 at 12 and 36 hpf after MTA-ASO when MII injection.

F. Boxplot showing expression levels of W1-3 genes at 12 and 36 hpf in NC and MTA-ASO samples when MII injection. p-values from Wilcoxon rank-sum test are shown.

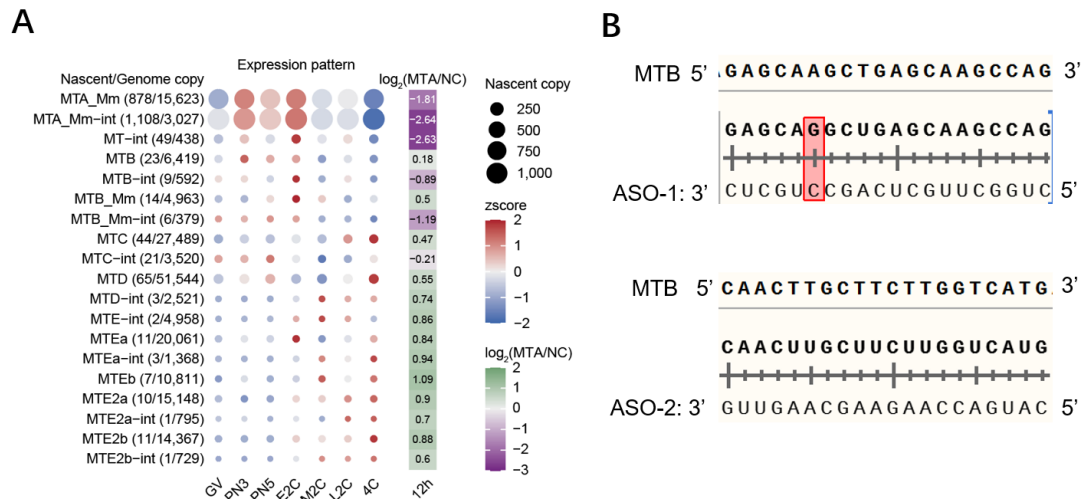
G. Boxplot showing expression change for the genes within indicated distances to the downregulated MTA_Mm-int upon MTA ASO at 12h (hpf) after MII injection. The number of expressed genes (RPKM >1 in Ctrl or ASO samples) in each distance category is annotated above the boxes.

9. Line 292 and Figure 4C: I am not sure the figures shows this. The KD just shows the MTA_Mm-int and MTA_Mm in the main figure while S7a-c just shows MT-INT goes down (can you also show MTB and MTC LTRs? This would be consistent with the upcoming data on the Nfya section. Or does just the MTB driving Nfya decrease expression (S7e)?

We thank the reviewer for this careful observation.

To address the specificity of ASO knockdown across MT family members, we examined the expression changes of all MT family members at 12 hpf after ASO injection into MII oocytes. The most pronounced downregulation was detected for MTA_Mm-int and MTA_Mm, followed by MT-int, while other MT families showed no significant changes (**MTB and MTC, did not show downregulation**, MTB-int and MTB_Mm-int trended downward). Besides, apart from MTA (MTA_Mm-int and MTA_Mm), other MT family members each had limited active copies, and their expression patterns were not identical to those of MTA (**Response Figure 15A**). Therefore, our ASO pool primarily affects MTA and MT-int, with no detectable impact on MTB or MTC under our experimental conditions.

Regarding the effect on MTB-Nfya (original Fig. S7e), we examined whether the MTB-Nfya chimeric transcript contains sequences complementary to our ASOs. Sequence alignment revealed that ASO-1 has a single-base mismatch with MTB, while ASO-2 is perfectly complementary (**Response Figure 15B**). This likely explains why we observed a decrease in Nfya expression in the original knockdown sample. However, we consider this an isolated case rather than representative of general MTB knockdown. To avoid potential misinterpretation, we have decided not to include this single data point (original Supplementary information, Fig. S7e) in the revised manuscript. The specificity results have been revised in the manuscript (**Supplementary information, Fig. S7c**).



Response Figure 15. Specificity of MTA-ASO.

(A) Expression patterns and knockdown levels of MT family members. Left numbers indicate nascent and genomic copy numbers. Bubble plot shows expression changes at different developmental stages (bubble size represents nascent copy number). Right heatmap shows RNA fold-change after MTA-ASO treatment at 12 h.

(B) MTA-ASO-1 and ASO-2 targeting MTB within MTB-Nfya.

10. Line 323: This result is somewhat unexpected. KD of MTA leads to an arrest, which could suggest other TEs would also be arrested or stalled, but instead you observe premature activation of subsequent TEs, suggesting they take on the TE transcriptional profile of later stage embryos, presumably 4C based on the ERVK levels. Can you speculate as to “how”? The DNASE I-TUNEL assay is not clear to me. I understand DNASE I sensitivity suggests open chromatin and TUNEL should label the newly formed breaks, but how does just TUNEL staining look in both conditions without DNase? The H2AX in S7I has a somewhat downward trend in the MTA-ASO but is N.S. ZGA is associated with opening of male and female pronuclei, which is a fairly quick process after fertilization, with the male pronuclei opening quicker. Might we be seeing the timing of this process altered due to the ASO interference? In the representative image in S7I, the female pronuclei appears “smaller” in the MTA-ASO vs NC-ASO. Can you go back and measure these ratios on the Zygotes used to quantify in S7M?

We thank the reviewer for these insightful questions and for the opportunity to clarify our results and interpretations.

Question 1: Why does MTA knockdown cause activation of downstream TEs (e.g., ERVK)?

We thank the reviewer for raising this important question, which is also of great interest to us. Further analysis of the MTA knockdown data revealed that MTA knockdown leads to downregulation of neighboring genes at 12 hpf (**Response Figure 16A**), which may occur through a cis-regulatory mechanism. However, the upregulation of 392 TE families at 12 hpf was not correlated with genomic distance (**Response Figure 16B**), suggesting that MTA may regulate TE silencing in trans via its RNA.

Preliminary data from Chromatin Isolation by RNA Purification sequencing (ChIRP-seq) experiments in embryonic stem cells (ESCs) suggest that MTA RNA directly binds to approximately thousands of retrotransposon loci with high enrichment (**Response Figure 16C,D**), potentially suppressing their transcription and modulating their chromatin state. Therefore, we currently propose that the increased chromatin accessibility upon MTA knockdown is primarily due to the loss of MTA RNA, which relieves the suppression of other TEs, leading to their widespread activation and subsequent chromatin opening.

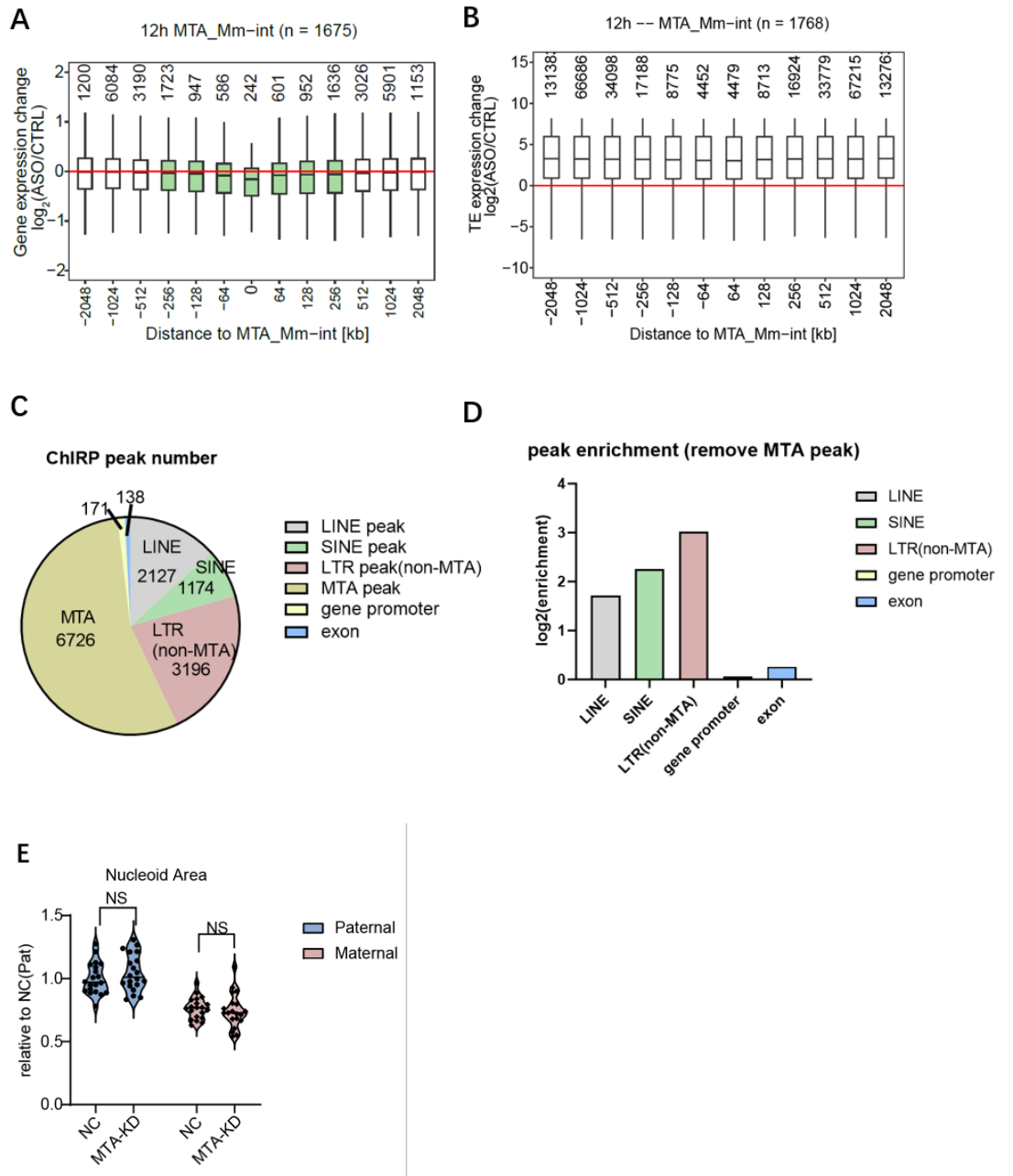
Gene and TE expression change results are supplement in **Fig.4j** and **Supplementary Fig.S8k**. Since these ChIRP-seq results from ESCs are unpublished, we have not included them in the current manuscript. Nevertheless, we have added a discussion of this hypothesis in the revised Discussion section.

Question 2: Clarification of the in vivo DNaseI-TUNEL assay.

We adapted this method from a previous study (Jachowicz et al., Nat. Genet. 2017; 49:1502–1510) to assess chromatin accessibility in small numbers of early embryos. Briefly, viable embryos are briefly permeabilized, then treated with DNase I for 5 minutes, which cleaves only open chromatin regions. After fixation, TUNEL labels the newly created DNA breaks, and fluorescence intensity reflects the extent of open chromatin. This assay measures accessibility, not apoptosis. In contrast, to assess potential DNA damage, we used γ H2AX staining (**Supplementary Fig. S8m,n**). Although TE over-activation might theoretically cause genomic instability, we observed no significant increase in γ H2AX signal upon MTA knockdown, indicating that developmental arrest in MTA-depleted embryos is not primarily caused by DNA damage.

Question 3: Effect of MTA knockdown on pronuclear size.

The reviewer noted that the female pronucleus appears “smaller” in the representative image. To address this, we quantified the areas of both male and female pronuclei at 12 hpf after MTA ASO injection. As shown in **Response Figure 16E**, no significant difference was observed between MTA-ASO and control groups. Therefore, we do not consider that MTA knockdown substantially affects pronuclear size. The specificity results have been revised in the manuscript (**Supplementary Fig. S8l**).



Response Figure 16. Effect of MTA knockdown.

A. Boxplot showing expression change for the genes within indicated distances to the downregulated MTA_Mm-int upon MTA ASO at 12h (hpf) after MII injection. The number of expressed genes (RPKM >1 in Ctrl or ASO samples) in each distance category is annotated above the boxes.

B. Boxplot showing expression change for the TEs (MT families excluded) within indicated distances to the downregulated MTA_Mm-int upon MTA ASO at 12hpf after MII injection. The number of expressed TEs (RPKM >0.3 in Ctrl or ASO samples) in each distance category is annotated above the boxes.

C. Peak number and peak ratio of MTA ChIRP-seq in ESCs (preliminary data).

D. Peak enrichment (remove MTA peaks) of MTA ChIRP-seq in ESCs.

E. Nucleoid area of paternal and maternal pronuclei at 12 hpf after MTA-ASO injection. Student t-

test; NS, not significant.

11. Line 355 and figure 5B: This is a little confusing. MTB appears to be an Oocyte (GV) family, which is supported by the Chimera's expression (Fig5C). The previous results suggested that after MTA KD, an accelerated TE profile was seen (we see 2C and 4C TEs prematurely). I am a little confused how MTB was now being focused on. Was the link meant to be from Chromatin accessibility (from the previous section)? The Chimera expression seems to vanish prior to ZGA. Does the protein persist into 2C (where the chromatin role was reported in line 354)? How do we know this is the Chimera or Canonical action? They appear to be equal to each other in terms of expression (5B and 5C).

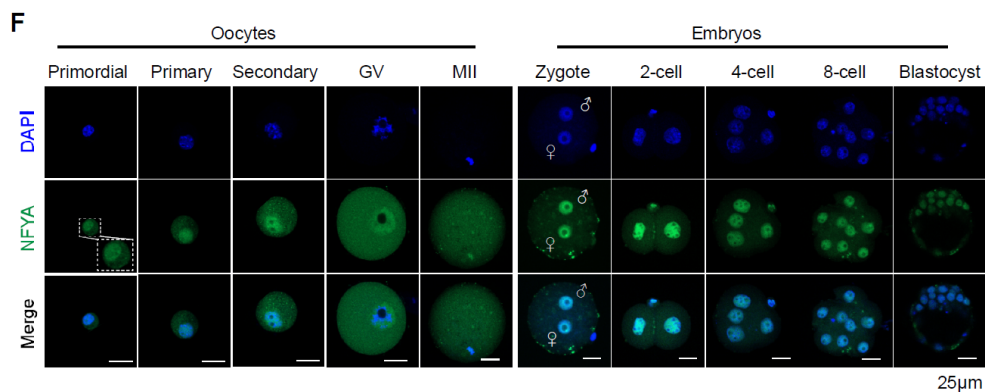
We thank the reviewer for these thoughtful questions and for the opportunity to clarify our rationale and findings regarding MTB-Nfya.

Question 1: How was MTB-Nfya focused on?

We identified the MTB-Nfya chimeric transcript during our systematic search for TE-derived transcription start sites that generate chimeric transcripts (**Supplementary information, Table 3**). Given the known role of NFYA as a pioneer factor, we were particularly interested in whether its TE-derived isoform might function during early embryogenesis, and thus chose MTB-Nfya as a candidate for functional validation. It was not the link from chromatin accessibility.

Question 2: Does NFYA protein Persist into 2C?

Our results showed that the MTB-Nfya chimeric transcript disappears after the PN5 stage, whereas the canonical transcript continues to be expressed. Using published data (PMID: 27259149), NFYA protein is present at the 2-cell stage. Also, a recent study from Yang et al., reported on BioRxiv (**showed below**) exhibited that Nfya is highly expressed during both oogenesis and ZGA, but at a very low level in later-stage of embryogenesis and mESCs (<https://doi.org/10.64898/2026.03.30.715371>), which provided the evidence that protein of NFYA persisted into 2C.

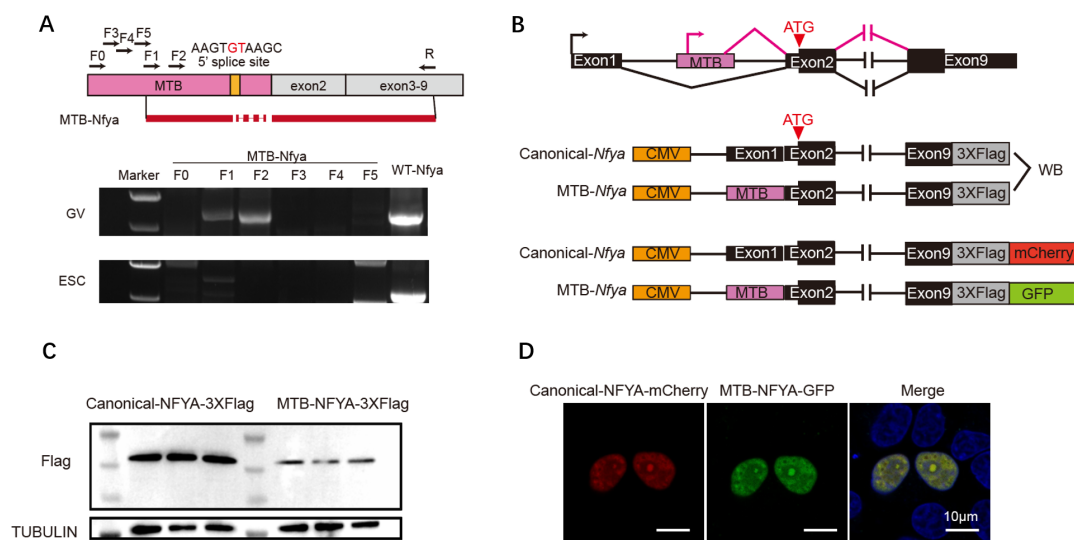


Question 3: How do we know this is the Chimera or Canonical action?

We performed a series of experiments to address whether chimera encode a protein. First, RT-PCR and Sanger sequencing confirmed the existence of the MTB-Nfya chimeric transcript in oocytes, in

which a segment of MTB replaces the canonical exon 1 (**Response Figure 17A**). ORF prediction and in vitro expression of the cloned chimeric transcript demonstrated that its open reading frame is unchanged; the protein produced is identical in size and subcellular localization to that of the canonical Nfya transcript (**Response Figure 17B–D**). These results were added to results section in **Supplementary Fig. S9e-h**. Thus, we propose that the MTB element primarily serves as an alternative promoter and 5' UTR to boost NFYA protein levels before the 2-cell stage, without altering the protein's structure or function. This “dual-promoter” system ensures adequate NFYA supply during the oocyte-to-embryo transition.

It remains to be determined whether the two different 5' UTRs lead to differences in translational efficiency, and whether the transposon-derived transcript serves as the predominant source of NFYA protein during the GV-to-1-cell stages or acts as a backup reservoir for the canonical transcript. We have made some speculations and discussions regarding the protein functions of the chimeric transcripts in Discussion section of revised manuscript.



Response Figure 17. Protein validation of MTB-Nfya chimeric transcripts.

(A) RT-PCR products obtained with different primer sets targeting MTB-Nfya, and the schematic alignment of Sanger sequencing results confirming the splice junction.

(B) Schematic diagram of the splicing patterns and predicted transcription start sites (red arrow, ATG) of canonical Nfya and MTB-Nfya, along with the expression vector design.

(C) Western blot showing the protein sizes produced by the canonical Nfya and MTB-Nfya expression constructs in human 293T cells.

(D) Fluorescence reporter assays showing that both canonical NFYA and MTB-NFYA exhibit the same nuclear localization pattern in 293T cells.

Minor comments:

1. In general, there is a lack of telling the number of samples in many experiments, especially in embryo survival graphs (percentages). Fig5f,5k S9f

We apologize for the omission of sample sizes in the survival plots. In the revised manuscript, we have now included the exact number of embryos analyzed for each data point directly in the figures.

2. Abstract:

Line 34: Sounds too similar to “RNAi”.

Line 36: TEs could also be coding. See MERVL-GAG and HERVK-GAG in mouse and human embryos. There are likely many others (RTs, for example).

Line 42: ERVK? Are there representative families like muERVL? IAP?

Line 48: TE Reprogramming. Is this the standard term? It makes sense to me but I haven’t seen it written this way before.

We thank the reviewer for these detailed and constructive suggestions on the abstract. We have rewritten the Abstract in the revised manuscript and have avoided the inappropriate wording mentioned above.

3. Introduction:

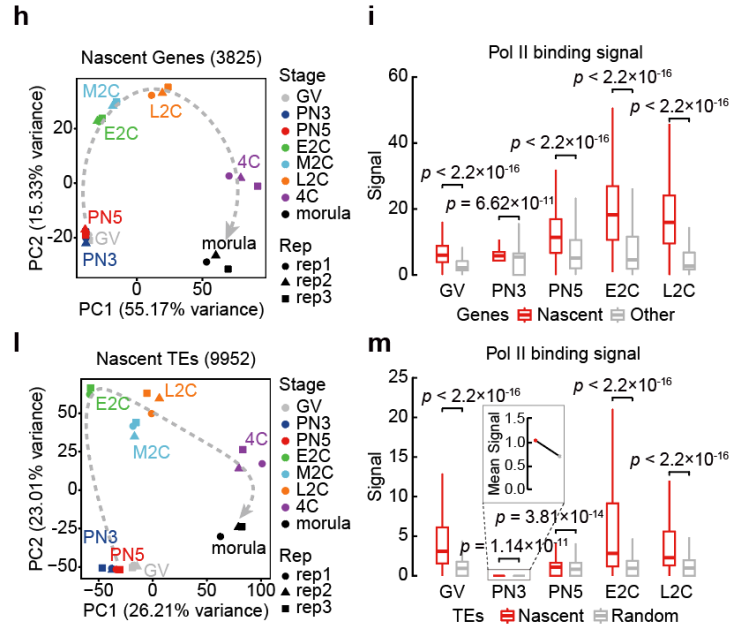
Line 72: The sentence should have “is”: “, which is transiently highly expressed”...

Line 73: This is a major motivation for the paper. The LINE and SINE examples should be explained a little bit more.

We thank the reviewer for these suggestions. We have corrected the sentence in line 72 by adding “is” as recommended. In addition, we have expanded the description of LINE and SINE examples in line 73 to better explain their relevance and transient expression dynamics, which further clarifies the major motivation of this study. The revised text has been highlighted in the manuscript.

4. In Fig. 1H/I/J/L/M/N, the GV groups are missing but should be included.

We appreciate the reviewer’s comment. In the revised manuscript, we have added the GV group to **Fig. 1h, 1i, 1l, and 1m** where data were available (**Response Figure18**). However, for panels Fig. 1j and 1n (LET-RNA data), the GV stage is not included in the original LET-RNA dataset; therefore, we are unable to add the GV group to those panels.

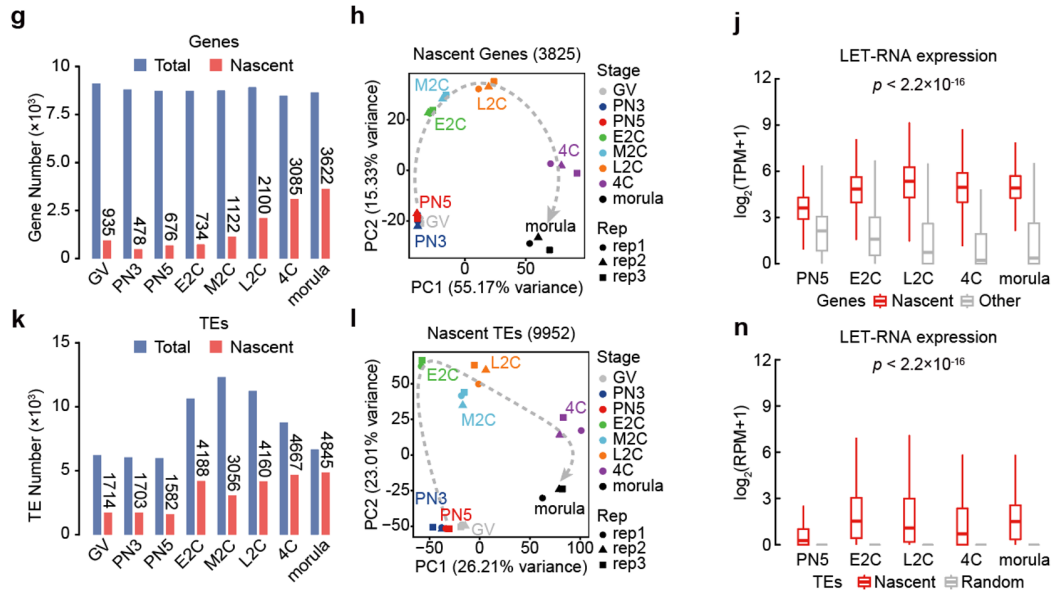


Response Figure 18 (corresponding to Fig.1h, 1i, 1l, and 1m) GV samples included.

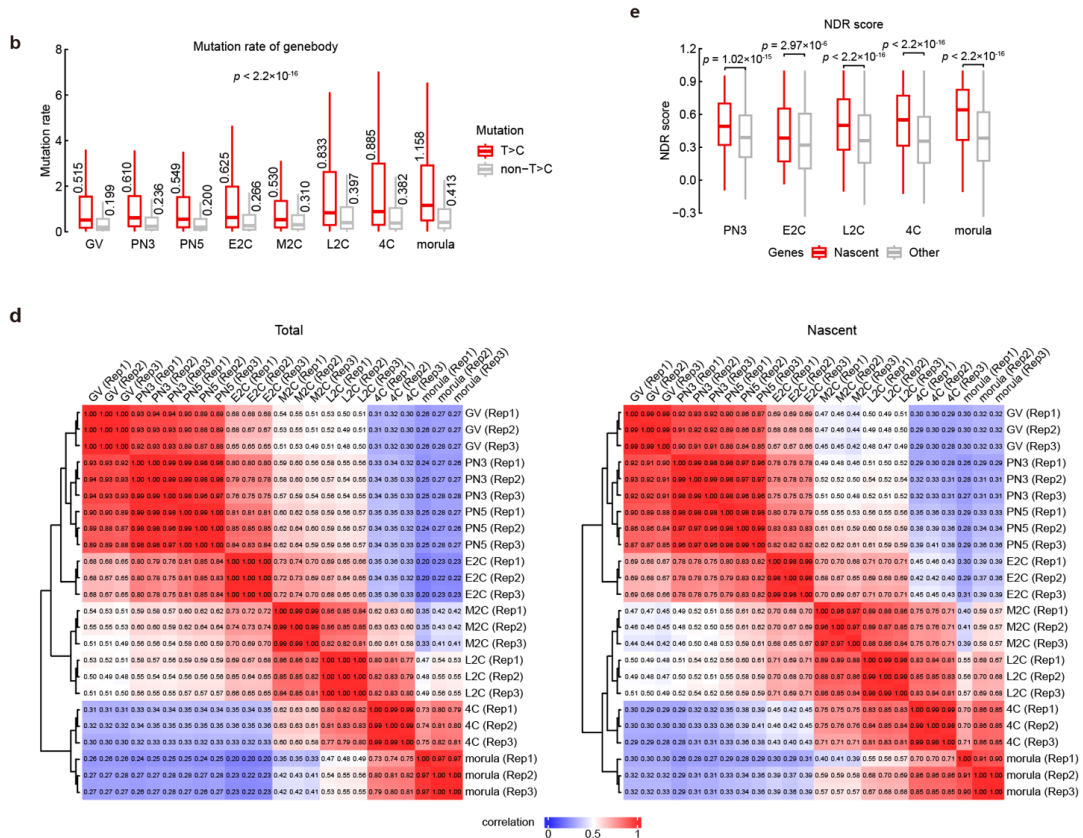
5. Fig. S2 and S3c, this study primarily focuses on ZGA, therefore it is unclear why the figures present the nascent ratio in morula. Furthermore, the morula data are not utilized elsewhere in the manuscript, even not mentioned in the schematic diagram in Fig. S3A. This dataset appears to be redundant, and it should be replaced with data from other stages.

We thank the reviewer for this comment. During method establishment, we initially used morula-stage embryos as a positive control because their transcriptional activity is much higher than that of earlier stages, making them ideal for validating the reliability of the nascent RNA detection method. In the original submission, these data were placed in supplementary figures. However, to better demonstrate the richness of the dataset and to address the reviewer's concern, we have now moved the morula data into the main figures. Specifically, morula results have been incorporated into **Fig. 1g, 1h, 1j, 1k, 1l, and 1n**, and **Supplementary Fig. 3b,d,e**. This integration not only removes redundancy but also provides a more comprehensive view of the transcriptional dynamics from early to late preimplantation stages.

Fig.1



Supplementary information, Fig.S3



Response Figure 19 (corresponding to Fig.1g,h,j,k,l and n, Supplementary Fig. S3b-e) Morula samples included.

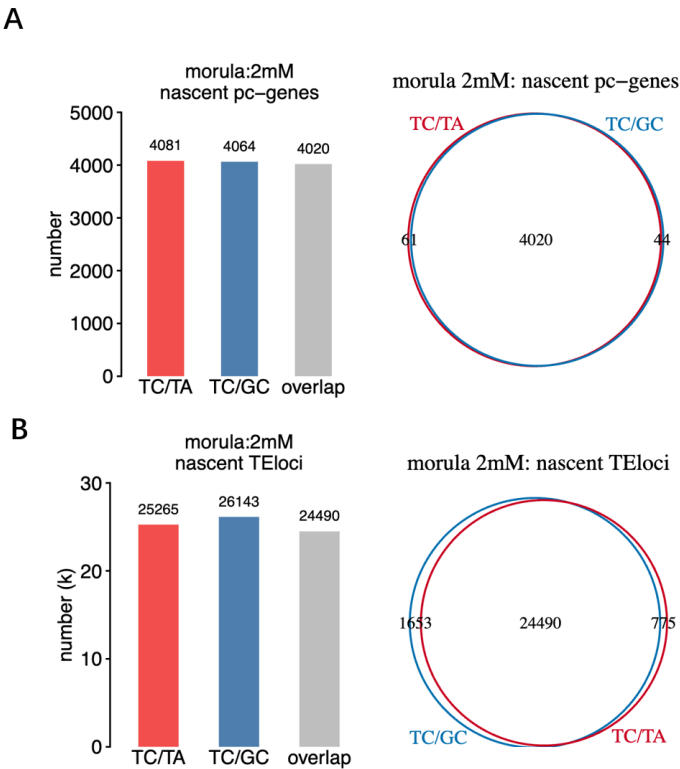
6. Line 126-127: “...we used a T to C / T to A > 3 cut off for nascent gene and TE definition...” I am wondering if the high concentration of 4sU can perturb the T > A mutation ratio that is

attributed to transcription. Considering this potential confounding effect, it may be more appropriate to use alternative mutation types, such as G > C, as the basis for the cutoff?

We thank the reviewer for this insightful comment. The concern that a high concentration of 4sU might perturb the T > A mutation ratio is certainly worth considering. Our choice of using T > A as the background was mainly based on the fact that the total amounts of T bases are comparable between conditions. This approach follows the computational strategy used in SLAM-seq as described by Pooja Bhat et al. (PMID: 36757845).

To directly address the reviewer’s concern, we additionally calculated the numbers of nascent genes and TEs using an alternative cutoff (T C / T G > 3). As shown in **Response Figure 20**, the results obtained with T C / T G > 3 were highly similar to those obtained with T C / T A > 3 in terms of both protein-coding genes and transposable elements (TEs) at the morula stage.

Therefore, we believe that, based on the current data, the T C / T A > 3 cutoff is reliable for defining nascent genes and TEs. If the reviewer wishes us to include these data in the manuscript, we are happy to provide them as additional information.



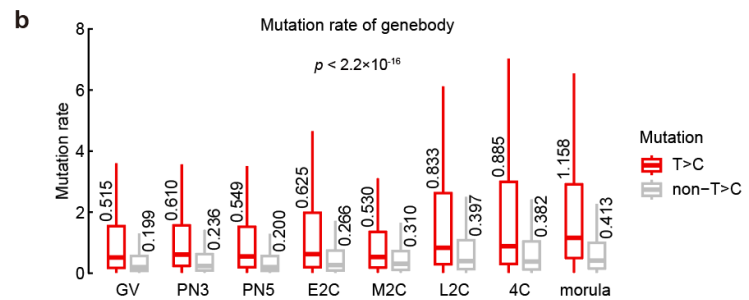
Response Figure 20. Basis for cut off.

A. Number and overlap of protein-coding genes with detectable transcriptional signals in morula defined by T to C / T to A > 3 versus T to C / T to G > 3.

B. Number and overlap of transposable elements (TEs) with detectable transcriptional signals in morula defined by the two cutoffs.

7. In Fig. S3b, the variation in the mutation ratio across developmental stages is not clearly presented. The authors should provide detailed values or fold changes for the T>C groups relative to the non-T>C groups.

We thank the reviewer for this suggestion. In the revised manuscript, we have added the exact values of the T > C mutation ratio relative to non-T > C groups directly in **Supplementary Fig. S3b**, so that the variation across developmental stages is clearly presented.



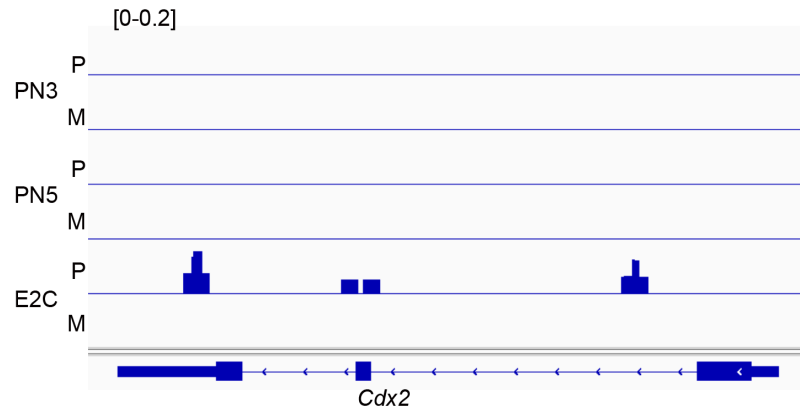
Response Figure 21. corresponding to Supplementary Fig. S3b

8. Line 202: The text begins to jump between 1C and PN3/5 without much explanation for when/how the cell is referred to either. Some clarification or sticking to one type of designation is helpful.

We apologize for the confusion caused by the inconsistent use of "1C" and "PN3/5". PN3 and PN5 are two time points during the gradual fusion of the male and female pronuclei after fertilization, and both belong to the 1-cell stage. In the revised manuscript, we have clarified the relationship between PN stages and the 1C stage to make the designation consistent and easier to follow.

9. Line 227: The Cdx2 hit is interesting and unexpected. This is associated with specification of the trophoblast lineages. Is this the earliest report of Cdx2 expression?

We thank the reviewer for the insightful comment. To address the question of whether this is the earliest report of *Cdx2* expression, we further examined the parental-origin assignment of nascent *Cdx2* transcripts. No *Cdx2* nascent transcription was detected at the PN stage. However, at the E2C stage, we observed low level of reads originating from the paternal allele, with no detectable reads from the maternal allele (**Response Figure 22**). To our knowledge, this represents the earliest detection of *Cdx2* transcription during preimplantation development (at the two-cell stage). Given the extremely low abundance of the detected *Cdx2* nascent transcripts, its transcriptional authenticity requires further validation.



Response Figure 22. IGV browser of paternal and maternal reads from *Cdx2* in PN and E2C stages.

10. Line 243: It is not clear what is meant by “excluding MaLR in this paper”.

We thank the reviewer for pointing out the ambiguity. The intended meaning was that MaLR is sometimes classified under the ERVL family, but in our study we discuss MaLR as a separate category; therefore, when we refer to ERVL elsewhere in the text, we specifically mean ERVL elements excluding MaLR. However, to avoid confusion, we have removed the confusing note “excluding MaLR in this paper” from the revised manuscript.

11. Line 264: Somewhat hard to read, please revise.

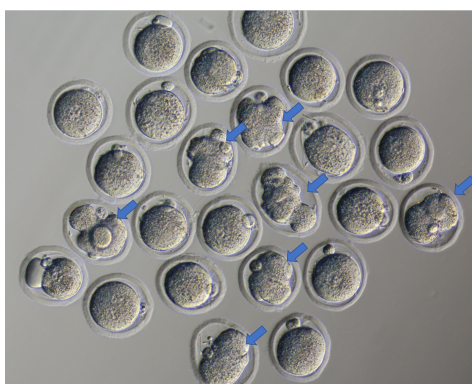
We have revised the sentence as suggested. The revised sentence now reads:

“To investigate the genomic features of these three transcriptionally active LTRs during ZGA, we found that active ERVK were preferentially located near gene exons, whereas active MaLR were predominantly intronic and intergenic.”

This change has been made in the revised manuscript.

12. In Fig 4h, the nascent genes of 36 hpf MTA-ASO embryos seem be closer to the M2C stage along PC1. If so, why these embryos arrest at the 1-cell stage rather than progressing to E2C stage?

We thank the reviewer for this important question. We do not consider the MTA-knockdown embryos arrested at the 1-cell stage as dead; rather, their first mitotic division is severely impaired (we observed incomplete cleavage in some embryos at 36 hpf, indicating a block to mitosis, **as shown in Response Figure 23**). Under such conditions, the overall transcriptional profile may gradually drift toward a state resembling the M2C stage over time, even though the embryos fail to complete the first cleavage. Moreover, this PCA was performed using expression levels of 3,825 Wave1–3 genes, and PC1 explains only 49% of the variance. And we found a lot of ZGA genes are dysregulated among W1–3, indicating a dramatic abnormal transcriptome of embryos at 36 hpf (Figure 4h,g). Thus, the PCA result should be interpreted as a reference for developmental potential rather than a precise predictor of the actual cell cycle stage.



Response Figure 23. Arrested embryos at 36 hpf after MTA-ASO injection in MII. The blue arrows indicate 1-cell embryos with abnormal division.

13. In Fig 4h, the nascent gene profiles of 36 hpf NC embryos are closer to the L2C stage, whereas in Fig 4l, the nascent TE profiles of the same samples are closer to the M2C stage. This suggests that TE activation lags behind gene activation. The authors should clarify whether this result is biologically reasonable.

We thank the reviewer for carefully noticing this interesting discrepancy. To be honest, we cannot provide a definitive explanation at this stage. However, we propose two possible reasons.

First, it may arise from technical issues in TE quantification. Because many TE copies share highly similar sequences, it is challenging to assign uniquely mapped reads to specific loci, and the estimation of copy-level expression is inherently imprecise. This could lead to stronger batch effects for TE expression than for gene expression, potentially causing the observed shift in PCA between gene- and TE-based profiles.

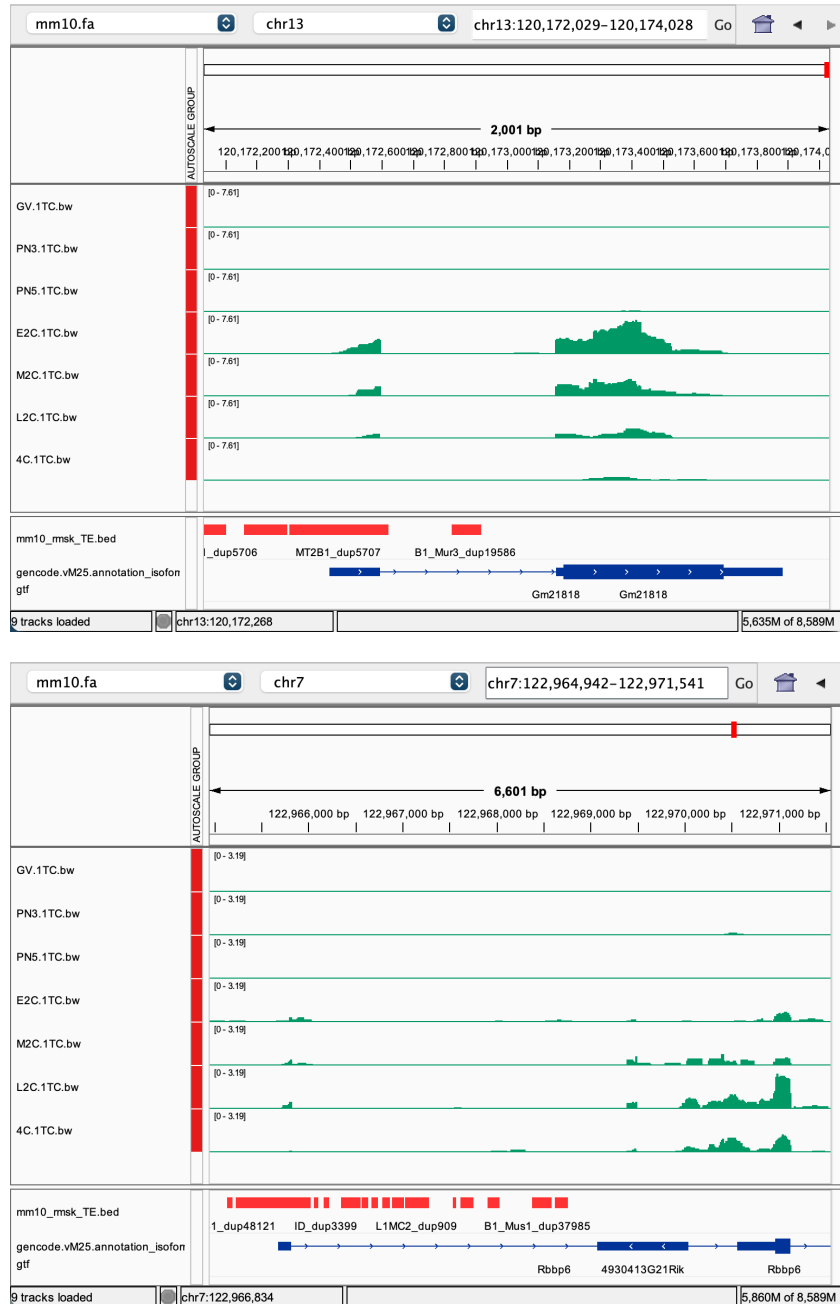
Second, ASO injection itself may have mild toxicity even at the optimized concentration. Although we chose a safe and effective concentration (15 μ M) based on our titration, we cannot completely rule out weak adverse effects. The NC group (injected with control ASO) might thus differ from un-injected WT embryos to some extent, possibly contributing to the apparent lag in TE activation.

14. Line 319: Typo, please revise.

We thank the reviewer for pointing out. We have revised this sentence.

15. Line 349 and Figure 5A: How does the analysis define “exon”? I understand a TSS originating from an intron (cryptic) or intergenic (upstream) like in the examples shown in 5B, but I cannot distinguish how “exon” can be a TSS. Can you elaborate or show examples? Most of this is explained in S8A, but I am not sure I see “exon”.

We thank the reviewer for this important question. In Fig. 5a, we categorized the genomic distribution of these TE-containing TSSs relative to host genes: a TE is classified as “exonic” if it overlaps with an exon of a gene (including cases where the TE resides within the exon, potentially in either the same or opposite transcriptional orientation). Such TE-exon overlaps are not uncommon, and two representative examples are provided in the response figure below. We have clarified this definition in the revised main text.



Response Figure 24. Genome browser showing examples of TE-exon overlaps.

16. In Fig. 5C, How are the junction reads determined? Directly above it in 5B is an example showing 217 vs 198 reads. In Panel 5C at PN5, we are shown 44 vs 49.

We thank the reviewer for the question. In Fig. 5b, the numbers shown are raw junction read counts, whereas in Fig. 5c, the values represent **normalized read counts**. To avoid confusion, we have clarified this distinction in the y-axis label of Fig. 5c.

17. Line 364 and figure 5E: I might be interpreting this wrong, but the “common” exon would indicate to me that after siRNA KD, only a very tiny amount of “total transcripts” went down, meaning the Canonical generates the majority of all Nfya? If this is correct, then the minor reduction in 5F and 5G might be explained. What are the N-Values for each experiment here (5F

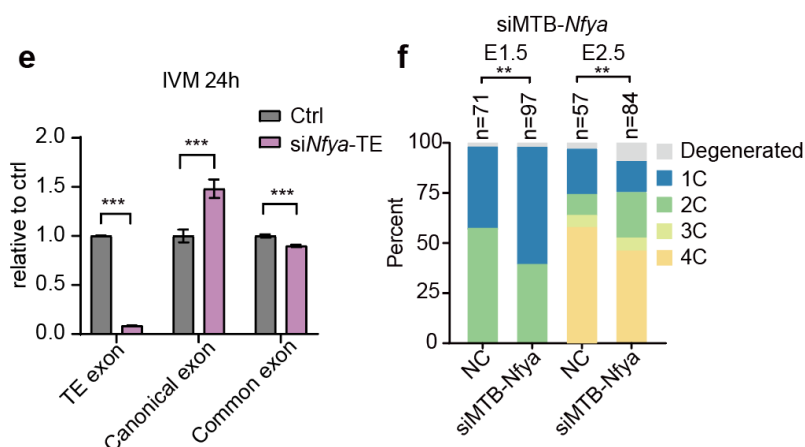
and 5G)? This is missing from legend. “Crucial” might be too strong of a term. (Line 369), as the blastocyst rate at 108hpf are relatively similar. Setd2 shows more of a dynamic range.

We thank the reviewer for these insightful comments.

Question 1: the canonical Nfya generates the majority of all Nfya ? (Fig. 5e): We fully agree with the reviewer’s concern. The reviewer noticed that the canonical transcript (represented by the common exon) did not decrease substantially after siRNA knockdown. In fact, we detected a compensatory increase in canonical transcripts. Because the expression levels of canonical and TE-chimeric transcripts are comparable at the GV and PN stages, the overall *Nfya* transcript level did not change dramatically. It indicates that the mouse embryo has evolved two sets of promoters to ensure high transcriptional activity of *Nfya* before the two-cell stage, underscoring the importance of NFYA as a pioneer factor for zygotic genome activation.

Question 2: What are the N-Values (Fig. 5f): We apologize for omitting the n-numbers. The exact numbers of embryos analyzed have now been added directly to the figures (Fig. 5f) as well as the corresponding legends.

Question 3: Crucial might be too strong of a term (Line 369): We agree with the reviewer that “crucial” may be too strong. We have revised the conclusion as “*However, a noticeable delay in progression to the 2C stage was evident, indicating that MTB-initiated Nfya transcripts promotes the ZGA process (Fig. 5f,g).*”



Response Figure 25. corresponding to Fig. 5e,f.

e. RT-qPCR showing expression levels of the indicated transcripts relative to control (non-targeting siRNA, NC) in GV oocytes after 24 h of in vitro maturation following siRNA injection. p-values from Student t-test are shown. n=3 technical replicates. ***p < 0.001.

f. Developmental ratio at E1.5 and E2.5 post-IVF after NC and siMTB-Nfya siRNA-injection groups. n = total embryo number tested from two independent injections.

18. Line 379 and Figure 5H: How was this done? The 5H schematic looks like it targets this locus 3 ways: Upstream, Downstream and Internal. I’d imagine the internal would have many off targets. Is that what is shown in 5I and 5J when “TE” is mentioned on the X Axis? As in: a large

portion of MT2 and ORR1B1 are reduced in this experiment, with these chimeras (and canonical as well?) being a part of that global downregulation? According to 5B, roughly half of the expression should be coming from the canonical Setd2 expression. Does the canonical compensate? (Primers can be designed against just the canonical isoform transcript to confirm, similar to what is shown in S8C and S8D). The details of this strategy are not clear to me in the methods.

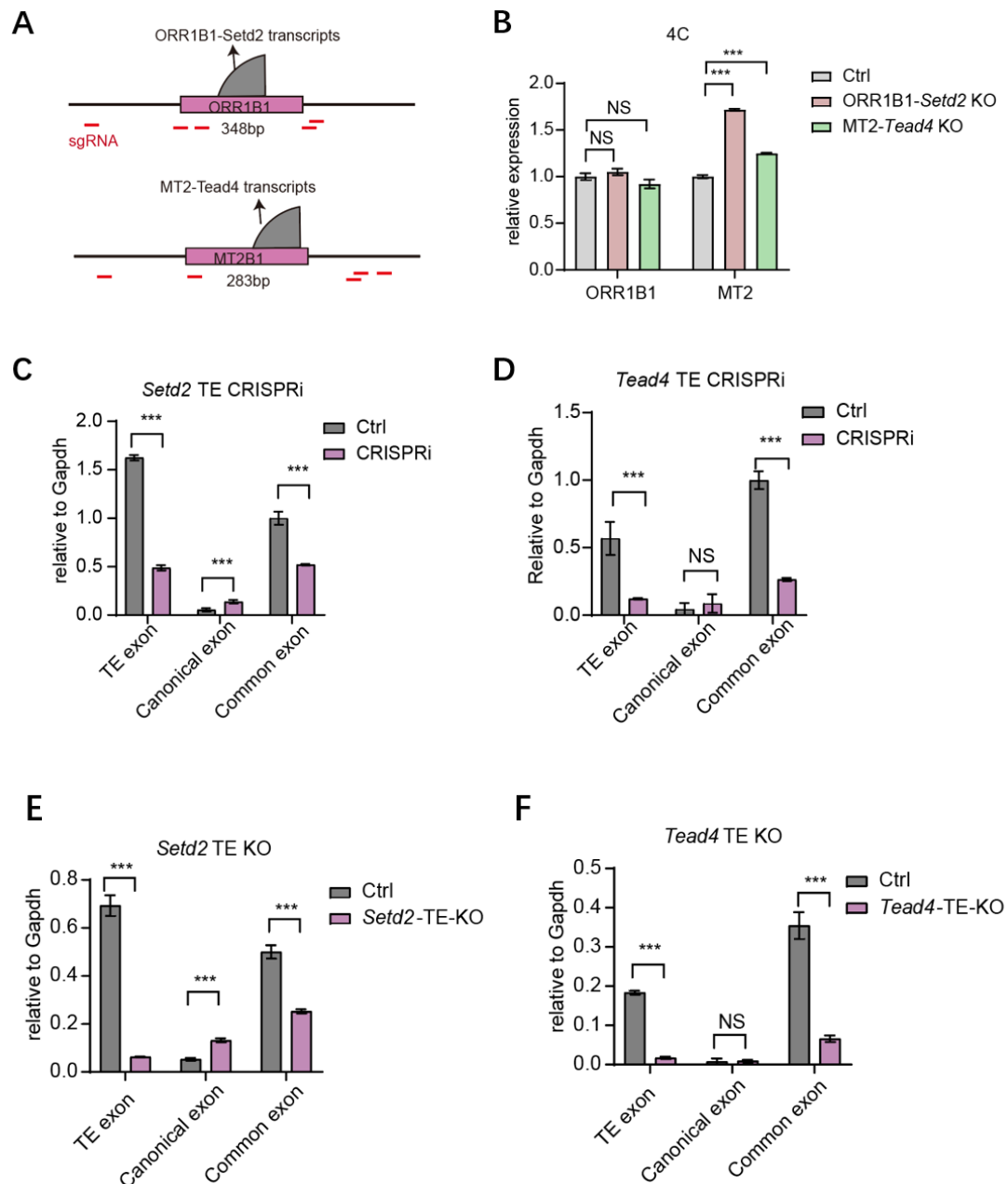
We thank the reviewer for the detailed questions regarding the CRISPR targeting strategy and potential off-target effects.

Question 1: sgRNA design and targeting strategy (Fig. 5h): For each targeted TE locus, we designed sgRNAs using the CRISPOR web tool (<https://crispor.gi.ucsc.edu>). We selected 5–8 top-scoring sgRNAs within approximately 600 bp around the TE element and then chose the best candidates based on genomic position. In total, we designed and pooled five sgRNAs for each knockout: two sgRNAs targeting ORR1B1 and one sgRNA targeting MT2B1 (**Response Figure 26A**). The schematic in [Fig. 5H and Supplementary Fig.S10b](#) illustrates the approximate positions of these sgRNAs (upstream, internal, and downstream relative to the TE).

Question 2: Off-target assessment: We acknowledge the concern that internal sgRNAs might target other copies of the same TE family. To evaluate this, we used general primers reported in the literature for MT2 (doi.org/10.1016/j.celrep.2024.113775) and for ORR1B1 ([doi: 10.3389/fchem.2017.00034](https://doi.org/10.3389/fchem.2017.00034)) to measure the expression of other family members. RT-qPCR on cDNA from KO embryos showed that MT2 and ORR1B1 families were not significantly downregulated (**Response Figure 26B**), indicating high specificity of our sgRNA pool.

Question 3: Canonical compensation (Fig. 5i, 5j and related): We also examined whether the canonical transcripts of Tead4 and Setd2 are upregulated to compensate for the KD or KO of TE-driven isoforms. For Tead4, the canonical transcript level was unchanged. For Setd2, the canonical transcript increased approximately 2-fold at the 4-cell stage; however, its absolute abundance is very low compared to the TE-driven isoform, and the total Setd2 expression was still significantly reduced (**Response Figure 26C-F**). Therefore, we conclude that at this stage, the majority of Setd2 and Tead4 transcription is driven by the TE promoters, and compensation by canonical transcripts is insufficient to rescue the total mRNA level.

These results have now been incorporated into the respective figures in the manuscript ([Fig5.i,j and Supplementary Fig. S10b-d](#)).



Response Figure 26. CRISPR targeting strategy and validation.

(A) Schematic of the *ORR1B1-Setd2* and *MT2-Tead4* chimeric transcripts, showing sgRNA numbers and target positions.

(B) Expression levels of universal *ORR1B1* and *MT2* elements in 4-cell embryos after *ORR1B1-Setd2* KO and *MT2-Tead4* KO, respectively (Student t-test; *** $p < 0.001$; ns, not significant).

(C) and (D). Expression levels of indicated transcripts relative to control after CRISPRi measured by qPCR. Student t-test p-values are shown. n=3 technical replicates. ** $p < 0.01$, *** $p < 0.001$. NS, not significant.

(E) and (F). Expression levels of indicated transcripts relative to control (only sgRNA without Cas9) after CRISPR KO, measured by qPCR. Student t-test p-values are shown. n=3 technical replicates.

****p < 0.01, ***p < 0.001. NS, not significant.**

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have significantly improved their analyses, and the presentation of their work has improved. We have the following additional comments that should be addressed.

We sincerely thank the reviewer for the thoughtful and constructive comments, which have helped us further improve the quality of our manuscript. Below we address each point in detail.

1. We have requested the authors to bring their findings in the context of published findings in the discussion, however, only few published papers have been referred to. Thus, we encourage the authors to work on the discussion.

Response: We appreciate the reviewer's suggestion. In the revised manuscript, we have substantially expanded the Discussion section by incorporating 15 additional references that are highly relevant to our work. These newly added citations cover recent advances in transposable element biology during early embryogenesis, TE-derived chimeric transcripts, and the regulatory roles of specific TE families in mammalian pre-implantation development. We believe these additions place our findings in a broader and more balanced context. The corresponding references are now cited as **Refs [30,31,33,35,43,54-63]** in the revised Discussion.

2. Regarding the requested ASO validations it is unclear whether the other MT family members are expressed in this context, making it difficult to interpret the absence of downregulation as evidence of ASO specificity.

Response: We thank the reviewer for raising this important specificity concern. In **Supplementary Fig. 7g**, we now provide a comprehensive view of the expression levels of all MT family members in our nascent RNA dataset. The figure displays both the number of expressed copies and the global expression levels (normalized counts) for each MT subfamily at the PN stage. As shown, the vast majority of MT family members are expressed at negligible levels compared to MTA, with only MTB and MTC showing detectable yet substantially lower signals.

3. Lines 323 - 325 / Fig. S7g: Please clarify what the z-score represents in this analysis.

Response: We apologize for the lack of clarity. The z-score shown in **Supplementary Fig. 7g** represents the standardized expression level of each nascent MT family member across the different stages examined. We have now added this explanation in both the figure legend (**Supplementary Fig. 7g**)

4. The new analysis distinguishing solo from full-length MTA elements is interesting and useful for the community. However, the methods lack detail on

how these classes were derived from RepeatMasker annotations, which do not directly define full-length elements, but provide separate annotations for LTR and internal regions. The authors should describe the analyses in detail.

Response: We thank the reviewer for this comment. We have added a detailed description of the classification of MTA elements based on RepeatMasker annotations to the Methods section. Specifically, for each MTA_Mm-int, we identified the nearest MTA_Mm on each side and calculated the distance between their adjacent boundaries. MTA_Mm and MTA_Mm-int annotations with adjacent boundaries within 10 bp of each other were considered associated. Based on the presence or absence of associated MTA_Mm on either side, MTA elements were classified into four structural classes: LTR-int-LTR (full-length MTA), int-LTR, LTR-int, and int (solo MTA_Mm-int).

5. Lines 311–312: The conclusion that “transcriptional activation of MTA is essential” is an overstatement based on this experiment. ASOs primarily degrade RNA and do not directly inhibit transcription, so the data do not distinguish between a requirement for the endogenous MTA RNA itself and transcriptional activation from the endogenous MTA loci.

Response: We thank the reviewer for this critical and insightful comment. We fully agree that our previous wording was an overstatement, as the ASO experiment specifically targets the RNA product rather than the transcriptional process per se. In the revised manuscript, we have modified the conclusion in the Results section to strictly reflect what the experiment can demonstrate. The original sentence has been changed to: “Exogenous full-length MTA RNA could not rescue the functions of MTA, suggesting that the presence of the mature RNA transcript alone is insufficient to compensate for the loss of endogenous MTA function (See discussion).” In the Discussion section, we have further elaborated on the possible explanations for the rescue failure.

6. Lines 452–456 / Fig. 6a: Please clarify whether motif enrichment was performed using all proteins detected in the embryonic proteomics dataset or a filtered subset, and if the latter, describe the selection criteria.

Response: We thank the reviewer for this comment. We have clarified the selection criteria for motif enrichment in the Methods section. Motifs from the JASPAR 2026 vertebrate database were used as references, and only motifs whose corresponding transcription factors were detected in proteomic data from mouse early embryos (MII, L1C, L2C, and 4C) were retained for motif enrichment analysis.

Reviewer #2 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

I appreciate the authors' detailed and constructive responses to my comments. Overall, I found the rebuttal to be thoughtful and responsive, and I think the manuscript has been substantially strengthened. In particular, the authors have added useful controls and clarifications regarding the sensitivity of scTL-seq, the effects of 4sU treatment, allele-specific and TE-mapping strategies, MTA-ASO specificity, MTA rescue, and the validation of TE-derived chimeric transcripts. I remain generally positive about the study. My main view is that the paper is now much improved, but that some of the mechanistic conclusions, especially regarding MTA function, should still be phrased conservatively in the revised manuscript.

The additional validation of the scTL-seq method addresses many of my concerns. I appreciate that the authors revised the interpretation of the 4sU-IP-specific genes and no longer describe them simply as background noise. The added PRO-seq comparison, the discussion of detection thresholds, and the overlap analysis between 4sU-IP and scTL-seq are helpful. I also appreciate the added developmental assays showing that 2 mM 4sU treatment for 4 hours does not have an obvious detrimental effect on oocyte maturation or blastocyst development. These data make the methodological foundation of the study more convincing.

The authors also responded directly to my concerns about MTA-ASO specificity and the need for a rescue experiment. The rescue experiment is informative, even though exogenous full-length MTA RNA did not rescue the developmental phenotype. In my view, this result argues against a simple model in which freely supplied MTA RNA is sufficient, and instead supports a more nuanced interpretation in which MTA function may depend on genomic context, local transcription, or cis-regulatory activity. I think this is an important point, and I would encourage the authors to make sure that the revised manuscript does not overstate the conclusion that MTA RNA itself acts directly in trans unless this is clearly presented as a hypothesis.

The MTB-Nfya section is also improved. The RT-PCR/Sanger validation of the chimeric transcript, together with the protein-size and localization experiments, provides stronger evidence that MTB-Nfya is a genuine transcript that encodes a protein indistinguishable from canonical NFYA. I also appreciate that the authors softened the developmental phenotype from "partial arrest" to a developmental delay and discussed the possibility of compensation from canonical Nfya. This makes the interpretation much more balanced.

I also appreciate the additional explanation of the TE and allele-specific

mapping strategy. The authors now provide a clearer description of whole-genome alignment, unique mapping, HISAT-3N usage, SNP masking, base-quality filtering, read-end filtering, paired-read filtering, and RepeatMasker-based TE assignment. This clarification is helpful. At the same time, because unique mapping necessarily captures only a subset of TE-derived reads, I think it would be useful for the revised manuscript to explicitly acknowledge this limitation and the possibility that some TE copies or families may be underrepresented.

Response: We are grateful for the reviewer's overall positive evaluation and for the encouragement to maintain appropriate caution in our mechanistic interpretations. We have carefully re-examined all statements related to MTA function throughout the manuscript and have revised them to ensure they are not overstated. Specific revisions addressing this point are detailed in our responses below.

First, in the Results section, we have revised the conclusion of the rescue experiment to strictly reflect what the data demonstrate: "Exogenous full-length MTA RNA failed to rescue the developmental defects caused by MTA depletion, suggesting that the presence of the mature RNA transcript alone is insufficient to compensate for the loss of endogenous MTA function (see Discussion)."

Second, in the Discussion section, we have revised the potential trans-regulatory role of MTA RNA as a hypothesis rather than a demonstrated conclusion. The original wording has been modified to: "The repression of other TE transcriptional activation by MTA does not depend on genomic distance and may be accomplished through RNA-mediated trans-regulation, although the precise mechanism needs further investigation."

Third, in the revised manuscript, we have modified the sentence describing the TE transcriptional quantification in the Results section (associated with Fig. 3a). The revised text in the manuscript now reads as follows:

"Next, we quantified the proportion of transcribed transposons at copy level using uniquely mapped reads from the GV to 4C stages (see Method). Recognizing that unique-mapping approach captures only a subset of the total TE-derived reads due to the exclusion of multi-mapping reads, we acknowledge that the transcriptional activities of certain TE copies or families may be underrepresented."

MINOR:

There are a few remaining points that I think should be addressed in the revised manuscript, mostly by ensuring that the relevant data and caveats are actually included rather than only described in the rebuttal. First, some useful validation data, including the EU-labeling and MII robustness controls, appear to be discussed in the response but may not be incorporated into the manuscript or supplement. Since these data help address important concerns, I think the key controls should be included. Second, the proposed mechanism by which MTA suppresses other TEs remains speculative, particularly because the ChIRP-seq

data are preliminary, from ESCs, and not included in the manuscript. Therefore, any model in which MTA RNA directly represses other retrotransposons should be clearly framed as a possible mechanism rather than a demonstrated conclusion. Third, although I understand the authors' explanation for why a reciprocal cross was not feasible, the strong asymmetry in informative SNP numbers and the lack of reciprocal cross still leave some room for allele-assignment bias. I do not think a reciprocal cross is necessary at this stage, but I would ask that this limitation be stated clearly. Finally, for the DNaseI-TUNEL chromatin accessibility assay, the authors clarified the rationale, but it would be helpful to know whether a no-DNase TUNEL control was performed or to include a brief statement addressing this point.

Overall, I think the authors have taken the comments seriously and have made substantial improvements. I remain supportive of the manuscript, provided that the revised version includes the promised controls and maintains appropriately cautious language around the mechanistic interpretation of MTA function. I would support publication after VERY minor revision.

We sincerely thank the reviewer for the thoughtful and constructive comments, and for the positive assessment of our revised manuscript. Specific revisions addressing these minor points are detailed in our responses below.

Point 1: First, some useful validation data, including the EU-labeling and MII robustness controls, appear to be discussed in the response but may not be incorporated into the manuscript or supplement.

Response: We thank the reviewer for this reminder. We have now incorporated these validation data into the revised manuscript and supplementary materials:

The EU-labeling validation data have been added to **Supplementary Fig. 3e,f**, demonstrating the specificity and robustness of our nascent RNA detection method.

The MII controls (including the MII nascent signal intensities with or without 4sU and conversion) have been included in **Supplementary Fig. 5a**. These additions are now described in the corresponding figure legends and briefly mentioned in the Results section.

Point 2: Second, the proposed mechanism by which MTA suppresses other TEs remains speculative, particularly because the ChIRP-seq data are preliminary, from ESCs, and not included in the manuscript. Therefore, any model in which MTA RNA directly represses other retrotransposons should be clearly framed as a possible mechanism rather than a demonstrated conclusion.

Response: We fully concur with the reviewer's assessment. In the revised Discussion, we have removed any potentially overconfident phrasing and have reframed the proposed trans-repression model as a speculative hypothesis. The relevant sentence now reads:

“The repression of other TE transcriptional activation by MTA does not appear to depend on genomic distance and may be accomplished through RNA-mediated trans-regulation, although the precise mechanism awaits further investigation.”

The ChIRP-seq data from ESCs mentioned in our rebuttal are not included in the manuscript, and we have ensured that no conclusions are drawn from them in the present study.

Point 3: Third, although I understand the authors’ explanation for why a reciprocal cross was not feasible, the strong asymmetry in informative SNP numbers and the lack of reciprocal cross still leave some room for allele-assignment bias. I do not think a reciprocal cross is necessary at this stage, but I would ask that this limitation be stated clearly.

Response: We thank the reviewer for this fair and constructive suggestion. In the revised Discussion, we have explicitly acknowledged this limitation. The following statement has been added:

“However, since we did not carry out reciprocal cross experiments (e.g., using maternal PWK crossed with paternal C57), the conclusions drawn from our current SNP-based classification of parental transcripts are subject to certain limitations, including potential biases arising from the asymmetric distribution of informative SNPs between the two parental genomes.”

Point 4: Finally, for the DNaseI-TUNEL chromatin accessibility assay, the authors clarified the rationale, but it would be helpful to know whether a no-DNase TUNEL control was performed or to include a brief statement addressing this point.

Response: We thank the reviewer for raising this important point regarding the experimental controls for the DNaseI-TUNEL assay. We completely agree that a no-DNase TUNEL control is essential for ruling out background signals arising from endogenous DNA breaks.

In our preliminary pre-experiments, we indeed performed TUNEL staining on embryos without DNase I treatment. Under these conditions, we observed no appreciable TUNEL signal above background, confirming that the level of endogenous DNA fragmentation in our embryo samples is negligible. Based on these validation results, we omitted this control from the main experimental cohorts to streamline the assay.

Following the reviewer’s suggestion, we have now added the statement in Methods section as: “In preliminary experiments, parallel TUNEL staining was performed on embryos without DNase I treatment, and no discernible TUNEL signal was detected, indicating a negligible background from endogenous DNA breaks. Therefore, this no-DNase control was not routinely included in the subsequent main experimental groups.”