## Cell Reports

## Complex regulation of Eomes levels mediated through distinct functional features of the Meteor long non-coding RNA locus

## Graphical abstract



## Highlights

- An array of perturbations targeting the Meteor long noncoding RNA locus
- Transcription elongation through Meteor is required for Eomes activation in mESCs
- Meteor is dispensable for the formation of beating cardiomyocytes
- Meteor promoter DNA is required for suppressing Eomes during neuronal differentiation


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## In brief

Using a wide array of perturbation techniques, Gil et al. dissect the locus encoding the Meteor long non-coding RNA and show that distinct cell-typespecific functional features within the locus fine-tune the spatiotemporal expression patterns of the nearby gene Eomes in mouse embryonic stem cells and during differentiation.

# Complex regulation of Eomes levels mediated through distinct functional features of the Meteor long non-coding RNA locus 

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## SUMMARY

Long non-coding RNAs (IncRNAs) are implicated in a plethora of cellular processes, but an in-depth understanding of their functional features or their mechanisms of action is currently lacking. Here we study Meteor, a IncRNA transcribed near the gene encoding EOMES, a pleiotropic transcription factor implicated in various processes throughout development and in adult tissues. Using a wide array of perturbation techniques, we show that transcription elongation through the Meteor locus is required for Eomes activation in mouse embryonic stem cells, with Meteor repression linked to a change in the subpopulation primed to differentiate to the mesoderm lineage. We further demonstrate that a distinct functional feature of the locus-namely, the underlying DNA element-is required for suppressing Eomes expression following neuronal differentiation. Our results demonstrate the complex regulation that can be conferred by a single locus and emphasize the importance of careful selection of perturbation techniques when studying IncRNA loci.

## INTRODUCTION

Long non-coding RNAs (IncRNAs) are RNA polymerase II transcription products emanating from thousands of loci in mammalian genomes. ${ }^{1-3}$ Concomitantly with the rise in the number of annotated IncRNAs, they have been implicated in a wide array of cellular processes. In particular, multiple IncRNAs have been shown to contribute to fine-tuning of exit from pluripotency and developmental programs. ${ }^{4,5}$

A substantial number of IncRNAs are transcribed from regions with enhancer characteristics ${ }^{6-8}$ and, accordingly, multiple studies suggested IncRNA contributions to enhancer functionality, for example through bridging enhancer-promoter chromatin loops, ${ }^{9,10}$ recruiting activating proteins, ${ }^{11}$ or affecting nuclear localization. ${ }^{12}$ Such IncRNA loci can operate through the use of distinct functional features within the IncRNA gene, with the functionality of most such IncRNAs apparently relying not on the mature RNA product but rather on the underlying DNA element or various aspects of the transcriptional process, ${ }^{13-15}$ consistent with the generally poor conservation of these loci. ${ }^{8,16}$

The study of enhancer-transcribed IncRNAs is proving to be particularly challenging, as the reciprocal interactions and interdependencies between transcription, chromatin modifications, chromatin architecture, and nuclear localization complicate ef-
forts to separate direct and indirect effects of IncRNA activity. Crucially, these processes vary in their sensitivity to both the on-target and side effects of different perturbation techniques. This is exemplified by the sometimes contradictory effects noted when using different perturbation techniques to study particular loci, ${ }^{17,18}$ emphasizing the need to interpret the consequences of IncRNA perturbations with great care.

One enhancer-transcribed IncRNA of substantial interest is Meteor, also known as Platr11, Gm26975, or linc1405.5,19,20 Meteor is transcribed from the MesEndoderm Transcriptional Enhancer Organizing Region, located $\sim 70 \mathrm{~kb}$ downstream of the gene encoding the EOMES transcription factor (TF). ${ }^{14,20}$ EOMES, also known as TBR2, is a member of the T-box family of TFs, and is a master regulator of many developmental processes. ${ }^{21,22}$ Eomes is first expressed in the trophectoderm lineage, where it is required for self-renewal and early differentiation of trophoblast stem cells, so that Eomes-null embryos arrest shortly after implantation. ${ }^{23-25}$ Within the inner cell mass, Eomes expression initially marks the region that will become the primitive streak, making it one of the earliest markers of gastrulation onset. While Eomes is downregulated prior to the end of gastrulation, it is again detected in the developing forebrain, ${ }^{26,27}$ and has been attributed functions in various neuronal cell types both during development and in the adult brain ${ }^{21,28}$ as well as
in several types of immune cells. ${ }^{29-31}$ Owing to its involvement in and regulation of these eclectic developmental processes, Eomes expression levels and domains are themselves subject to tight regulation through cis-regulatory elements, some of which have been characterized. ${ }^{32}$

As befits its initial identification as a pluripotency-associated transcript, ${ }^{5,19}$ Meteor expression is highly associated with the pluripotent state, ${ }^{14,20}$ although one study indicated that its expression is upregulated in the embryonic heart and in mouse embryonic stem cell (mESC)-derived mesoderm precursors. ${ }^{33}$ Meteor's function is also subject to conflicting evidence. Two studies found that Meteor knockout (KO) caused reduction in Eomes expression in mESCs, ${ }^{14,34}$ suggesting that Eomes is the direct target of the Meteor locus. In contrast, another study, using a similar deletion scheme (Figure S1A), did not note any effect on Eomes expression in mESCs, but rather changes in expression of other genes, possibly indicating an Eomes-independent function of the Meteor locus. ${ }^{20}$ Nonetheless, all three studies agree that in mESCs, targeting of Meteor's transcription or RNA product does not have any noticeable effects on Eomes expression or otherwise, ${ }^{14,20,34}$ pointing to an RNA-independent function of the Meteor locus. In contrast, two additional studies argue in favor of an RNA-dependent Meteor function, showing that Meteor repression by either insertion of an early polyadenylation site (pAS) ${ }^{20,33}$ or short hairpin RNAs (shRNAs) ${ }^{33}$ caused significant reduction in the ability of mESCs to differentiate to functional cardiac mesoderm, though with conflicting evidence as to whether or not Eomes expression was the mediator of this activity. Specifically, one report suggested that during the process of cardiac mesoderm differentiation, Meteor functions in trans, as overexpression of one of the Meteor exons could rescue the phenotype of Meteor repression. ${ }^{33}$ The reduced ability to differentiate to cardiac mesoderm was suggested to prime the mESCs to differentiate to the ectoderm lineage, exemplified by increased efficiency of neuronal differentiation. ${ }^{20}$

In this study, we set out to address some of the key unanswered questions regarding Meteor biology. What is the effect of Meteor repression in mESCs, and to what extent is it mediated through Eomes? What is the role of the Meteor locus in priming cardiac mesoderm differentiation, and what is the contribution of the IncRNA to this activity? What are the roles, if any, of the Meteor locus during neurogenesis? Finally, what is the functional feature within Meteor that carries out these activities? We use a variety of perturbation techniques to show that two distinct functional features within the Meteor locus-transcriptional elongation through the Meteor locus, and the underlying DNA element-modulate Eomes expression levels in opposing manners in distinct cellular states, illustrating the complexity of the roles played by IncRNA loci within gene-regulatory networks.

## RESULTS

A suite of perturbation methods for interrogating the Meteor locus
To probe Meteor functionality and identify its functional features, we employed a suite of methods for perturbation of the Meteor locus in mESCs (Figures 1A and S1A-S1D). First, we used CRISPR-Cas9 and a pair of guide RNAs (gRNAs) targeted
to the two sides of the Meteor transcription start site (TSS). This generated clones with homozygous KO of the Meteor promoter (pKO) that resulted in complete ablation of Meteor expression, and a clone with a homozygous inversion of the promoter (plnv) that showed very low and inefficient transcription to the other side of the Meteor promoter (Figures 1A-1C). The deletion/ inversion encompassed $\sim 550 \mathrm{bp}$, a more targeted region than the 1- to $3-\mathrm{kb}$ region deleted in previous Meteor KO models (Figure S1A). ${ }^{14,20}$ We also received cell lines in which the entire Meteor locus was removed (full KO, or fKO lines) ${ }^{34}$ (Figure 1A). As a complementary approach that targets Meteor transcription without changing the DNA sequence, we infected vectors expressing gRNAs targeting the Meteor TSS into mESCs stably expressing a catalytically dead Cas9 (dCas9) protein (dCas9-KD [knockdown]), ${ }^{35}$ which reduced Meteor levels by $\sim 90 \%$ (Figure 1B). In addition, we generated cell lines which introduced a polyadenylation site (pAS) into the first intron of Meteor so as to reduce transcription through the Meteor locus, and similarly employed a cell line in which Meteor expression was destabilized by insertion of a self-cleaving ribozyme (Rz) sequence in its second exon ${ }^{34}$ (Rz-KD, Figure 1A). Lastly, to separate the effects of locus transcription from the DNA sequence at the promoter, we generated promoter exchange ( pEX ) cell lines in which the constitutive PGK promoter was knocked in downstream of the Meteor promoter, so that the transcription from the Meteor promoter is blocked by a pAS and the Meteor region is transcribed from the PGK promoter instead (Figure 1A).

## Transcription into the Meteor gene body enhances Eomes expression in mESCs

As previously noted, ${ }^{14,34}$ KO of either the entire Meteor gene body (fKO) or only the promoter region (pKO) caused a significant reduction of $\sim 50 \%$ in Eomes levels (Figures 1C and 1D). However, it is noteworthy that here and throughout, we saw a wide variability in both Meteor and Eomes expression levels among wild type (WT) and (in the case of Eomes) Meteor KO clones, especially when the cells were grown in serum, as can also be noted from the expression levels of these genes in the ESpresso single-cell RNA-sequencing (scRNA-seq) dataset (Figure S1E). This hindered our ability to detect a significant change in Eomes levels when assayed by qRT-PCR, which might explain contradicting previous reports. ${ }^{20}$ While alkaline phosphatase staining confirmed that the Meteor pKO lines were pluripotent (Figure S1F), we suspected that even small changes in pluripotency state could affect expression of these genes and therefore cultured the cells in serum-free medium supplemented with PD0325901 and CHIR99021, selective inhibitors of MEK and GSK3 (" 2 i " medium). 2 i conditions shield the cells from differentiation signals, resulting in a morphologically and transcriptionally uniform "ground state" of pluripotency, ${ }^{36,37}$ which indeed resulted in more uniform reduction in Eomes expression upon Meteor KO (Figures 1C and 1D). This effect of Meteor on Eomes expression appears to be specific to the naive state of pluripotency, as Meteor pKO cells cultured in the presence of fibroblast growth factor and activin, conditions which favor the generation of a "primed" epiblast stem cell-like state, did not exhibit changes in Eomes levels (Figure 1E). These cells are


Figure 1. Meteor transcriptional elongation promotes Eomes expression in naive mESCs
(A) Perturbations used to analyze the Meteor locus in this study. Shown are the locations of gRNAs used for deleting or inverting the Meteor promoter (pKO) or gene body (fKO) or for inhibiting transcription (dCas9-KD); the locations of insertion of ribozyme (Rz) or polyadenylation (pAS) sequences; and the scheme for knocking in the PGK promoter downstream of the Meteor promoter ( pEX ). The fKO and Rz-KD mESC lines are the same as in Tuck et al. ${ }^{34}$
(B) RNA-seq tracks showing Meteor expression in the various pKO and plnv lines (grown in serum-free 2i/LIF conditions) and dCas9-KD lines (serum/LIF conditions). All tracks are normalized to the same scale. Orange denotes transcription on the plus strand, and blue denotes transcription on the minus strand. (C) RNA-seq quantifications of Meteor and Eomes in Meteor pKO and plnv cell lines grown in serum/LIF (left) or serum-free 2i/LIF (right) conditions. Amounts normalized to WT1. Bars represent standard errors; $n=3$. ${ }^{*} p<0.05,{ }^{* *} p<0.005$.
(D) qRT-PCR quantifications of Meteor and Eomes levels in Meteor fKO and Rz-KD mESCs grown in serum-free 2i/LIF conditions. Levels were normalized to WT4 and Ppib for internal control. Bars represent standard errors; $n=8$. ${ }^{*} p<0.05$, **p $<0.005$.
(E) Same as (D), for Meteor pKO and plnv mESCs grown in primed conditions, normalized to WT1. $\mathrm{n}=4$.
(F) Same as (D), for pAS clones grown in serum-free 2i/LIF conditions. Levels were normalized to WT7. $\mathrm{n}=3$.
(G) Same as (C), for Meteor dCas9-KD lines grown in serum/LIF conditions. Amounts normalized to Ctrl. $\mathrm{n}=3$. The dCas9-KD efficiencies of Meteor were $85 \%$ and 94\% for KD1 and KD2, respectively.
(H) Same as (D), for pEX clones grown in serum-free 2i/LIF conditions. Levels were normalized to WT9. $n=3$.

See also Figure S1.
found at a more advanced state of differentiation representative of the post-implantation embryo, in which lineage-specific markers, including Eomes, are induced compared with naive mESCs. ${ }^{38,39}$ The lack of effect of Meteor repression on Eomes levels in primed cells suggests that Eomes induction at the onset
of differentiation is not directly dependent on Meteor but rather is likely a consequence of altered cell state at the naive mESC stage (see below).

We were not successful in achieving substantial silencing of Meteor RNA levels using infections of shRNA-expressing
vectors, possibly due to the strong chromatin association of Meteor IncRNA (Figure S1G). Nonetheless, no significant changes in Eomes expression were noted when Meteor expression became undetectable through Rz-KD (Figure 1D), nor when a partially effective pAS was inserted into the first intron of Meteor (Figure 1F), suggesting that the Meteor RNA product likely does not contribute to Eomes regulation in mESCs. Furthermore, Eomes expression was not substantially affected when Meteor transcription was reduced by $80 \%-90 \%$ using dCas9-KD (Figure 1G). Interestingly, however, inversion of the Meteor promoter caused significantly reduced expression of Eomes, albeit to a slightly lower extent than the pKO (Figure 1C). As transcription from the Meteor promoter is initiated in this cell line, with evidence for inefficient elongation to the opposite direction (Figure 1B), this could indicate that transcription elongation into the Meteor locus-and specifically, the minimal level of elongation that still occurs in the Meteor dCas9-KD cells-is required for proper Eomes regulation in mESCs. Alternatively, the functional feature of the Meteor enhancer could be the underlying DNA sequence, which is left intact in the Rz-KD, pAS, and dCas9-KD cells lines, with the promoter inversion interfering with proper functionality of this DNA, for example by disrupting the integrity, arrangement, or orientation of TF binding sites (TFBSs). To examine this possibility, we used the JASPAR database ${ }^{40}$ and $\mathrm{FIMO}^{41}$ to systematically compare the predicted TFBSs in the WT and the inverted sequence. While only few differences were identified (Figure S1H), we cannot exclude the possibility that disruption of binding of one of these TFs plays a role in regulation of Eomes levels. Replacing the Meteor promoter with another constitutively expressed promoter increased expression of Meteor, albeit with a variable effect between different clones, but had no consistent effect on Eomes levels (Figure 1H). Combined, results from these various cell lines and perturbations suggest that the functional feature of the Meteor locus in mESCs is minimal transcription initiation or elongation into the Meteor gene body.

## Reduced Eomes expression is associated with altered

 chromatin organization in mESCsAs Meteor is transcribed from an enhancer, ${ }^{20}$ we next wanted to examine whether Meteor perturbations that are associated with Eomes downregulation are also manifested in an altered chromatin organization of the locus. We therefore probed the topology of the Meteor locus using targeted chromosome conformation capture (4C) ${ }^{42}$ and CCCTC-binding factor (CTCF) chromatin immunoprecipitation sequencing (ChIP-seq). 4C analysis using either the Meteor or Eomes promoters as bait demonstrated spatial proximity between their respective loci, as has been shown previously ${ }^{14}$; deletion of either the entire Meteor gene body or the promoter region resulted in reduced contacts between the region surrounding Meteor and the Eomes locus, as did inversion of the Meteor promoter (Figures 2A, S2A, and S2B). In accordance with their lack of effect on Eomes levels, no significant changes between the Meteor and Eomes loci were noted in the dCas9-KD or Rz-KD mESC lines (Figures S2A and S2C).

These altered contacts between the Eomes and Meteor loci were not associated with any evident differential CTCF occu-
pancy in the region (Figure 2A). It was shown that in mESCs, PRC2, the chromatin remodeler responsible for H3K27me3 deposition, modulates chromatin interactions between poised enhancers and their targets, thus promoting a chromatin topology required for proper induction of the target genes throughout differentiation processes. ${ }^{44-46}$ Indeed, while the Meteor promoter region is associated with active chromatin, the region downstream to the Meteor gene body displays chromatin marks associated with both active (H3K27ac) and repressive (H3K27me3) states (Figure S2D). We therefore employed Cut\&Run to map H3K27me3 deposition around the Meteor locus in mESCs carrying various Meteor perturbations. We noted an increase in H3K27me3 levels surrounding Meteor and its downstream enhancer region in the Meteor pKO lines (Figures 2B and S2E). These increased H3K27me3 levels upon Meteor repression would suggest that Meteor acts in preventing, rather than promoting, PRC2 recruitment and H3K27me3 deposition, in line with some indications that the binding of PRC2 to DNA and RNA is mutually antagonistic. ${ }^{47-50}$ Accordingly, analysis of previously published PRC2 RNA immunoprecipitation (RIP) data ${ }^{43}$ revealed that Meteor is within the top $5 \%$ of transcripts bound by EZH2, the catalytic subunit of PRC2 (Figure 2C), with data from an independent mESC RIP experiment ${ }^{51}$ confirming an above-background association between Meteor and EZH2 but not with SUZ12, another PRC2 subunit (Figure S2F). The increased H3K27me3 deposition was also noted when the Meteor promoter was inverted (Figure 2B), altogether supporting a negative correlation between H3K27me3 deposition at the Meteor locus and Eomes expression in mESCs (Figures 1B and 2B). Combined, these results suggest that transcription through the Meteor gene body is required for restricting the spread of H3K27me3 in the Meteor enhancer region and enabling proper expression of Eomes in naive mESCs, with the nascent RNA likely mediating this function during its transcription while being dispensable after its production (see discussion).

## Meteor repression does not alter the cardiac mesoderm differentiation potential of mESCs

We next wanted to examine the effects of Meteor transcription on the cardiac mesoderm differential potential of mESCs. To this end, we utilized the hanging-drop embryoid body (EB) differentiation system. ${ }^{52}$ Consistent with its pluripotency-specific expression and with one previous study, ${ }^{20}$ Meteor expression was quickly reduced in the early days of the EB differentiation protocol (Figure S3A). Markers for the three germ layers were induced throughout the differentiation, with a relatively low induction of ectoderm markers such as Pax6 and Otx2, consistent with reduced efficiency of differentiation to the ectodermal lineage previously reported in this system for differentiation in leukemia inhibitory factor (LIF)-free and retinoic acid-free medium (Figure S3B). ${ }^{53-55}$ Expression of Eomes, as well as additional early mesoderm markers such as $T$ (Brachyury) and FoxA2, peaked at day 4 (Figure S3B).

Using qRT-PCR and RNA-seq, we monitored the effect of Meteor repression on mesodermal gene induction and cardiac mesoderm formation in this differentiation system. Perturbation of Meteor expression by either KO of its promoter or dCas9KD did not lead to any significant reduction in the formation of


Figure 2. Meteor depletion induces chromatin changes in mESCs
(A) (Top) Genome browser image of the region surrounding the Meteor locus. Shown are representative transcript models; RNA-seq tracks where orange denotes transcription on the plus strand and blue denotes transcription on the minus strand; and ENCODE mESC ChIP-seq tracks. (Middle) 4C analysis in the indicated mESC lines using the Meteor or Eomes promoters as viewpoints. Domainograms showing mean contact per fragment end for a series of window sizes are placed below smoothed trend lines and raw counts of the contact profiles. (Bottom) ChIP-seq tracks of CTCF in the indicated mESC lines. All tracks are normalized to the same scale.
(B) (Top) Genome browser image of the region surrounding the Meteor locus. RNA-seq track is the same as shown in (A); Cut\&Run analysis of H3K27me3 levels in the indicated mESC lines grown in serum-free 2i/LIF conditions. (Bottom) Bar plot shows quantification of signal in the highlighted region, normalized to WT1 and to a H3K27me3-rich region near the Ppib gene (see STAR Methods). Bars represent standard errors; $\mathrm{n}=3$. *p $<0.05$, one-sided t test.
(C) Distribution of reads per kilobase per million (RPKM) of all transcripts identified in an EZH2 RNA immunoprecipitation (RIP) dataset taken from Zhao et al. ${ }^{43}$ RPKM of Meteor indicated by a red line.
See also Figure S2.
beating foci in EBs at either day 9 or day 12 (Figures 3A and 3B). This is in stark contrast to previous reports in which Meteor repression led to severe inhibition of functional cardiac mesoderm, with only $0 \%-20 \%$ of EBs exhibiting beating foci. ${ }^{20,33}$ Accordingly, and likewise in contrast to previous reports, we did not note any consistent changes in expression of Eomes or other mesodermal marker genes in either the pKO or dCas9KD cells throughout the differentiation process (Figures 3C, S3C, and S3D). Specifically, no decrease in Eomes expression was noted at day 4, which is the peak of Eomes induction during differentiation of WT cells.

This deviation from previous reports might suggest that Meteor regulation of Eomes throughout EB differentiation depends on the genetic background of the mESCs. However, we could not find any consistent effect on either the levels or timing of activation of Eomes or additional mesoderm marker genes in mESCs derived from another background (129-C57BI/6 mESCs), in which the full Meteor gene body was removed (fKO) or Meteor RNA was destabilized by insertion of a selfcleaving ribozyme sequence (Rz-KD) ${ }^{34}$ (Figures 1A and S3E).

Nevertheless, we cannot rule out that accumulated genomic or karyotypic aberrations in our cell lines affect their pluripotency status or differentiation potential, a general concern with longterm culture of pluripotent stem cells in general and those undergoing clonal selection in particular. ${ }^{56,57}$

The most likely explanation for the inconsistency with previous reports stems from the difference in the growth medium in which the mESCs were kept prior to onset of the differentiation. Specifically, while in previous reports the mESCs were cultured in serum-based medium, ${ }^{20,33}$ we cultured the cells in $2 i$ medium pre-differentiation. mESCs grown in either serum or 2i conditions are considered to be found in a "naive" state of pluripotency and retain the ability to differentiate into the three germ layers, although both protocols are associated with some alterations to differentiation potential. Specifically, serum-cultured cells exhibit high transcriptomic variability, with a subset of the population found at a more differentiated state with elevated expression of lineage-determination genes. ${ }^{36,37,58,59}$ Therefore, and given the discrepancy in cardiac mesoderm differentiation when Meteor is depleted despite similar effects on Eomes levels


Figure 3. Embryoid body differentiation of Meteor-depleted cells
(A) Percentage of EBs that have beating foci at day 9 and day 12 of EB differentiation in the indicated cell lines. Bars represent standard errors; $\mathrm{n}=3$.
(B) Same as (A), for Meteor dCas9-KD cells.
(C) $3^{\prime}$ RNA-seq tracks of the Eomes locus on day 4 of EB differentiation in the indicated cell lines. All tracks are normalized to the same scale.

See also Figure S3.
in mESCs, we reasoned that Meteor repression affects cardiac mesoderm differentiation indirectly through affecting the cellular state at the naive mESC stage, an effect that is particularly apparent when mESCs are cultured in serum.

## Meteor and Eomes are expressed in distinct mESC subpopulations

To test this hypothesis, we performed scRNA-seq to characterize the effect of Meteor KO on Eomes expression and cellular state in serum-grown mESCs. Approximately 750 cells from either one WT or one Meteor pKO line were assayed. scRNAseq data showed that both Meteor and Eomes are variably expressed in mESCs, with Meteor detected ( $\geq 1$ reads) in just $\sim 9 \%$ of the WT cells (Figure S4A). About a quarter of WT cells had detectable expression of Eomes, mostly at low levels of 14 reads/cell, although a small population ( $\sim 2.5 \%$ of all cells) expressed Eomes at higher levels (Figure S4A).

We analyzed the scRNA-seq data using the MetaCell package, ${ }^{60}$ which partitions the cells into groups of metacells (MCs) that represent a single putative cellular state. Interestingly, despite their spatial proximity and the regulatory link offered by the reduced expression of Eomes in Meteor KO cells (Figure 1C), Meteor and Eomes were predominantly expressed in non-overlapping MCs (Figures 4A, 4B, S4B, and S4C) ( $p=0.046$; onesided Fisher's test considering only WT cells). Eomes was enriched primarily in MCs that express genes associated with a more differentiated state, such as $T, L h x 1$, and Otx2. ${ }^{61-63}$ Accordingly, gene ontology (GO) term analysis of the top 100 genes correlated with Eomes expression in this dataset yielded terms such as "anterior/posterior axis specification" and "gastrulation" (Figure S4D). Meteor, on the other hand, was enriched in a subset of MCs that are associated with high expression of pluripotency genes, such as Esrrb and Sox2 (Figures 4B, S4B, and S4C).

## Altered expression of pluripotency-associated

 programs of Meteor-depleted mESCsscRNA-seq was performed in parallel on Meteor WT and pKO cells. The total number of cells occupying MCs associated with high levels of pluripotency markers (MCs 5-15; Figures 4B, S4B, and S4C) was similar in the WT and pKO line (Figure S4E).

However, among those MCs, there was a shift of pKO cells from MCs 6-8 to MCs 12-14 (Figures 4C and S4E). Examination of the genes differentially expressed between these MC groups indicates that MCs 12-14, which are more dominant in the Meteor pKO cells, preferentially expressed pluripotency-related and primordial germ cell-related genes such as Dppa3, Zbtb10, and Nanos3, whereas MCs 6-8, which are more prominent in WT cells, preferentially expressed genes related to differentiation and development processes, such as Emp1, Tgm3, and Adap1 (Figure 4D). Accordingly, while we did not note any general effect of Meteor KO on the levels of classic pluripotency markers such as Oct4, Sox2, and Nanog in bulk RNA-seq, we did observe upregulation of genes normally associated with the pluripotent state and downregulation of genes that are normally associated with more differentiated states (Figure S4F).

The total number of cells occupying the more differentiated subset of the Meteor pKO population (MCs 2-4; Figure S4B) did not significantly change. However, similarly to the shift within the pluripotency-related population, there was a shift within these MCs, with Meteor pKO cells occupying MC 4 rather than MCs 2-3, which were populated predominantly by WT cells (Figures 4C, S4B, and S4E). The expression of Eomes itself was highest in MC 2 (Figures 4B and S4B), which is associated with high expression of genes involved in mesoderm specification such as $T$, Mesp1, Mixl1, and Gsc (Figure 4E). The reduction of cells occupying MC 2 was thus the likely cause for both the reduction of total Eomes levels seen in the bulk RNA-seq (Figure 1C) as well as the reduced capacity of serum-grown mESCs to differentiate into cardiac mesoderm genes previously noted. ${ }^{20,33}$ The fact that Meteor KO caused a decrease in cells from MCs in which Meteor itself was not expressed at significant levels (Figures 4B, 4C, S4B, and S4E) suggests that either Meteor RNA is dispensable for the establishment of the cellular states represented by these MCs-making the DNA element the functional feature of Meteor in this system-or, alternatively, that cells cycle back and forth between the different states, with Meteor repression in the plu-ripotency-related states preventing their entering the state represented by MCs 2 and 3, possibly mediated by epigenetic changes that we observed in the Meteor locus (Figures 2A and 2B) (see discussion).


Figure 4. Distinct subpopulations of mESCs in Meteor WT and KO mESCs (A) 2D projection of the MetaCell ${ }^{60}$ adjacency graph. Cells are shown as small dots, whose location indicates similarity to the adjacent cells and metacells (MCs). Color was assigned to each MC according to the ratio of WT and pKO cells that comprise it, with darker shades representing MCs comprised mostly of WT cells and lighter shades representing MCs comprised mostly of pKO cells.
(B) Correlation between the log of the fold enrichment values (expression enrichment over the median MC) for the indicated gene pairs, separated by MCs.
(C) Same as (A), separately for WT1 and pKO1 cells.
(D) Scatterplot comparing gene expression between the indicated groups of MCs. Highlighted are the 20 most differentially expressed genes in each group.
(E) Same as (D), for MCs 2 and 4.

See also Figure S4.

## A distinct functional feature in Meteor represses Eomes in NPCs

As opposed to MC 2, MC 4, which was substantially more populated in the Meteor KO population compared with WT cells, did not express predominantly mesoderm-related genes but was rather defined by an enrichment of genes associated with neurogenesis such as Pou3f1, Sall2, Trh, and Rab15 (Figure 4E). It was previously suggested that the reduced mesodermal differentiation capacity of Meteor KO cells drives them toward the neuronal lineage. ${ }^{20}$ We therefore examined whether Meteor repression had an effect on the ability of mESCs to differentiate into neurons. In contrast to the EB differentiation system, where Meteor was rapidly reduced to undetectable levels (Figure S3A), Meteor continued to be expressed throughout neuronal differentiation (using a two-step differentiation protocol described by Ying et al. ${ }^{64}$ ), albeit with reduced expression levels, an expression pattern that correlated with Eomes expression in this system
(Figures 5A and 5B). The chromatin organization of the locus also changed throughout the differentiation system, with Meteor-Eomes contacts remaining stable in neural progenitor cells (NPCs) but decreasing at day 8 of neuronal differentiation (Figure 5A). Active regions within the locus also shifted throughout the differentiation system, with the Golga4 pro-moter-which is found in significant spatial proximity to Eomes in neurons (Figure 5A)-being the most active region in NPCs, as indicated by H3K27ac ChIP-seq we performed in these cells (Figure 5C). This draws a correlation between Eomes expression and spatial proximity to the Meteor locus throughout neuronal differentiation and suggests that distinct cis elements regulate Eomes expression in this system, with the Meteor IncRNA or its underlying enhancer possibly contributing to this regulation.

Accordingly, we tested the ability of Meteor KO mESCs to undergo neuronal differentiation. Both Meteor pKO and plnv mESCs were able to differentiate to neurons, as seen by their

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Figure 5. A distinct functional feature of the Meteor locus represses Eomes throughout neuronal differentiation
(A) (Top) Genome browser image of the region surrounding the Meteor locus. Shown are representative transcript models, and RNA-seq tracks taken from Hezroni et al. ${ }^{65}$ where orange denotes transcription on the plus strand and blue denotes transcription on the minus strand. All tracks are normalized to the same scale. (Bottom) 4C analysis in the indicated cells using the Meteor or Eomes promoters as viewpoints. Domainograms showing mean contact per fragment end for a series of window sizes are placed below smoothed trend lines and raw counts of the contact profiles.
(B) RSEM quantifications of Meteor and Eomes expression levels in the indicated cell types; RNA-seq data are the same as shown in (A).
(C) ENCODE ChIP-seq tracks in mESCs (top), and H3K27ac ChIP-seq and H3K4me3 Cut\&Run tracks in NPCs (bottom). Genomic coordinates are aligned to (A).
ability to form Nestin-expressing NPCs and Tuj1-expressing neurons (Figures S5A and S5B). Surprisingly, however, when Meteor pKO cells were differentiated to NPCs, a strong upregulation of Eomes expression was noted (Figure 5D). Many genes associated with Eomes were also upregulated in the Meteor KO NPCs, including genes related to differentiation (such as Nodal, Fgf4, Fgf8, and FoxA2). The only GO term category significantly associated with Eomes upregulation in these cells was neuronal differentiation, in accordance with the known roles of EOMES in the regulation of neuronal development and differentiation. ${ }^{21}$

Eomes was upregulated in NPCs only when the Meteor promoter was deleted, but not inverted (Figure 5D). This is in contrast to the reduced level of Eomes seen in mESCs with an inverted Meteor promoter (Figure 1C), suggesting that a distinct functional feature mediates Meteor activity in mESCs as compared with NPCs, and also indicating that altered Eomes expression in NPCs is not a consequence of the differential Eomes expression in mESCs, which was noted in both pKO and plnv cells. Furthermore, Eomes levels were not increased when the pEX lines, in which transcription into the Meteor gene body was initiated at a constitutive PGK promoter rather than the Meteor promoter, were differentiated to NPCs (Figure 5E). A plausible explanation for this observation is competition between the Eomes and Meteor promoters over the activity of a shared enhancer (see discussion). Consistent with this model, Meteor dCas9-KD cells also did not show significant upregulation of Eomes upon differentiation to NPCs (Figure 5D), although it is noteworthy that there was some non-significant upregulation of Eomes compared with the control in one of the cell lines in which the KD was somewhat more efficient. It thus appears that the DNA element underlying the Meteor transcriptional activity is important for proper regulation of Eomes levels in NPCs.

## DISCUSSION

The low sequence conservation of enhancer-transcribed IncRNAs ${ }^{8,16,66}$ suggests that as a group, the RNA products of these genes likely do not play an important role in enhancer activity. Rather, if functional, these loci are proposed to act mostly via their underlying DNA element or by the act of their transcription. ${ }^{15}$ Distinguishing between these levels of functionality is notoriously difficult, complicating efforts to reconcile results from different studies on the same gene. Here, we show that the Meteor locus encodes for two distinct, cell-type-specific functions, one of which is DNA mediated and the other dependent on the act of transcription through the locus (Figure 5F). In naive mESCs, elongation through the Meteor gene body appears
to be necessary for maintaining Eomes levels, as KO or inversion of the Meteor promoter are sufficient to reduce Eomes levels by $\sim 50 \%$, whereas dCas9-KD, introduction of Rz or pAS sequences, or promoter replacement-all of which allow some level of transcription through the locus-do not have this effect on Eomes levels (Figures 1C-1H). It is noteworthy that the effects of "the act of transcription" may be specific to transcription through a particular region within the IncRNA gene, as has been demonstrated for the Airn IncRNA. ${ }^{67}$ The effect of Rz-mediated cleavage on the distance and dynamics of transcription after the Rz sequence has been encountered is not yet clear and might depend on local sequence attributes that dictate folding dynamics. Similarly, the transcription machinery has been suggested to continue transcribing for a few kilobases after encountering a pAS. ${ }^{68}$ Thus, the lack of a significant effect on Eomes levels caused by insertion of a pAS or Rz sequence into the first intron or second exon of Meteor, respectively (Figures 1A, 1D, and 1F) does not necessarily indicate that transcription through the remainder of the locus is dispensable for full functionality, nor does it contradict the phenotype observed upon insertion of a pAS further downstream of the Meteor TSS. ${ }^{20}$

The reduced Eomes expression in Meteor pKO lines is accompanied by H3K27me3 accumulation around the broad enhancer region within the Meteor locus (Figures 2B and S2E). Combined with the association of Meteor IncRNA with PRC2 (Figures 2C and S2F), this suggests that as Meteor is being transcribed its association with PRC2 evicts the latter from the DNA and prevents H3K27me3 deposition, in accordance with previous findings that the binding of PRC2 to DNA and RNA is mutually exclusive. ${ }^{47-50}$ Importantly, functionality of the act of transcription may also include the nascent RNA produced in the process-for example, by recruiting or evicting proteins such as PRC2 from the chromatin, as has been proposed ${ }^{69}$ - with the lingering RNA present at the end of the transcriptional process being dispensable for the activity. It is not yet clear in what way the increased H3K27me3 accumulation around the Meteor enhancer is translated into reduced Eomes expression and whether this involves PRC2's role in mediating spatial chromatin organization in mESCs, ${ }^{44-46}$ as might be suggested by the reduced contacts between the Eomes and Meteor regions in Meteor KO cells (Figures 2A and S2B). Regardless of the exact mechanism, however, the association of PRC2 with Meteor does not necessarily imply a sequence-specific function of Meteor, as the selectivity and specificity of PRC2's interaction with RNA is unclear. ${ }^{70-74}$

Intriguingly, inhibition of Meteor transcription elongation in mESCs causes reduction in Eomes levels despite the two transcripts being expressed in largely distinct cells within the mESC population (Figures 4B, S4B, and S4C), suggesting that

Meteor transcription indirectly regulates Eomes by affecting the state at which the cells are found. This model can also explain the apparently different effects of Meteor inhibition on the ability of mESCs to differentiate to beating EBs, which was unaffected in our hands when growing mESCs in 2i but severely affected in Meteor-null cells grown in serum. ${ }^{20,33}$ This indicates that Meteor repression affects the differentiation potential of the population when grown under specific conditions rather than the hindered differentiation ability being a direct consequence of reduced Eomes levels. This altered differentiation potential can be linked to the altered epigenetic landscape in Meteor pKO and plnv cells (Figures 2A and 2B), which could itself prevent mESCs from entering or remaining in a cellular state that requires Eomes expression, affecting the overall level of Eomes expression detected in bulk RNA-seq. This exemplifies how transcription of IncRNA loci could participate in priming differentiation or developmental processes during which they are no longer required to be expressed. Interestingly, an additional pluripo-tency-associated IncRNA, Platr4, was recently shown to act in trans to promote mesoderm differentiation in vitro and in vivo while itself being rapidly downregulated during differentiation, suggesting that such priming activities by IncRNAs might be common in early development. ${ }^{75}$

In contrast to the situation in mESCs, during neuronal differentiation the Meteor locus acts to repress, rather than enhance, Eomes expression (Figures 5D and 5F). Also contrary to its function in mESCs, the functional feature of Meteor in this system appears to be the DNA element-specifically, the short region that encompasses the TSS and first exon of the IncRNA - rather than elongation through the Meteor enhancer, as no changes in Eomes expression in NPCs are noted when the Meteor promoter is inverted (Figure 5D). The mechanism through which Meteor represses Eomes in this system is not yet clear, although considering the dependency of the phenotype on the DNA rather the RNA element and the lack of effect when the promoter sequence is severely disrupted by insertion of an alternative promoter and a hygromycin gene (Figure 5E), a particularly appealing possibility is competition between the Eomes and Meteor loci over binding to enhancers, as has been shown for other IncRNA loci. ${ }^{76} \mathrm{~A}$ possible candidate for the limiting enhancer is the region downstream of Eomes with which the Eomes locus is associated following neuronal differentiation (Figure 5A). Regardless of the mechanism, the consequence of the heightened Eomes expression in Meteor-null NPCs, and specifically whether it affects the efficiency of neuronal differentiation, remains to be seen. Alternatively, as Eomes is expressed only at specific times and compartments within the developing brain, ${ }^{21}$ increased Eomes expression in NPCs could affect the composition of the neuronal types acquired throughout the differentiation process, implicating the Meteor enhancer in fine-tuning neuronal differentiation.

The study of Meteor illustrates the importance of careful selection of perturbation techniques for the study of IncRNA loci, as both the suggested function and functional feature of the locus appear to depend on the employed perturbation method. Taken together, the emerging picture is that Meteor controls Eomes levels through distinct, cell-type-specific functional features, but not through its RNA product. While our findings do not
exclude the possibility that the Meteor locus has additional functions in other systems or through additional functional features, as has been suggested, ${ }^{33}$ we could not detect any substantial Meteor expression in systems other than mESCs, testes, and throughout neuronal differentiation (Figures S3A, 5A, and 5B; https://bis.zju.edu.cn/MCA/search3.html), which makes an RNA-dependent Meteor function in other cell types unlikely. The lack of an RNA-based function is also consistent with our inability to identify sequence-similar homologs of the Meteor IncRNA outside of rodents. The human IncRNA LINC01980 is transcribed from a syntenic region and has a similar expression pattern, ${ }^{20}$ but when its sequence is projected to the mouse genome through a whole-genome alignment it does not overlap with the Meteor locus.

The intricate, cell-type-specific regulation of Eomes through distinct features of the Meteor locus serves as an illustration of how complex regulation of master regulator TFs can be finetuned through the use of IncRNA transcriptional units. This can be achieved through combinatorial activity of multiple IncRNA units, as has been described for the regulatory network of the Hand2 $\mathrm{TF}^{17,77}$ or, as described here, through distinct functions mediated by the same locus in different cellular contexts. It is expected that in-depth study of additional IncRNA loci, using multiple perturbations in various biological systems, will uncover additional loci with complex phenotypical output and unveil other mechanisms through which IncRNA loci contribute to regulation of gene expression.

## LIMITATIONS OF THE STUDY

In contrast to several previous studies of Meteor IncRNA, we show here that Meteor-null mESCs can efficiently differentiate to cardiac mesoderm. To ascertain whether different initial growth conditions of the mESCs, and not clonal selection, indeed explain these variations, further experiments would be needed, including differentiation of our KO cells after growing them in serum-based media. Furthermore, we currently compare the effects of Meteor depletion on early mesoderm differentiation and on NPCs, which comprise a more differentiated state along the ectoderm lineage. In the future, it would be interesting to look at earlier stages of ectoderm differentiation, potentially with sin-gle-cell resolution, so as to directly compare the results with findings relating to mesoderm differentiation and to ascertain whether the changes in the Eomes-expressing subpopulation of the mESCs have an effect on differentiation to additional germ layers.

Furthermore, additional cellular models can help validate and identify other functional features of the Meteor locus. Specifically, replacing the Meteor promoter with a different, sequence-diverged yet transcriptionally active promoter might help validate our findings regarding the DNA element being the functional feature of Meteor in NPCs. Nonetheless, as clean separation of the effects of a DNA from any transcriptional activity is challenging, and as another promoter might contain some sequence elements in common with the Meteor promoter, a library of mutated Meteor promoter sequences would enable zeroing in on the exact sequence elements that confer the promoter activity. Lastly, this study focused on the roles of different
functional features within the Meteor locus in vitro; further studies are needed to delineate their contribution to early embryonic development in vivo.

## STAR太METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j. celrep.2023.112569.

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## AUTHOR CONTRIBUTIONS

N.G. and I.U. conceived the study. N.G. and R.B.-T.P. carried out experiments. N.G. analyzed all high-throughput data. Z.M. assisted with generating scRNAseq libraries. A.T. and M.B. generated the Meteor fKO and Rz-KD model. N.G. and I.U. wrote the manuscript with input from the other authors.

## DECLARATION OF INTERESTS

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## STAR太METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| :---: | :---: | :---: |
| Antibodies |  |  |
| Rabbit monoclonal anti-H3K27ac (D5E4) | Cell Signaling Technology | Cat\#8173; RRID AB_10949503 |
| Rabbit monoclonal anti-CTCF (D31H2) | Cell Signaling Technology | Cat\#3418; RRID AB_2086791 |
| Rabbit monoclonal anti-H3K27me3 (C36B11) | Cell Signaling Technology | Cat\#9733; RRID AB_2616029 |
| Rabbit monoclonal anti-H3K4me3 (C42D8) | Cell Signaling Technology | Cat\#9751; RRID AB_2616028 |
| Critical commercial assays |  |  |
| SENSE mRNA-Seq Library Prep Kit V2 | Lexogen | Cat\#001.96 |
| QuantSeq 3' mRNA-Seq Library Prep Kit FWD | Lexogen | Cat\#015.96 |
| Xfect mESC Transfection Reagent | Takara Bio | Cat\#631320 |
| Deposited data |  |  |
| Raw and analyzed RNA-seq data | This study | GEO: GSE223445 |
| Raw and analyzed 3' RNA-seq data | This study | GEO: GSE223449 |
| Raw and analyzed scRNA-seq data | This study | GEO: GSE223452 |
| Raw and analyzed 4C data | This study | GEO: GSE223451 |
| Raw and analyzed ChIP-seq data | This study | GEO: GSE223447 |
| Raw and analyzed Cut\&Run data | This study | GEO: GSE223448 |
| Analyzed data of gene expression levels in different mESC compartments | GEO record GSE99366 | GEO: GSE99366 |
| Raw data of control mESCs undergoing neural differentiation | Stryjewska et al. ${ }^{78}$ | GEO: GSE75616 |
| Raw data of EZH2 RIP-seq | Zhao et al. ${ }^{43}$ | GEO: GSE17064 |
| Raw data of EZH2 and SUZ12 RIP-seq in WT mESCs | Garland et al. ${ }^{51}$ | GEO: GSE137491 |
| Experimental models: Cell lines |  |  |
| M. musculus: WT1: R1 wild type | This study | N/A |
| M. musculus: WT2: R1, wild type | This study | N/A |
| M. musculus: WT3: R1, wild type | This study | N/A |
| M. musculus: pKO1: R1, homozygous KO of Meteor promoter | This study | N/A |
| M. musculus: pKO2: R1, homozygous KO of Meteor promoter | This study | N/A |
| M. musculus: plnv: R1, homozygous inversion of Meteor promoter | This study | N/A |
| M. musculus: WT4: 129-C57BI/6, wild type | Laboratory of Marc Bühler ${ }^{34}$ | N/A |
| M. musculus: WT5: 129-C57BI/6, wild type | Laboratory of Marc Bühler ${ }^{34}$ | N/A |
| M. musculus: WT6: 129-C57BI/6, wild type | Laboratory of Marc Bühler ${ }^{34}$ | N/A |
| M. musculus: fKO1: 129-C57BI/6, homozygous KO of Meteor gene | Laboratory of Marc Bühler ${ }^{34}$ | N/A |
| M. musculus: fKO2: 129-C57BI/6, homozygous KO of Meteor gene | Laboratory of Marc Bühler ${ }^{34}$ | N/A |
| M. musculus: Rz-KD: 129-C57BI/6, homozygous insertion of a Rz sequence into Meteor | Laboratory of Marc Bühler ${ }^{34}$ | N/A |
| M. musculus: Ctrl: R1 stably expressing dCas9 | This study | N/A |
| M. musculus: KD1: R1 stably expressing dCas9 and gRNA for KD1 | This study | N/A |
| M. musculus: KD2: R1 stably expressing dCas9 and gRNA for KD2 | This study | N/A |
| M. musculus: WT7: R1, wild type | This study | N/A |

(Continued on next page)

| Continued |  |  |
| :---: | :---: | :---: |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| M. musculus: WT8: R1, wild type | This study | N/A |
| M. musculus: pAS1: R1, homozygous insertion of pAS/MAZ into Meteor | This study | N/A |
| M. musculus: pAS2: R1, homozygous insertion of pAS/MAZ into Meteor | This study | N/A |
| M. musculus: WT9: R1, wild type | This study | N/A |
| M. musculus: WT10: R1, wild type | This study | N/A |
| M. musculus: pEX1: R1, homozygous insertion of a Hyg/pAS/PGKp into Meteor | This study | N/A |
| M. musculus: pEX2: R1, homozygous insertion of a Hyg/pAS/PGKp into Meteor | This study | N/A |
| M. musculus: pEX3: R1, homozygous insertion of a Hyg/pAS/PGKp into Meteor | This study | N/A |
| Oligonucleotides |  |  |
| For a list of oligonucleotides used in this study see Table S1 | This study | N/A |
| Recombinant DNA |  |  |
| pCas9-GFP | Ding et al. ${ }^{79}$ | Addgene plasmid \#44719 |
| pKLV-U6gRNA | Koike-Yusa et al. ${ }^{80}$ | Addgene plasmid \#50946 |
| lenti MS2-P65-HSF1_Hygro | Konermann et al. ${ }^{81}$ | Addgene plasmid \#61426 |
| CSII-U6-gRNA-CBh-3xFLAG-PA-dCas9-P2A-Puro | Deposited by the laboratory of Tohru Kimura | Addgene plasmid \#83306 |
| Software and algorithms |  |  |
| STAR | Dobin et al. ${ }^{82}$ | https://github.com/alexdobin/STAR |
| RSEM | Li and Dewey ${ }^{83}$ | https://deweylab.github.io/RSEM/ |
| DEseq2 | Love et al. ${ }^{84}$ | https://bioconductor.org/packages/ release/bioc/html/DESeq2.html |
| MetaCell | Baran et al. ${ }^{60}$ | https://tanaylab.github.io/metacell/ |
| KentUtils | Kent et al. ${ }^{85}$ | https://github.com/ENCODE-DCC/kentUtils |

## RESOURCE AVAILABILITY

## Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Igor Ulitsky (igor.ulitsky@weizmann.ac.il).

## Materials availability

This study did not generate new unique reagents.

## Data and code availability

- All RNA-seq, $3^{\prime}$ RNA-seq, scRNA-seq, 4C, ChIP-seq, and Cut\&Run data generated in this study have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. This paper also analyzes existing, publicly available data. These accession numbers for the datasets are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.


## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

R1 and 129-C57BI/6 mESCs were routinely grown on gelatin-coated plates on MEFs, in one of the following media:
Serum-based medium: DMEM (Gibco) containing 15\% ES-grade fetal calf serum (Biological Industries), 1X Sodium pyruvate (Gibco), 1X Nonessential amino acids (Gibco), $0.1 \mathrm{mM} \beta$-mercaptoethanol (Sigma), $100 \mathrm{U} / \mathrm{ml}$ Penicillin and $0.1 \mathrm{mg} / \mathrm{mL}$ Streptomycin
(Biological Industries), and $10 \mathrm{ng} / \mathrm{ml}$ LIF (Weizmann Proteomics Unit). Cells were passaged with $0.25 \%$ Trypsin (Biological Industries) every 2-3 days.

2i medium: a 1:1 mixture of Neurobasal medium (Gibco) and Knockout DMEM (Gibco), supplemented with 1X B27 (Gibco), 1X N2 (Gibco), 1X Glutamax (Gibco), 1X Sodium pyruvate (Gibco), 1X Nonessential amino acids (Gibco), $0.1 \mathrm{mM} \beta$-mercaptoethanol (Sigma), $52.5 \mu \mathrm{~g} / \mathrm{ml}$ BSA, $100 \mathrm{U} / \mathrm{ml}$ Penicillin and $0.1 \mathrm{mg} / \mathrm{mL}$ Streptomycin (Biological Industries), $1 \mu \mathrm{M} \mathrm{PD} 0325901$ (Axon Medchem), $3 \mu$ M CHIR 99021 (Axon Medchem), and 10 ng/ml LIF (Weizmann Proteomics Unit). Cells were passaged with $0.25 \%$ Trypsin (Biological Industries) every 2-3 days. Trypsin was quenched with serum-based medium, which was removed prior to re-plating.

Primed medium: DMEM/F12 (Sigma) supplemented with 1\% Knockout serum replacement (Gibco), 1X B27 (Gibco), 1X N2 (Gibco), 1X Glutamax (Gibco), 1X Sodium pyruvate (Gibco), 1X Nonessential amino acids (Gibco), $0.1 \mathrm{mM} \beta$-mercaptoethanol (Sigma), 100U/ ml Penicillin and $0.1 \mathrm{mg} / \mathrm{mL}$ Streptomycin (Biological Industries), $12 \mathrm{ng} / \mathrm{ml}$ FGF2 (Peprotech), and $20 \mathrm{ng} / \mathrm{ml}$ Activin A (R\&D). Cells were passaged with $0.05 \%$ Trypsin (Biological Industries) every 3 days. Trypsin was quenched with serum-based medium, which was removed prior to re-plating. $10 \mu \mathrm{~m}$ ROCK inhibitor (Axon Medchem) was added to the medium the day before and after each split.

## METHOD DETAILS

## Generation of promoter KO (pKO) and inversion (plnv) cell lines

To generate Meteor promoter KO lines, mESCs were depleted from MEFs by 20 min incubation on gelatin-coated plates and seeded on 6-wells ( $5 \times 10^{5}$ cells/well). mESCs were transfected with pCas9-GFP and two pKLV-U6gRNA plasmids containing gRNAs targeting the two sides of the Meteor TSS (see Table S1). Transfections were carried out using Xfect mESC Transfection Reagent (Takara Bio) according to manufacturer's instructions. MEFs were added 3h post-transfection. $0.8 \mu \mathrm{~g} / \mu \mathrm{L}$ Puromycin was added to the medium 24 h after transfection for selection. Six days after transfection, mESCs were seeded at a very low density on a 10 cm plate. Colonies were picked, expanded, and the genomic deletion was verified using PCR and single molecule FISH (see Figures S1B-S1C and Table S1). Lack of Meteor expression was verified by qRT-PCR and RNA-seq. Clones that underwent a similar process but did not exhibit the genomic deletion were expanded alongside and used as WT controls.

Generation of polyA insertion (pAS) cell lines
Meteor polyA insertion mESCs were generated by transfection with a pKLV-U6gRNA plasmid containing gRNA 2 that was used for generating Meteor pKO lines (see Table S1), pCas9-GFP, and a ssODN (200nt) containing two homology arms (50nt each), a short poly(A) site (49nt), and two MAZ sites. ${ }^{86}$ Colonies were picked, expanded, and the poly(A)/MAZ insertion was confirmed by PCR amplifications and sequencing (Table S1). Clones without an insertion were expanded alongside and used as WT controls.

Generation of PGK promoter exchange ( pEX ) cell lines
The PGK promoter knock in vector (pBlueScript-KS+ vector (Stratagene)) was generated by insertion of Meteor $5^{\prime}$ homology arm (Meteor promoter, 985 bp in length, mm9 coordinates chr9:118,315,278-118,316,262) and $3^{\prime}$ homology arm (Meteor first exon, $362 \mathrm{bp}, \mathrm{mm} 9$ coordinates chr9:118,314,889-118,315,250) upstream of a Hygromycin resistance gene and a pAS (taken from Addgene plasmid \#61426) and downstream of a PGK promoter (taken from pKLV-U6gRNA), respectively (see Figure 1A). ${ }^{87}$ The targeting vector was co-transfected with pCas9-GFP and pKLV-U6gRNA plasmids containing gRNA pEX (see Table S1) which targets immediately upstream of Meteor TSS. $0.8 \mu \mathrm{~g} / \mu \mathrm{L}$ Puromycin and $4 \mu \mathrm{~g} / \mu \mathrm{L}$ Hygromycin were added to the medium 24 h after transfection. Six days after transfection, mESCs were seeded at a very low density on a 10 cm plate. Colonies were picked, expanded, and the Hy gromycin resistance and PGK insertion was verified using PCR (Table S1). Clones without an insertion were expanded alongside and used as WT controls.

## Generation of dCas9-KD cell lines

Viruses were produced by transfecting HEK293T cells with a 1:0.65:0.35:0.25 ratio of CSII-U6-gRNA-CBh-3xFLAG-PA-dCas9-P2APuro, pMDL, pVSVG, and pRev plasmids, respectively, using PEI transfection. ${ }^{88}$ Medium was collected 48 h after transfection, filtered, and kept at $-80^{\circ} \mathrm{C}$.

These viruses were used to create mESCs stably expressing dCas9: mESCs were depleted from MEFs by 20 min incubation on gelatin-coated plates, and $6 \times 10^{5}$ cells were incubated in 1.5 mL of virus-containing medium for 30 min at $37^{\circ} \mathrm{C}$, and then seeded on two 6 -wells. $0.8 \mu \mathrm{~g} / \mu \mathrm{L}$ Puromycin was added to the medium 24 h after transfection. Surviving colonies were seeded at a very low density on a 10 cm plate. Colonies were picked, expanded, and the presence and expression of dCas9 was assayed by PCR and Western Blot.

Meteor dCas9-KD mESCs were created by infecting the dCas9-expressing mESCs with viruses prepared as described above from pKLV-U6gRNA plasmids containing gRNAs targeting the vicinity of the Meteor TSS (see Table S1). 48h post-infection, mESCs were sorted for BFP expression using FACSAria II (BD Biosciences), and screened for Meteor KD using qRT-PCR and RNA-seq.

## EB differentiation

mESCs were depleted from MEFs by 20 min incubation on gelatin-coated plates, and seeded at $500 \mathrm{cells} / 27 \mu \mathrm{~L}$ drop in differentiation medium (serum-based mESC medium without LIF), on the lids of 10 cm plates with PBS. On d2 drops were collected, combined, and
transferred to non-adherent 10 cm petri dishes in differentiation medium. On d7 individual EBs were transferred to separate gelatincoated 24 -wells and grown in differentiation medium for five additional days. EBs were screened for beating foci at day 9 and day 12 of differentiation. RNA expression throughout the differentiation was assayed using 3' mRNA-Seq or qRT-PCR (see Table S1 for primer sequences).

## Neuronal differentiation

Neuronal differentiation was based on a protocol taken from. ${ }^{89} \mathrm{mESCs}$ were grown for two passages in serum-based medium on MEF-free, gelatin-coated plates. Cells were then seeded on gelatin-coated plates ( $2 \times 10^{5}$ cells $/ 6-$ well $)$ in neuronal differentiation medium: 1:1 mixture of DMEM/F12 (Sigma) and Neurobasal medium (Gibco), supplemented with 0.5X B27 (Gibco), 0.5X N2 (Gibco), 1X Glutamax (Gibco), $0.1 \mathrm{mM} \beta$-mercaptoethanol (Sigma), $25 \mu \mathrm{~g} / \mathrm{ml} \mathrm{BSA}$, and $100 \mathrm{U} / \mathrm{ml}$ Penicillin and $0.1 \mathrm{mg} / \mathrm{mL}$ Streptomycin (Biological Industries). After four days, the cells were dissociated with $0.05 \%$ Trypsin (Biological Industries) and re-plated ( $3.5 \times 10^{5} / 6-$ well) on Poly-D-Lysine (Sigma) and Laminin (Life)-coated plates, in neuronal differentiation medium also containing $20 \mathrm{ng} / \mathrm{ml}$ FGF2 (Peprotech). FGF2 was removed after 24 hr and the cells were grown for an additional three days.

For stainings, ${ }^{65}$ cells were fixed in $4 \%$ paraformaldehyde for 15 min , and incubated in blocking buffer ( $5 \%$ donkey serum, $2 \%$ BSA, and $0.1 \%$ Triton X-100 in PBS). Primary antibodies (mouse anti-Nestin (Abcam ab6142) or rabbit anti-Beta III Tubulin (Tuj1, Abcam ab18207)) were diluted in permeabilization buffer (same as above, without Triton X-100) and incubated with the cells at $4^{\circ} \mathrm{C}$ overnight. Secondary antibodies (donkey anti-mouse Alexa 594 (Molecular Probes A21203) or goat anti-rabbit Alexa 594 (Abcam ab150080)) were diluted in permeabilization buffer and incubated with fixed cells for 2 h at RT. Imaging was done using the EVOS FL Cell Imaging System.

## RNA sequencing

$0.5-1 \mu \mathrm{~g}$ total RNA was used to prepare RNA-seq libraries using either the SENSE mRNA-Seq Library Prep Kit V2 (Lexogen) or the QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (FWD) (Lexogen), according to the manufacturer's protocols, and sequenced on either a NextSeq 500 or Novaseq 6000 machine. Reads were mapped to the mouse genome ( mm 9 ) using STAR. ${ }^{82}$ Expression levels were quantified using RSEM, ${ }^{83}$ and differential expression analysis was performed using DESeq $2 .{ }^{84}$ For $3^{\prime}$ RNA-seq, RSEM was run on a gene annotation GTF file where the last exon of each gene was extended by 2 Kb to the $3^{\prime}$ direction if no other gene was found on the same strand within that distance, to ensure that reads falling outside the annotated genes were assigned correctly.

For scRNA-seq, cells were sorted into 384-well plates containing 2 nM barcodes and lysis buffer. After MARS-seq barcoding and library preparation ${ }^{90}$ the libraries were sequenced on a NextSeq 500 machine. UMI matrices were generated, summarizing the expression levels of all genes across all WT and Meteor pKO cells, ${ }^{91,92}$ and metacells were constructed and analyzed using the MetaCell package. ${ }^{60}$

## Chromatin immunoprecipitation (ChIP)

ChIP was performed ${ }^{93}$ by crosslinking $1 \times 10^{7}$ cells in $1 \%$ formaldehyde for 10 min at RT, and the reaction was quenched by addition of 125 mM glycine and 5 min incubation at RT. Cells were centrifuged 5 min at $2000 \mathrm{xg}, 4^{\circ} \mathrm{C}$, washed three times in PBS containing protease inhibitors (PI), and lysed in 1 mL lysis buffer ( 5 mM PIPES $\mathrm{pH} 8.0,85 \mathrm{mM} \mathrm{KCI}, 1 \%$ Igepal, and 1X PI) for 15 min on ice. Cells were centrifuged 5 min at $250 \mathrm{Xg}, 4^{\circ} \mathrm{C}$, supernatant was discarded, and nuclei were lysed by incubation in nuclei lysis buffer ( 50 mM Tris-HCl pH 8.1, 10 mM EDTA pH 8.0, $1 \%$ SDS, and 1 XPI ) for 30 min on ice, followed by flash freezing in liquid nitrogen and re-thawing. Pellets were sonicated using a Bioruptor for either 12 (mESCs) or 15 (NPCs) cycles in high setting, 30 s ON, 30 s OFF, centrifuged 10 min at $14,000 \mathrm{rpm}, 4^{\circ} \mathrm{C}$, and supernatant was transferred to a new tube and diluted $\times 10$ in IP dilution buffer ( 50 mM Tris- $\mathrm{HCl} \mathrm{pH} 7.4,150 \mathrm{mM} \mathrm{NaCl}, 1 \%$ Igepal, $0.25 \%$ Deoxycholic acid, 1 mM EDTA pH 8.0, and 1X PI). $50 \mu \mathrm{~L}$ protein A/G magnetic beads (GenScript, Cat \#L00277) per IP were washed twice in binding/washing buffer ( $0.5 \%$ BSA and $0.5 \%$ Tween 20 in PBS), resuspended in 1 mL binding/washing buffer with either anti-H3K27ac (D5E4) antibody (Cell Signaling \#8173) or anti-CTCF (D31H2) antibody (Cell Signaling, \#3418), and incubated $>1 \mathrm{~h}$ at RT with rotation. Beads were washed with 1.5 mL binding/washing buffer, and $\sim 60 \mu \mathrm{~g}$ chromatin per IP was added and incubated overnight at $4^{\circ} \mathrm{C}$ rotation. IPs were washed on ice six times with RIPA buffer ( 10 mM Tris- $\mathrm{HCl} \mathrm{pH} 8.0,140 \mathrm{mM} \mathrm{NaCl}, 1 \mathrm{mM}$ EDTA pH 8.0, $1 \%$ Triton X-100, $0.1 \%$ SDS, $0.1 \%$ Deoxycholic acid; first wash also contained 1X PI), twice with RIPA-500 buffer (same, except with 500 mM NaCl), twice with LiCl wash buffer ( 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 250 mM LiCl, $0.5 \% \mathrm{NP}-40$, and $0.5 \%$ Deoxycholic acid), once with TE ( 10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0 ), and then resuspended in $50 \mu \mathrm{~L}$ of direct elution buffer ( 10 mM Tris- HCl pH 8.0 , 5 mM EDTA $\mathrm{pH} 8.0,300 \mathrm{mM} \mathrm{NaCl}$, and $0.5 \%$ SDS). Decrosslinking was done by incubating with $1 \mu \mathrm{~g}$ RNAse for 30 min at $37^{\circ} \mathrm{C}$, followed by addition of $20 \mu \mathrm{~g}$ Glycogen and $50 \mu \mathrm{~g}$ proteinase K and incubation for 2 h at $37^{\circ} \mathrm{C}$ and overnight at $65^{\circ} \mathrm{C}$. DNA was cleaned with $2.3 x$ AMPure XP breads and eluted in $60 \mu \mathrm{~L}$ of 10 mM Tris- HCl pH 8.0 .

Library preparation was done by end repair, A-tailing, and adapter ligation, ${ }^{94}$ followed by sequencing on either a NextSeq 500 or NovaSeq 6000 instrument.

## Cut\&Run

For Cut\&Run analysis, ${ }^{95} 2 \times 10^{5}$ cells were pelleted 3 min at 600 xg , washed twice in 1.5 mL Wash buffer ( 20 mM Hepes-Naoh pH 7.5 , $150 \mathrm{mM} \mathrm{NaCl}, 0.5 \mathrm{mM}$ Spermidine, and 1 X protease inhibitors), and resuspended in $300 \mu \mathrm{~L}$ Wash buffer. $10 \mu \mathrm{~L}$ of BioMag Plus

Concanavalin A beads (\#BP531, WeisScientific) were washed twice in Binding buffer ( 20 mM Hepes-KOH pH 7.9, $10 \mathrm{mM} \mathrm{KCl}, 1 \mathrm{mM}$ CaCl 2 , and $40 \mathrm{mM} \mathrm{MnCl}_{2}$ ) and added to the cells. Cell-bead mixture was rotated $5-10 \mathrm{~min}$ at RT , magnetized, resuspended in $150 \mu \mathrm{~L}$ Antibody buffer (Wash buffer with 0.15\% Digitonin, 2mM EDTA, and $1.5 \mu \mathrm{~L}$ of either H3K27me3 antibody (C36B11, Cell Signaling \#9733) or H3K4me3 antibody (C42D8, Cell Signaling \#9751), and incubated overnight at $4^{\circ} \mathrm{C}$.

Cells were washed twice in Dig-Wash buffer (Wash buffer also containing $0.15 \%$ Digitonin), resuspended in $150 \mu \mathrm{~L}$ Dig-Wash buffer containing $350 \mathrm{ng} / \mathrm{ml}$ pA-MNAse (Weizmann Proteomics Unit), and rotated 1 h at $4^{\circ} \mathrm{C}$. Cells were then washed twice with Dig-Wash buffer, resuspended in $100 \mu \mathrm{~L}$ Dig-Wash buffer, and placed in ice water for a few minutes. MNAse was activated by adding $2 \mu \mathrm{~L}$ of 100 mM CaCl 2 and incubating 30 min on ice. Reaction was stopped by adding $100 \mu \mathrm{~L} 2 \mathrm{X} \mathrm{STOP} \mathrm{buffer} \mathrm{( } 340 \mathrm{mM} \mathrm{NaCl}, 20 \mathrm{mM}$ EDTA, 4 mM EGTA, $0.15 \%$ Digitonin, $100 \mu \mathrm{~g} / \mathrm{ml}$ RNAse A, and $50 \mu \mathrm{~g} / \mathrm{ml}$ glycogen). Chromatin fragments were released by incubating 30 min at $37^{\circ} \mathrm{C}$, transferring the supernatant to new tubes, and incubating 1 h at $50^{\circ} \mathrm{C}$ with $1 \mu \mathrm{~L} 20 \%$ SDS and $2.5 \mu \mathrm{~L} 20 \mathrm{mg} / \mathrm{ml}$ Proteinase K. DNA was isolated using phenol-chloroform extraction. Library construction was done by end repair, A-tailing, adaptor ligation, and amplification ${ }^{95,96}$ and libraries were sequenced on either a NextSeq 500 or NovaSeq 6000 instrument.

## Targeted chromosome conformation capture (4C)

mESC cultures were depleted from MEFs by 20 min incubation on gelatin-coated plates. $3 \mathrm{C}^{92}$ was carried out on $5 \times 10^{6}$ cells, with the following slight modification from the protocol. ${ }^{42,97}$ permeabilization buffer constitution was $10 \mathrm{mM} \mathrm{Tris-HCl} \mathrm{pH} \mathrm{8} 10 \mathrm{mM} \mathrm{NaCl},, 0.5 \%$ NP-40, supplemented with protease inhibitors. For a list of primers used for the 4C analysis, see Table S1. Libraries were sequenced on either a NextSeq 500 or NovaSeq 6000 instrument, and analyzed using the UMI-4C package. ${ }^{42}$

## QUANTIFICATION AND STATISTICAL ANALYSIS

## Statistical analyses

The statistical analyses and software used are indicated in the Method Details section. Statistical tests used, including replicate numbers, are indicated in the figure legends, and all computed $p$ values are indicated in the figures.

## Cut\&Run data analysis

H3K27me3 signal was quantified using the bigWigAverageOverBed tool of the kentUtils package as mean signal of three repeats at the following regions: the Meteor gene body ( mm 9 coordinates chr9:118,306,226-118,315,298), a region encompassing the Meteor locus and downstream enhancer ( mm 9 coordinates chr9:118,286,700-118,314,400), and an H3K27me3-rich control region at the HoxB locus ( mm 9 coordinates chr11:96,149,799-96,216,130). Signal was normalized to the WT1 cell line and to a control region near the Ppib gene (mm9 coordinates chr9:65,914,278-65,932,710).

