NuRD-dependent DNA methylation prevents ES cells from accessing a trophectoderm fate

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Summary

Embryonic Stem (ES) cells are able to give rise to the three germ layers of the embryo but are prevented from contributing to the trophoblast. The molecular nature of this barrier between embryonic and trophectodermal cell fates is not clear, but is known to involve DNA methylation. Here we demonstrate that the Nucleosome Remodeling and Deacetylation (NuRD) co-repressor complex maintains the developmental barrier between embryonic and trophectodermal cell fates by maintaining transcriptional silencing of trophectoderm determinant genes in ES cells. We further show that NuRD activity facilitates DNA methylation of several of its target promoters, where it acts non-redundantly with DNA methylation to enforce transcriptional silencing. NuRD-deficient ES cells fail to completely silence expression of the trophectoderm determinant genes *Elf5* and *Eomes*, but this alone is not sufficient to induce transdifferentiation towards the trophectoderm fate. Rather this leaves ES cells capable of activating expression of trophectoderm-specific genes in response to appropriate extracellular signals, enabling them to commit to a trophectodermal cell fate. Our findings clarify the molecular nature of the developmental barrier between the embryonic and trophoblast cell fates, and establish a role for NuRD activity in specifying sites for de novo DNA methylation.

Introduction

The first lineage commitment event during early mammalian development results in the segregation of two distinct cell types in the early blastocyst: the inner cell mass (ICM), which will go on to form all cell types in the embryo, and the trophectoderm (TE), which will give rise to placental tissues (Johnson and McConnell, 2004). Embryonic stem (ES) cells and trophoblast stem (TS) cells can be derived from implantation-stage embryos and maintained indefinitely in culture, while retaining many features of early embryonic cells (Evans and Kaufman, 1981; Martin, 1981; Ralston and Rossant, 2005; Tanaka et al., 1998). ES cells can differentiate further into the three germ layers: mesoderm, endoderm and ectoderm, but are excluded from the trophoblast fate. In contrast, TS cells can give rise to trophoblast derivatives but are prevented from differentiating into the three germ layers.

A specific combination of epigenetic modifications and transcription factors orchestrates the distinctive potentials of ES cells and TS cells, imposing a barrier that prevents trans-differentiation once fate is established (Niwa, 2007; Ralston and Rossant, 2005). This potency barrier faced by ES cells can be overcome, however, through forced expression of TS cell-specific transcriptional factors, which allows ES cells to convert into TS cells (Gu et al., 2011; Ng et al., 2008; Niwa et al., 2005). Similarly, ES cells mutant for the DNA methyltransferases, and therefore deficient for DNA methylation-mediated transcriptional silencing, are able to commit to the TE lineage (Ng et al., 2008; Sakaue et al., 2010). These observations indicate that the barrier between ES and TS cell fates is maintained through control of gene expression.

DNA methylation is associated with transcriptional repression and is essential for embryonic development (Li et al., 1992; Okano et al., 1999). While dispensable for ES cell self-renewal, TS cell self-renewal and TS cell differentiation, DNA methylation is essential for ES cell differentiation (Jackson et al., 2004; Lei et al., 1996; Okano et al., 1999; Sakaue et al., 2010). Vertebrate genomes are globally methylated, with the most notable exceptions being CpG-rich stretches of DNA, termed CpG islands, located at the 5' ends of many genes (Bird, 2002). In ES cells the vast majority of CpG islands remain methylation-free, with the few exceptions including genes

predominantly expressed in the germline (Fouse et al., 2008; Meissner et al., 2008; Mohn et al., 2008). Exactly how de novo DNA methylation is targeted to specific regions of DNA is not well understood, although chromatin-remodeling proteins have been shown to be important for the establishment and/or maintenance of DNA methylation patterns (Bourc'his and Bestor, 2002; Klose and Bird, 2006).

The Nucleosome Remodeling and Deacetylation (NuRD) complex is a multiprotein co-repressor complex that regulates developmental transitions in embryos and ES cells (McDonel et al., 2009). ES cells lacking Mbd3, a structural NuRD component protein, are viable but are unable to commit to differentiation upon withdrawal of self-renewal signals (Kaji et al., 2006). NuRD-mediated deacetylation can direct the activity of the polycomb repressive complex 2 (PRC2) to silence gene expression (Reynolds et al., 2011), and NuRD has also been implicated in directing DNA methylation to a target gene in leukaemia cells (Morey et al., 2008). Mbd3-deficient ES cells have been shown to inappropriately express trophectoderm-specific genes in certain conditions (Kaji et al., 2006; Zhu et al., 2009). The extent to which NuRD-deficient ES cells can cross the ES-TS barrier, or whether NuRD and DNA methylation act upon the same genes to prevent misexpression of TE markers have not previously been explored.

Here we undertake a molecular investigation into the nature of the NuRD-dependent block that normally restricts ES cells away from a TE cell identity. We find that NuRD activity facilitates DNA methylation of a number of its target genes and repetitive elements in ES cells, including the TE determinant gene *Elf5*. We further show that NuRD-dependent transcriptional silencing of both *Elf5* and *Eomes* renders ES cells insensitive to TE-inducing extracellular signals. These experiments show that NuRD activity and DNA methylation function in a non-redundant manner to restrict the developmental potential of pluripotent cells, effectively constructing a barrier between ES cell and TS cell fates.

Results

Reversible hypomethylation at promoters and constitutive heterochromatin in NuRD-deficient ES cells

A number of genes reportedly regulated by DNA methylation show elevated expression levels in $Mbd3^{-/-}$ ES cells (Fig. 1A,B) (Reynolds et al., 2011). This included genes normally expressed in the germ line (*Sohlh2*, *Dazl*, and *Tex13*) and the trophectoderm determinant gene *Elf5* (Maatouk et al., 2006; Ng et al., 2008). Bisulphite sequencing near the transcription start site of each gene verified that all of these genes show a considerable degree of DNA methylation in wild type ES cells (Fig. 1C). In contrast DNA methylation levels were much reduced at all of these promoters in Mbd3-null ES cell lines, consistent with the increased expression levels seen for the corresponding genes (Fig. 1C). The majority of genes showing NuRD-dependent silencing in ES cells do not normally show DNA methylation at their promoters in ES cells (Reynolds et al., 2011) (e.g. Fig. 1D; *Htra1* and *Ppp2r2c*). Thus we conclude that DNA methylation is directed to a relatively small subset of NuRD target genes.

Ectopic expression of Mbd3b in $Mbd3^{-/-}$ ES cells restores NuRD function (Kaji et al., 2006) and results in reimposition of transcriptional silencing at NuRD target genes (Fig. 1A). Restoring NuRD function to mutant cells also resulted in the re-establishment of DNA methylation to near wild type levels (Fig. 1C). The fact that promoters re-gain DNA methylation

levels indicates that restoration of NuRD activity targets them for de novo DNA methylation. Thus the ability of ES cells to methylate these promoter sequences depends more on NuRD activity than on the existing DNA methylation pattern.

DNA methylation at constitutive heterochromatic sequences such as major satellite, minor satellite, and IAP elements, was also found to be much reduced in NuRD-deficient ES cells using methylation-sensitive restriction digests and Southern blotting (Fig. 2A,B). As with the euchromatic sequences, this loss of methylation was rescued upon reintroduction of Mbd3 to the $Mbd3^{-/-}$ ES cells, demonstrating that the ability of ES cells to methylate satellite sequences is intimately linked to their ability to form the NuRD complex. Despite being important for methylation of these heterochromatic sequences, absence of NuRD activity does not have a detectable effect upon global levels of 5-methylcytosine (Fig. 2C).

Global gene expression analysis (Reynolds et al., 2011) indicated reduced levels of *Dnmt3b* transcripts and elevated levels of *Dnmt3l* transcripts in Mbd3-null ES cells (Fig. 1B). In contrast no significant changes in the levels of Dnmt3a or Dnmt1 transcripts were detected. While decreased levels of Dnmt3b protein could explain the observed hypomethylation of minor satellite repeats (Okano et al., 1999), the fact that we also see hypomethylation at major satellites, IAPs, and single-copy sequences indicates that this is unlikely to be the only explanation for the demethylation seen in $Mbd3^{-/-}$ ES cells. Therefore, as NuRD does not control global DNA methylation levels in ES cells (Fig. 2C), we conclude that loss of DNA methylation is a particular feature of satellite sequences and a relatively small subset of genes subjected to NuRD-dependent transcriptional control in ES cells.

NuRD represses early trophectoderm markers in ES cells

One of the genes demethylated and inappropriately expressed in the absence of NuRD activity was *Elf5*. *Elf5* has been reported to serve as an epigenetically regulated gatekeeper between embryonic and trophoblast fates in embryonic stem cells (Ng et al., 2008). This fact, combined with observations that ES cells lacking *Mbd3*, or in which *Mbd3* has been knocked down, are able to express markers of TE (Kaji et al., 2006; Zhu et al., 2009), led us to further investigate the relationship between NuRD function, *Elf5* misexpression, and the barrier between embryonic and TE cell fates.

While expression of trophoblast markers in NuRD-deficient ES cells has been reported by us and by others (Kaji et al., 2006; Zhu et al., 2009), in neither study was it demonstrated whether this was due to actual transdifferentiation of ES cells towards a TE fate, or simply due to a failure of transcriptional silencing of TE markers. $Mbd3^{-/-}$ ES cells can be maintained for several years in self-renewing conditions without adopting a trophoblast morphology, ruling out any suggestion that Mbd3 is required to prevent transdifferentiation of ES cells to TE (B.H. lab, unpublished observations). Indeed, we only detect expression of markers of differentiated TE in Mbd3-null ES cells after more than five days' culture as embryoid bodies (Kaji et al., 2006). Therefore we assessed TE differentiation in wild type, $Mbd3^{-/-}$ ES cells and Mbd3b-rescued $Mbd3^{-/-}$ ES cells subjected to an extended differentiation protocol. ES cells were cultured in suspension for five days and then plated on gelatinized dishes in ES media without LIF, and then monitored for the emergence of cells showing trophoblast giant cell morphology. Three weeks after plating, cells containing very large nuclei and expressing the
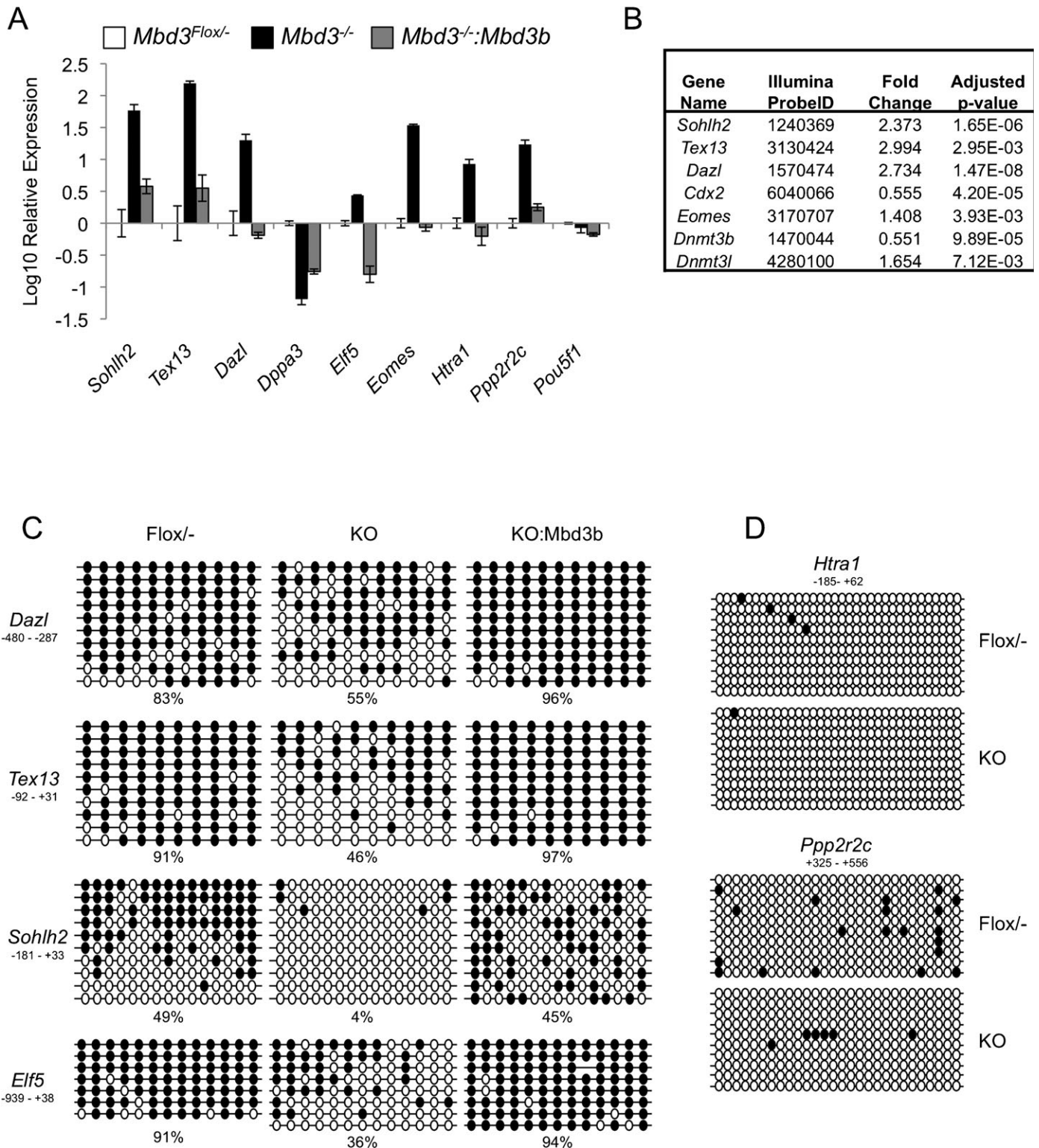
Fig. 1. Gene expression and DNA methylation changes in *Mbd3*^{−/−} ES cells. (A) Expression levels of indicated genes in *Mbd3*^{−/−} ES cells (black) and *Mbd3*^{−/−} ES cells rescued with an Mbd3b transgene (grey) are displayed relative to that seen in *Mbd3*^{Flox/-} ES cells. Error bars represent SEM from ≥3 biological replicates. All genes shown except *Pou5f1* show significant misexpression in null cells (p<0.001 using a two tailed t-test). **(B)** Expression data from Illumina bead arrays as reported (Reynolds et al., 2011). **(C)** Summary of bisulphite sequencing results at the promoters of the indicated genes in *Mbd3*^{Flox/-} ES cells (Flox/-), *Mbd3*^{−/−} ES cells (KO), and *Mbd3*^{−/−} ES cells in which Mbd3 expression was restored with an Mbd3b-expressing transgene (KO:Mbd3b). Sequences covered by the bisulphite analysis are indicated below the gene name, relative to the major transcription initiation site. Filled circles indicate methylated CpG dinucleotides, open circles represent unmethylated CpG dinucleotides. The percentage of methylated CpG dinucleotides is indicated below each panel. **(D)** Results of bisulphite sequencing in *Mbd3*^{Flox/-} ES cells (Flox/-) and *Mbd3*^{−/−} ES cells (KO) at the promoters of *Htra1* and *Ppp2r2c*. Sequences covered by the bisulphite analysis are indicated below the gene name, relative to the major transcription initiation site.
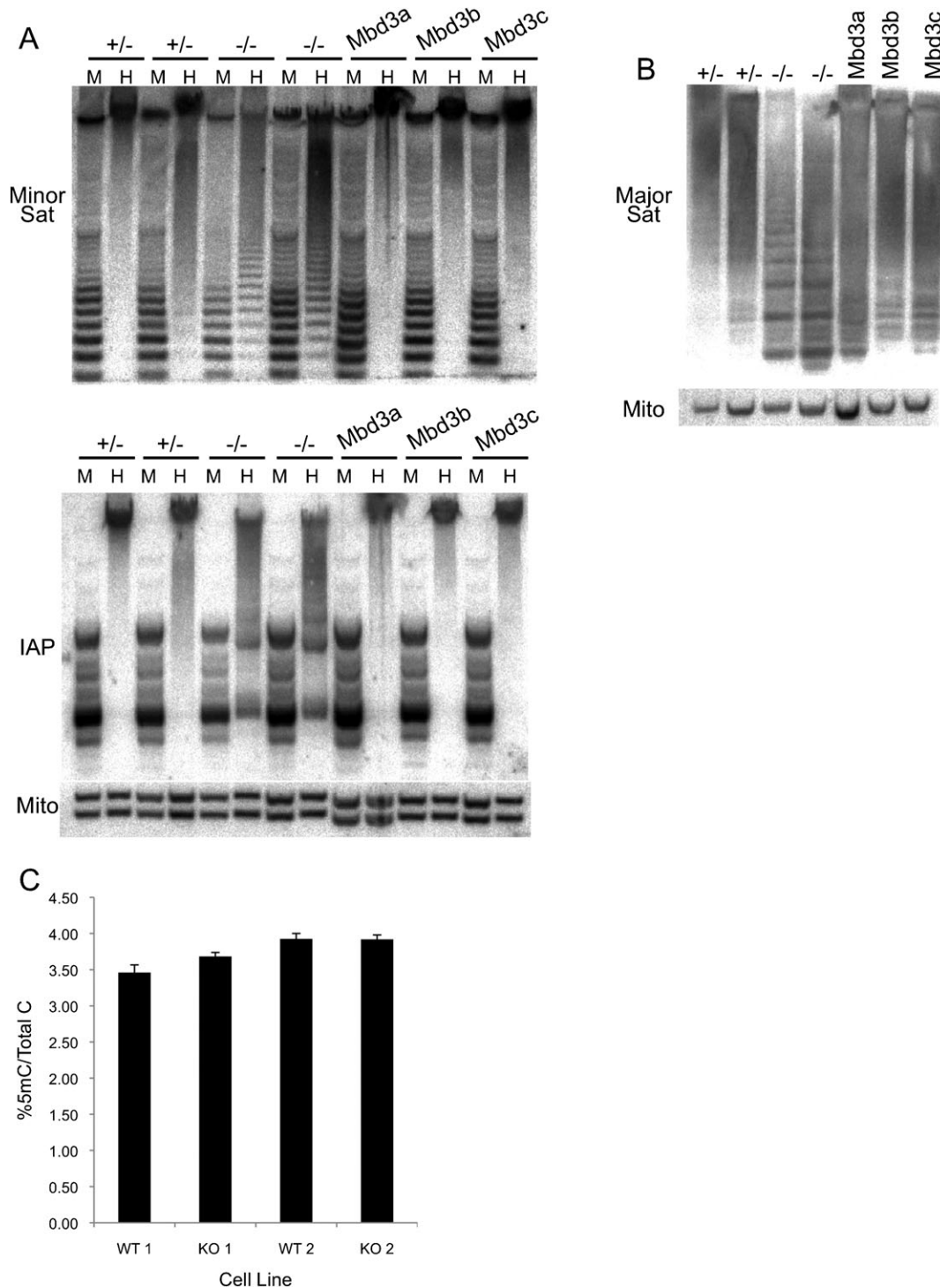
Fig. 2. Global analysis of DNA methylation in *Mbd3*$^{-/-}$ ES cells. Genomic DNA from parental ES cells (+/−), *Mbd3*$^{-/-}$ ES cells (−/−) or from ES cells expressing only Mbd3a, Mbd3b or Mbd3c was digested with MspI (M) and HpaII (H) **(A)** or with HpyCH4IV **(B)** before being Southern blotted and hybridised with probes for the minor (A) or major satellite DNA repeats (B), or for IAP LTRs (A). Mito: mitochondrial DNA probe used as a loading and digestion control. **(C)** Total 5-methylcytosine levels were quantitated in two different *Mbd3*$^{Flox/-}$ ES cell lines (WT1 and WT2) and two different *Mbd3*$^{-/-}$ ES cell lines (KO1 and KO2) by HPLC and mass spectrometry. Error bars represent SEM of three technical replicates.

trophoblast markers Elf5, Prl3d1 (PL-1) and Prl3b1 (PL-2) could be readily identified in *Mbd3*$^{-/-}$ cultures, consistent with the formation of trophoblast cells, but not in cultures made from wild type or Mbd3b rescued ES cells (Fig. 3A,B). These observations are consistent with a model in which Mbd3-null ES cells are

capable of transdifferentiation towards a trophoblast fate after prolonged exposure to differentiation conditions.

We suspected that a failure of appropriate transcriptional silencing at *Elf5*, and possibly other genes, left Mbd3-null ES cells permissive for trophoblast differentiation. Consistent with

this hypothesis, misexpression of *Eomes,* another early TE determinant, was also detectable in undifferentiated *Mbd3*$^{-/-}$ ES cells by microarray (Fig. 1B) (Reynolds et al., 2011). In contrast expression of *Cdx2* was found to be reduced in *Mbd3*$^{-/-}$ ES cells below the already very low level seen in wild type ES cells (Fig. 1B). These data were verified by quantitative RT-PCR (Fig. 3C). Culturing ES cells in media used to support TS cell maintenance (fibroblast conditioned media including Fgf4 and heparin (Himeno et al., 2008)) resulted in a slight increase of *Eomes* expression and substantial, further increase of *Elf5* expression only in the mutant cultures, but no activation of *Cdx2* was detected (Fig. 3C–E). Immunofluorescence revealed widespread co-expression of Oct4 and Eomes in Mbd3-null cultures subjected to TS conditions, consistent with a widespread conversion from an ES to TS cell identity (Fig. 3D). As expected for TS cells (Tanaka et al., 1998), withdrawal of Fgf4 resulted in expression of markers of differentiated trophoblast *Prl3d1* and *Prl3b1* in *Mbd3*$^{-/-}$ cultures but not in wild type or Mbd3b rescued cells (Fig. 3F), indicating that the TS-like cells derived from *Mbd3*$^{-/-}$ ES cells are able to differentiate into trophoblast derivatives. We conclude that NuRD blocks the very first steps in TE differentiation in ES cells by maintaining transcriptional silencing of the early trophoblast specification genes *Elf5* and *Eomes*.

Forced expression of *Elf5* in ES cells has been reported to result in activation of both *Cdx2* and *Eomes* (Ng et al., 2008), yet in our cultures *Cdx2* activation did not parallel *Elf5* expression. To investigate this discrepancy we overexpressed an epitope tagged version of *Elf5* in wild type ES cells using the strong CAG promoter (supplementary material Fig. S1). This resulted in a robust induction of *Eomes* but not of *Cdx2*, demonstrating that *Elf5* overexpression does not necessarily lead to *Cdx2* induction. Thus conversion of *Mbd3*$^{-/-}$ ES cells towards a TE fate is most likely driven by *Elf5* and *Eomes*, while *Cdx2*, which has been shown to be dispensable for the ES-to-TS conversion (Niwa et al., 2005), is not involved.

Depletion of *Elf5* or *Eomes* restores the ES-TS barrier in *Mbd3*$^{-/-}$ cells

In order to determine whether the loss of the NuRD-mediated transcriptional silencing of *Elf5* and *Eomes* underlies the loss of the barrier normally preventing ES cells from adopting a trophoblast cell fate seen in *Mbd3*$^{-/-}$ ES cells, we reduced the transcript levels of *Elf5* and *Eomes* using short hairpin RNAs (shRNAs). Levels of *Elf5* transcripts were depleted by approximately 85%, and those of *Eomes* by approximately 60% compared to levels seen in cells transfected with a scrambled shRNA control (Fig. 4A). Control and knock-down lines were maintained in TS media for 6 days, followed by 12 days of Fgf4 depletion. As shown in Fig. 4B, *Mbd3*$^{-/-}$ cells in which *Elf5* levels were depleted showed significantly reduced levels of markers of differentiated trophoblast (*Prl3d1* and *Tpbpa*) compared to *Mbd3*$^{-/-}$ cells transfected with the scrambled control shRNA. *Mbd3*$^{-/-}$ cells in which *Eomes* levels were decreased showed intermediate effects (Fig. 4B). These results indicate that the inappropriate expression of *Elf5,* and to a lesser extent *Eomes*, in the *Mbd3*$^{-/-}$ cells is responsible for the ability of these cells to differentiate into trophoblast.

Failure of gene silencing, but not precocious transcriptional activation in *Mbd3*$^{-/-}$ ES cells

In contrast to *Elf5*, activation of *Eomes* in the *Mbd3*$^{-/-}$ ES cells cannot be explained by the loss of DNA methylation, since the *Eomes* promoter is not methylated in ES cells (Ng et al., 2008). NuRD has been shown to control levels of acetylation at lysine 27 of histone H3 (H3K27ac) at target promoters, which indirectly regulates the levels of methylation at this same residue (H3K27Me3) (Reynolds et al., 2011). Therefore we next assessed the status of chromatin at the promoters of *Eomes* and *Elf5* in wild type and Mbd3-mutant ES cells. Chromatin immunoprecipitation (ChIP) across the *Eomes* locus revealed a significant loss of H3K27me3 and an increase in H3K4me3 and H3K27ac in the *Mbd3*$^{-/-}$ ES cells compared to both wild type and Mbd3b rescue ES cells (Fig. 5A), consistent with a loss of silencing and gain of transcription at this locus. Similarly, a substantial gain of the active histone marks H3K4me3 and H3K27ac was observed around the *Elf5* promoter in *Mbd3*$^{-/-}$ ES cells compared to the wild type and Mbd3b rescue ES cells (Fig. 5A). Further, the NuRD component protein Mi2β was found to associate with both the *Eomes* and *Elf5* promoters in wild type ES cells (Fig. 5B), indicating that NuRD physically associates with these promoters to maintain silent chromatin.

In self-renewing conditions, expression of *Elf5* is elevated approximately five-fold in the absence of a functional NuRD complex, but after exposure to TS cell conditions for four days expression increases a further ten-fold (Fig. 3C). This observation led us to hypothesise that Mbd3-null ES cells display a failure of silencing at *Elf5*, but not inappropriate transcriptional activation. If this is the case, then transcriptional activation of *Elf5* should require the cells to begin to adopt a TE fate, something that is critically dependent upon Fgf4 (Tanaka et al., 1998). To test this idea, wild type and *Mbd3*$^{-/-}$ ES cells were cultured in ES or TS conditions for 12 or 24 hours in the presence of an Fgf4 receptor (Fgf4r) inhibitor (SU5402), as well as in an inhibitor of Mek (PD184352), a downstream effector of the Fgf4 signaling pathway (Davies et al., 2000; Mohammadi et al., 1997) or carrier only (DMSO) (Fig. 5C). Western blotting was used to demonstrate that treatment of cells with either inhibitor for 12 hours was sufficient to completely block Erk phosphorylation (supplementary material Fig. S2). The selective inhibition of Fgf4r or Mek in TS media without Fgf4 does not alter *Elf5* expression levels in either wild type or Mbd3-null cultures. In contrast, supplementing the TS media with Fgf4 results in an approximately two-fold further increase in *Elf5* transcript levels in *Mbd3*$^{-/-}$ ES cells after just 12 hours, and this effect is blocked by either inhibitor (Fig. 5C). In contrast *Eomes* induction in *Mbd3*$^{-/-}$ ES cells is not responsive to Fgf4 signaling (data not shown). These data indicate that the *Elf5* expression seen in *Mbd3*$^{-/-}$ ES cells represents a basal expression level resulting from a failure of silencing, rather than inappropriate induction, as expression can be induced further by addition of Fgf4 and activation of the Mek/Erk pathway. Thus we suggest that transcriptional silencing by NuRD at the *Elf5* and *Eomes* promoters, which involves both histone modification and DNA methylation, normally prevents ES cells from being able to respond to external signals that would otherwise induce differentiation along the TE lineage.

Discussion

ES cells, and the inner cell mass cells from which they are derived, are able to differentiate into mesoderm, endoderm and ectoderm but are normally prevented from forming trophoblast. Ectopic expression of normally silent transcription factors has been shown to allow ES cells to overcome this differentiation
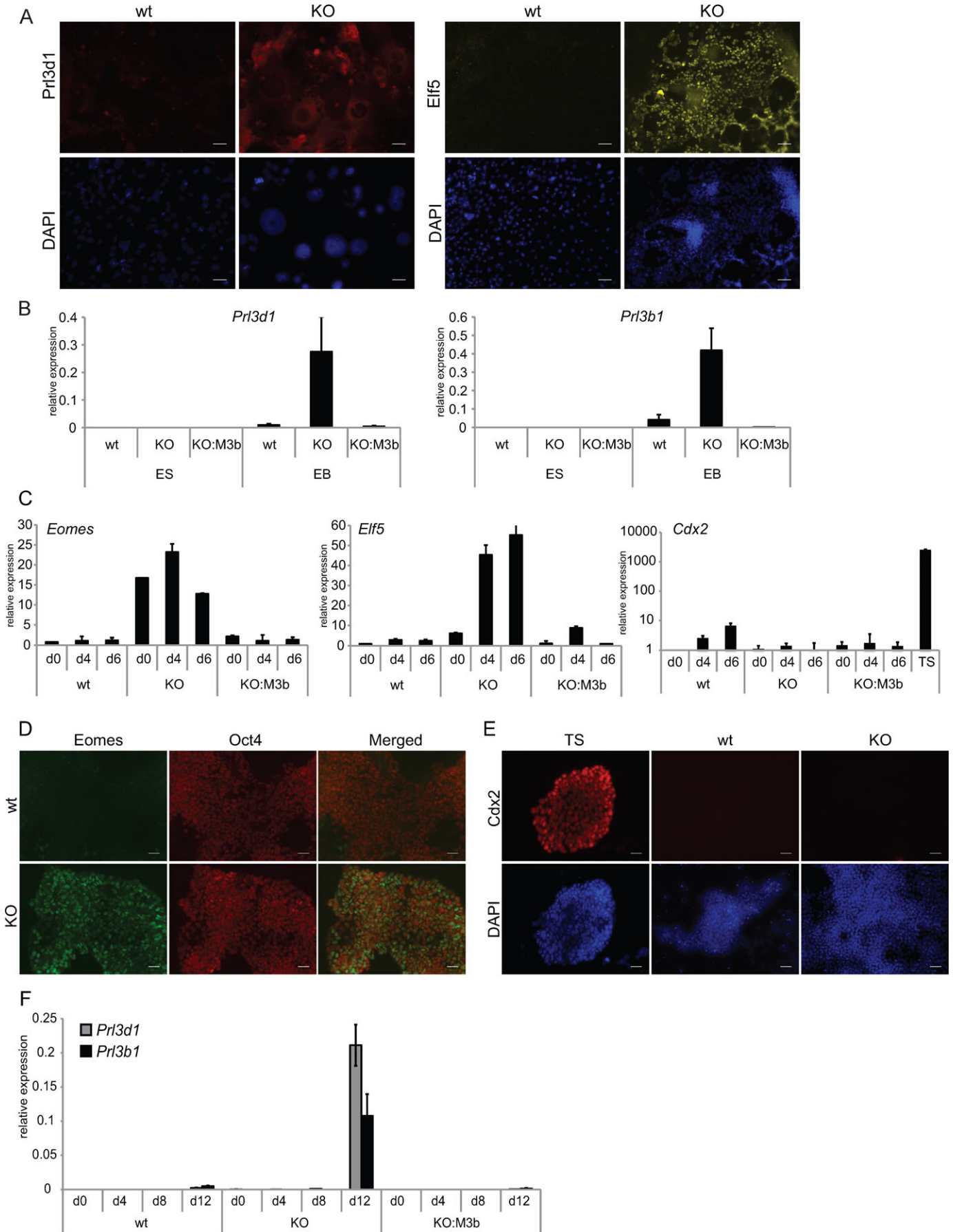
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Fig. 3. See next page for legend.
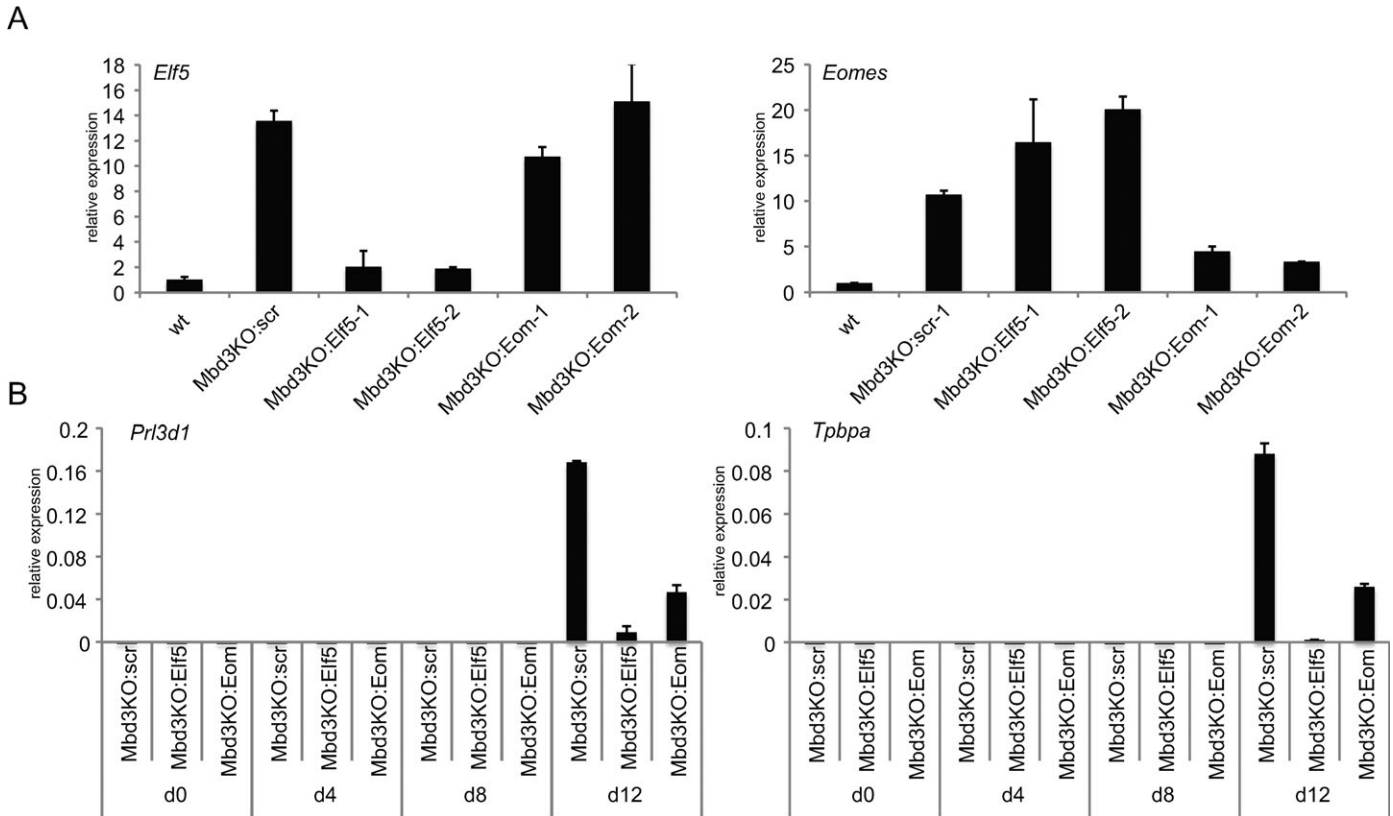
A

B

Fig. 4. Depletion of *Elf5* or *Eomes* in *Mbd3*^{−/−}ES cells prevents trophoblast differentiation. (A) Expression levels of *Elf5* (left panel) and *Eomes* (right panel) in wild type ES cells (wt) or in *Mbd3*^{−/−} ES cells (Mbd3KO) expressing control shRNA (scr), shRNA directed against *Elf5* (Elf5) or *Eomes* (Eom) are displayed relative to expression levels in wild type ES cells. **(B)** Expression levels of *Prl3d1* and *Tpbpa* in control (scr), *Elf5* depleted (Elf5) and *Eomes* depleted (Eom) Mbd3-null ES cell lines grown for 6 days in TS media and then for further 12 days in absence of Fgf4. Note that d0 refers to the cells kept for 6 days in full TS media.

barrier and adopt the normally inaccessible TE fate (Gu et al., 2011; Ng et al., 2008; Niwa et al., 2005). This provides evidence that the nature of the differentiation barrier faced by ES cells is likely to be maintained by the ability of those cells to repress expression of certain genes. Consistent with this idea, Hemberger and colleagues found that ES cells in which the maintenance DNA methyltransferase Dnmt1 is mutated lack this differentiation barrier (Ng et al., 2008). Here we demonstrate that transcriptional silencing by the NuRD complex, which at some genes involves specification of DNA methylation, also contributes to this differentiation barrier by maintaining repression of the TE-specific transcription factor genes *Elf5* and *Eomes*.

We draw a distinction between failure of silencing and activation of TE determinant genes. While ES cells lacking Mbd3 show precocious transcription of both *Elf5* and *Eomes*, expression levels remain much lower than is seen when TS

differentiation is induced with Fgf4. In contrast Fgf4 stimulation does not induce TE differentiation in wild type cells in which *Elf5* silencing is maintained (Fig. 5C). It has been reported that Mek/Erk signaling plays a crucial role in the trophoblast compartment where it promotes expression of *Cdx2* and then in turn of *Elf5* (Krueger et al., 2009; Lu et al., 2008). As we observe *Elf5* up-regulation but do not detect expression of *Cdx2* upon Fgf4 stimulation in *Mbd3*^{−/−} cells (Fig. 3C), we speculate that *Elf5* may be a direct target of Mek/Erk signaling. Our findings are consistent with a model in which NuRD-mediated, DNA methylation-dependent silencing at *Elf5* can act as a switch (Fig. 6): in the on state (i.e. absence of *Elf5* silencing) cells are able to respond to external trophoblast-inducing differentiation cues (i.e. Fgf4). However when in the off state (*Elf5* expression completely repressed) cells can no longer respond to this signal and do not initiate differentiation towards TE.

Fig. 3. *Mbd3*^{−/−} ES cells differentiate into trophoblast. (A) Immunofluorscence labeling of wild type (wt) and *Mbd3*^{−/−} (KO) embryoid bodies for Prl3d1 (Pl1, a giant cell marker) and Elf5 (a diploid trophoblast marker). Scale bars represent 113μm. **(B)** Expression levels of giant cell markers *Prl3d1* and *Prl3b1* (*Pl2*) in wild type (wt), *Mbd3*^{−/−} (KO) and *Mbd3*^{−/−} line rescued with a *Mbd3b* transgene (KO:M3b) grown as ES cells (ES) and embryoid bodies (EB). Error bars represent S.E.M of three biological replicates. **(C)** Expression levels of TS markers *Eomes*, *Elf5* and *Cdx2* in wild type (wt), *Mbd3*^{−/−} (KO) and *Mbd3*^{−/−} rescued with a *Mbd3b* transgene (KO:M3b) ES lines grown in ES (d0) and TS (d4, d6) conditions. Wild type TS line (TS) was used as a positive control for *Cdx2* expression. Error bars represent S.E.M of three biological replicates. **(D)** Double immunofluorescence labeling for Eomes and Oct4 in wild type (wt) and *Mbd3*^{−/−} (KO) ES lines grown for 4 days in TS conditions. Scale bars represent 35μm. **(E)** Immunofluorescence staining of Cdx2 in wild type (wt) and *Mbd3*^{−/−} (KO) ES lines grown for 4 days in TS conditions in comparison to wild type TS line (TS). Scale bars represent 35μm. **(F)** Expression levels of *Prl3d1* and *Prl3b1* in wild type (wt), *Mbd3*^{−/−} (KO) and *Mbd3*^{−/−} rescued with a *Mbd3b* transgene (KO:M3b) ES lines grown for 6 days in TS media and then for further 12 days in absence of Fgf4. (Note that d0 refers to the cells kept for 6 days in full TS media). Error bars represent S.E.M of three biological replicates.
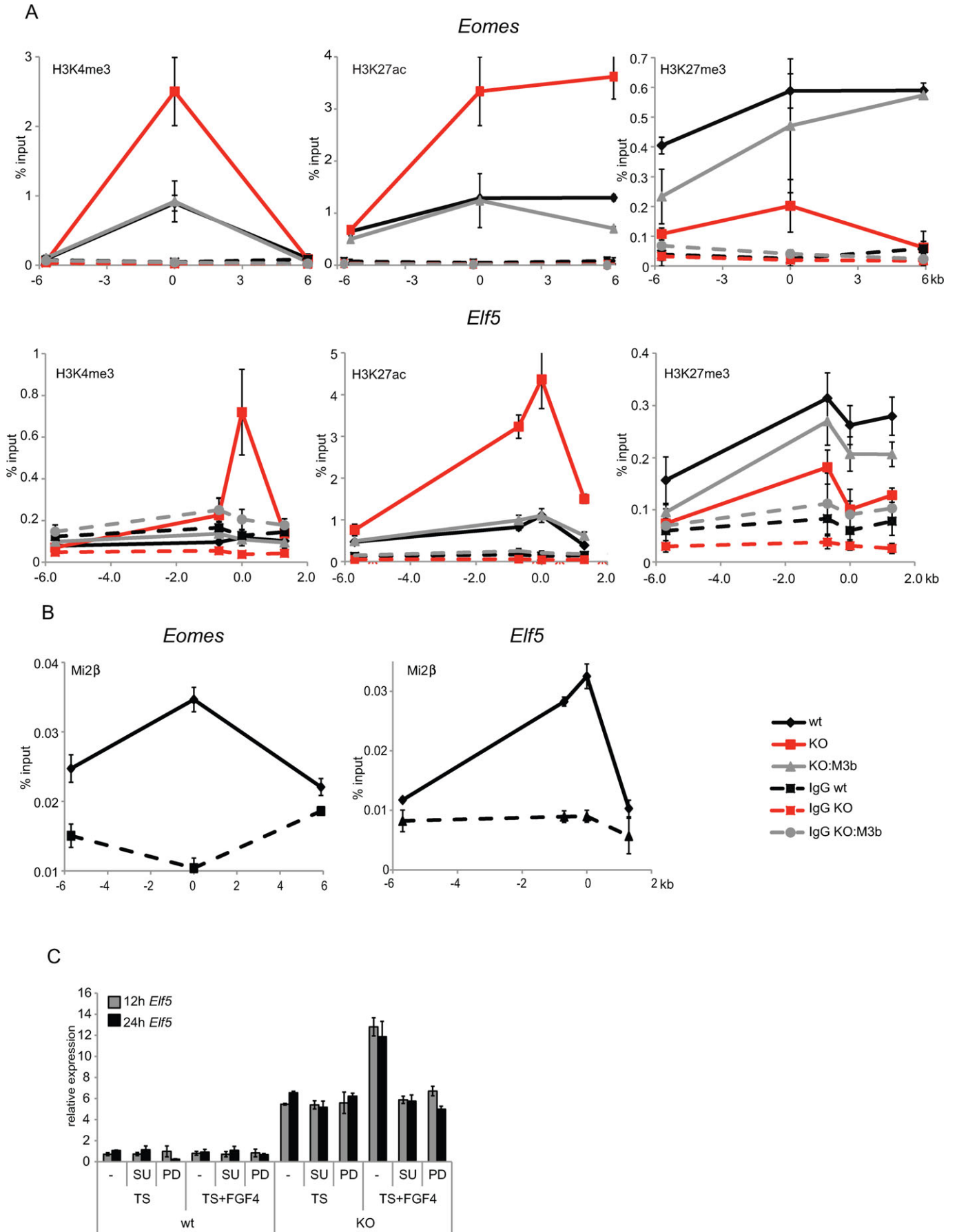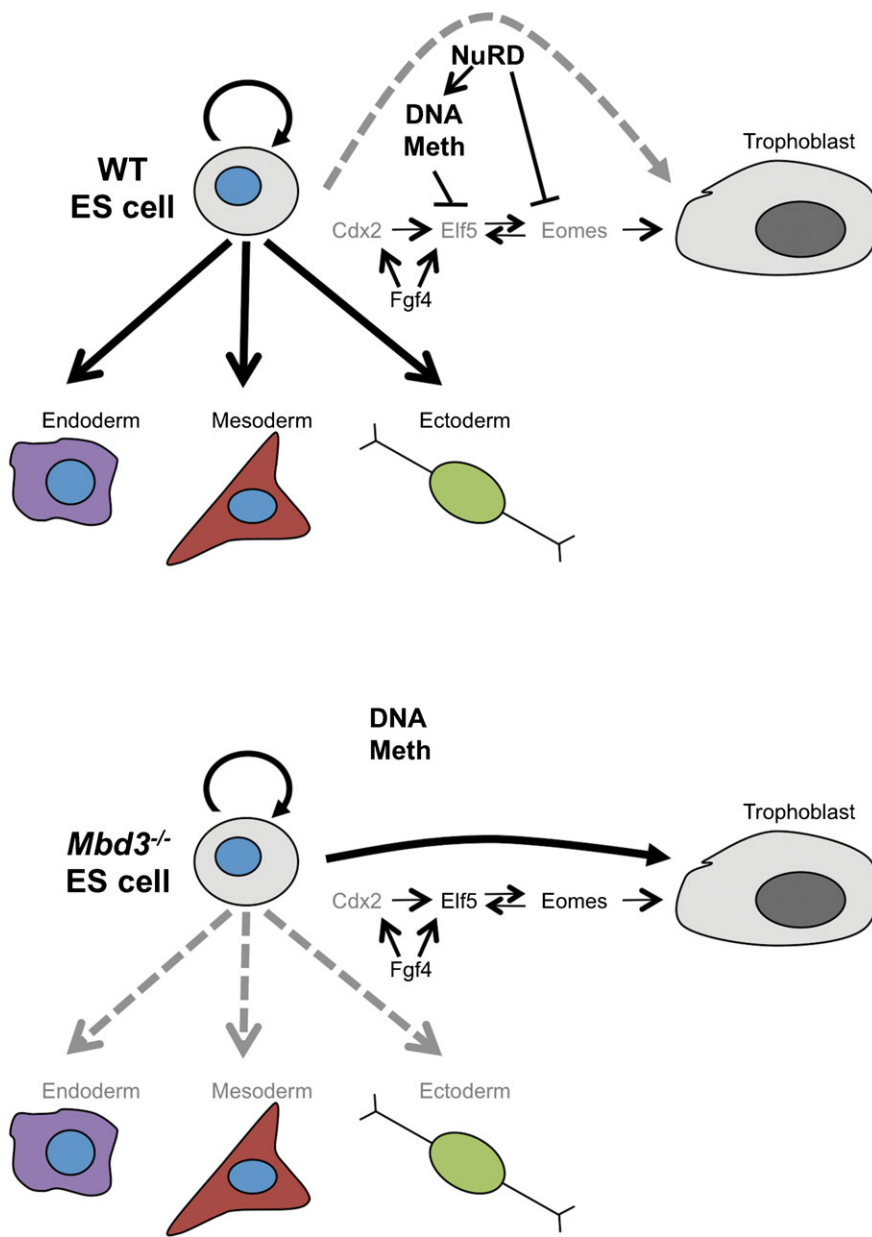
Fig. 5. See next page for legend.

Fig. 6. Model of NuRD function in ES cell self-renewal and differentiation. ES cells (top) can self-renew (circular arrow) or differentiate into one of the three germ layers (endoderm, mesoderm, or ectoderm). The path towards trophectoderm differentiation is normally blocked through the action of the NuRD complex and DNA methylation, which together maintain silencing of trophectoderm specific genes. In the wild type state trophectoderm determinant genes *Elf5* and *Eomes* are not expressed so ES cells are not responsive to the presence of trophectoderm-inducing extracellular signals such as Fgf4. *Mbd3*$^{-/-}$ ES cells (bottom) can also self-renew but cannot commit to differentiate along embryonic lineages. These cells lack a functional NuRD complex and hence fail to completely silence *Elf5* and *Eomes.* Although the DNA methylation machinery is largely intact, it is not directed towards *Elf5* in the absence of NuRD activity. While still capable of long-term self renewal, the failure of *Mbd3*$^{-/-}$ ES cells to completely silence *Elf5* and *Eomes* expression leaves them responsive to the presence of trophectoderm-inducing extracellular signals such as Fgf4. When exposed to such signals mutant cells can commitment towards a trophoblast fate.

It was surprising that overcoming the developmental barrier between ES cells and TS cells in *Mbd3*$^{-/-}$ cultures did not involve activation of the canonical TE marker *Cdx2*. *Mbd3*$^{-/-}$ embryos express *Cdx2* appropriately in early TE cells, demonstrating that Mbd3 is not required for *Cdx2* expression in vivo (Kaji et al., 2007) (B.H., unpublished), are able to implant in vivo and to outgrow ex vivo, indicating functionality of early

Mbd3$^{-/-}$ TE (Hendrich et al., 2001; Kaji et al., 2007). Similarly, *Mbd3*$^{-/-}$ TS cells show no abnormalities in *Cdx2* expression patterns (P.A.L., K. Kaji and B.H., unpublished). The absence of *Cdx2* induction in Mbd3-null ES cells was intriguing as it has been reported that Elf5, Eomes and Cdx2 regulate each other in a positive feed-back loop (Ng et al., 2008). Nevertheless it has been shown that forced expression of *Eomes* in ES cells can give rise

Fig. 5. *Mbd3*$^{-/-}$ **ES cells show active chromatin marks around** *Elf5* **and** *Eomes* **promoters. (A)** Chromatin immunoprecipitation (ChIP) using anti-histone H3 tri-methylated at lysine 4 (H3K4me3), anti-histone H3 acetylated at lysine 27 (H3K27ac), anti-histone H3 tri-methylated at lysine 27 (H3K27me3) and control IgG antibodies performed on wild type (wt; black line), *Mbd3*$^{-/-}$ (KO; red line) and rescued (KO:M3b; grey line) ES cells. Identically color-coded dashed lines indicate control IgG immunoprecipitation in the three ES lines. Immunoprecipitates were probed with primer pairs located at indicated positions relative to the transcriptional start site (X-axis). The Y-axis represents the results plotted as percentage of input. Error bars represent S.E.M of three biological replicates. **(B)** Chromatin immunoprecipitation using α-Mi2β antibody and control IgG antibodies in wild type ES cells. Immunoprecipitates were probed with primer pairs located at indicated positions relative to the transcriptional start site (X-axis). The Y-axis represents the results plotted as percentage of input. Error bars represent S.E.M of three biological replicates. **(C)** Expression levels of *Elf5* in wild type (wt) and *Mbd3*$^{-/-}$ (KO) ES lines grown for 12 h (grey bar) or 24 h (black bar) in complete TS media (TS+Fgf4) or without Fgf4 (TS) in presence of either 10 μM Mek1 inhibitor PD184352 (PD), 10 μM Fgf4 receptor inhibitor SU5402 (SU) or DMSO control (−). Error bars represent S.E.M of three biological replicates.

to trophoblast differentiation without the activation of *Cdx2*, and that *Cdx2* is not required to convert ES cells to TS cells in culture (Niwa et al., 2005) indicating that Eomes may function downstream of Cdx2 during TE formation (Fig. 6). Our results are in agreement with the latter report suggesting that activation of either *Eomes* or *Elf5* does not necessarily lead to activation of *Cdx2* in ES cells.

Both NuRD activity and DNA methylation function to maintain the lineage barrier between embryonic and trophoblast cell fates. NuRD has previously been reported to be important for de novo DNA methylation at the *Rarb* locus in leukemic cells and, together with Dnmt3a, to mediate repression of the *Pou5f1* gene in differentiating ES cells (Gu et al., 2011; Morey et al., 2008). Here we show that NuRD activity facilitates DNA methylation of a number of promoters and repetitive elements in ES cells. Despite showing a loss of DNA methylation at both major and minor satellite DNAs, quantification HPLC-MS/MS revealed no overall difference in total 5-methylcytosine levels between Mbd3-null and wild type ES cell lines. Such a directional loss of DNA methylation, i.e. loss at satellite sequences but no detectable change in overall DNA methylation levels, has been described previously in human cancer genomes (Feber et al., 2011), and may reflect some degree of redistribution of DNA methylation across the genome.

Histone deacetylation by the NuRD complex has been shown to direct the activity of PRC2 in ES cells (Reynolds et al., 2011). We detect a direct association of a NuRD component on the *Elf5* and *Eomes* promoters in wild type ES cells, which are normally silent and embedded in chromatin displaying hallmarks of transcriptional inactivity, such as DNA methylation and histone hypoacetylation. Thus it is conceivable that the class I HDAC activity contained within the NuRD complex normally serves to maintain these genes in a hypoacetylated state, directing (or allowing) both PRC2 and DNA methyltransferase activity to act upon these target promoters.

That DNA methylation patterns are re-established upon reformation of the NuRD complex indicates NuRD activity makes some of its target sequences high probability targets for de novo DNA methylation. Exactly how this works is not clear. NuRD is known to influence histone methylation levels at both H3K9 and H3K27 (Fig. 5; Reynolds et al., 2011), two marks that have been implicated in recruiting de novo DNA methyltransferase activity (Leung et al., 2011; Mohn et al., 2008). Why NuRD activity can specify the *Dazl* promoter to be methylated, for example, while the *Ppp2r2c* promoter remains unmethylated irrespective of expression status, must lie in either the DNA context or NuRD-independent chromatin context in which these genes reside. No clear correlation exists between the presence of DNA methylation, H3K27 trimethylation, or H3K4 trimethylation at these genes in wild type ES cells. Similarly, strict CpG density is unlikely to explain specific methylation of these genes, as the CpG islands associated with both *Dazl* and *Ppp2r2c* have a similar observed over expected CpG ratio (0.76, http://www.ensembl.org/Mus_musculus/Info/Index). The presence or absence of transcription factor binding sites within CpG islands has recently been shown to influence the probability of de novo DNA methylation (Lienert et al., 2011), although how such binding would be NuRD-dependent also remains to be elucidated.

NuRD's role in maintaining the barrier between embryonic and trophectodermal cell fates in ES cells exists side by side with its function in facilitating embryonic lineage commitment (Fig. 6). There is no evidence, however, that NuRD facilitates embryonic lineage commitment by maintaining the barrier to TE differentiation (Hargreaves and Crabtree, 2011). *Mbd3*$^{-/-}$ ES cells are capable of long term self-renewal despite lacking the ES-TS barrier, and after restoration of Mbd3 activity can contribute to embryonic development (Kaji et al., 2006) demonstrating that the ES-TS barrier is not required to prevent TE differentiation of ES cells. Together our findings clarify the nature of the epigenetic barrier between epiblast and trophoblast cell fates, and define the role played by NuRD-mediated transcriptional silencing in maintaining that barrier.

Materials and Methods

ES cell culture
Mbd3-null ES cell lines and floxed controls, Mbd3a-rescued and Mbd3b-rescued ES cell lines have been described (Kaji et al., 2006). Mbd3c-expressing ES cells were made as described (Aguilera et al., 2011). Mbd3b-rescued ES cells used for trophectoderm experiments were made by expressing an Mbd3b transgene fused to GFP in *Mbd3*$^{-/-}$ ES cells. ES cells were cultured on gelatin-coated dishes in standard conditions. For embryoid body differentiation cells were first grown for 5 days in suspension in ES media without LIF and then plated on gelatin-coated dishes for another 16 days. For transdifferentiation experiments 1×10^5 cells were plated on 6-well gelatin coated dishes and grown in standard TS media (RPMI, 20% serum, 1 mM sodium pyruvate, 2 mM glutamine, 100 μM β-mercaptoethanol, 25 ng/ml Fgf4, 1 μg/ml heparin) (Himeno et al., 2008), with 70% of the media preconditioned on mouse embryonic fibroblasts (MEFs). For differentiation, cells were grown on gelatin in TS media without Fgf4 and heparin.

Analysis of genomic DNA methylation
10 μg of genomic DNA was digested with HpyCH4IV, MspI or HpaII (New England Biolabs), blotted and hybridised using standard methods. For bisulphite sequencing, DNA was treated with sodium bisulphite as described (Herman et al., 1996; Tremblay et al., 1997). Modified DNA was amplified by PCR (supplementary material Table S1), cloned and sequenced. Primers were designed using the Meth Primer programme: (http://www.urogene.org/methprimer/index.html). At least ten sequences were examined for each CpG site, except for the *Elf5* promoter where at least 7 sequences were examined for each site.

For quantification of global 5-methylcytosine content, genomic DNA samples were boiled, treated with nuclease P1 (Sigma) for 16 h at 37℃, and with alkaline phosphatase (Sigma) for an additional 2 h at 37℃. After hydrolysis, total cytosine and 5mC content were measured by HPLC-MS in all samples. The LC-ESI/MS system consisted of an Agilent Serie 1100 HPLC system coupled to an Agilent LC/MSD VL mass spectrometer equipped with an electrospray ionization source (Agilent Technology, Palo Alto, CA). 50 ml of the hydrolyzed-DNA solution were injected onto an Atlantis dC18 column (2.1×150 mm; 5 mm particle size) protected by an Agilent guard column (2.1×20 mm; 5 mm particle size) at a constant flow of 0.220 ml min^{-1}. Two buffers, 0.1% formic acid in water (Solvent A) and 0.1% formic acid in 50% water: 50% methanol (Solvent B), were used, with a initial gradient of 5% solvent B, then an increase of solvent B to 50% within 9 min and an isocratic gradient (50% of solvent B) during 25 min. Electrospray source conditions were as described (Friso et al., 2002) with minor modifications. A drying gas flow of 10.0 l.min^{-1} was employed, with auxiliary 35 psis gas to assit with nebulization and a drying temperature of 350℃. The mass spectrophotometer was operated at a capillary voltage of 4000 V, and spectra were collected in positive ion mode. Identification of 2′-deoxycytidine (dC) and 5-methyl-2′-deoxycytidine (5mdC) was obtained by UV detection at A$_{254}$ and A$_{280}$. Global DNA methylation levels were calculated from integration peak areas of 5mdC relative to global cytidine (5mdC + dC).

Immunofluorescence
Cells were fixed in 4% paraformaldehyde/PBS for 20 minutes at 4℃, permeabilised and blocked for 30 minutes in 5% donkey serum/0.1% Triton X-100/PBS. The following primary antibodies with given dilutions were used: α-Cdx2 (1:200, Cell Marque), α-Eomes (1:200, ab23345, Abcam), α-Elf5 (1:200, sc-9645, Santa Cruz), α-Pl1 (1:200, sc-34713, Santa Cruz) (supplementary material Table S2). Alexa Fluor-conjugated secondary antibodies (Life Technologies) were applied at 1/1000 in blocking solution. Cells were imaged using a Zeiss AxioVert 200 microscope.

RNA knock-down and overexpression

Knock-down constructs containing shRNAs against *Elf5* or *Eomes* were purchased from Open Biosystems. Catalogue numbers and sequences targeted by the constructs are listed in supplementary material Table S3. To establish stable knock-down lines (KD), 1×10^6 $Mbd3^{-/-}$ ES cells were transfected with 4.5 µg of plasmid using Lipofectamine 2000 (Invitrogen) and after 24 hours subjected to 1 µg/ml puromycin selection. Single clones were picked and analysed. For *Elf5* overexpression, the *Elf5* open reading frame was cloned into the pCAG-Avi-3xFlag-ires-puro vector and the construct was elctroporated into $Mbd3^{+/-}$ ES cells. Stable transfectants were recovered following puromycin selection.

Quantitative PCR

Total RNA was prepared using Trizol® reagent (Life Technologies) and treated with DNaseI (Promega). First-strand cDNA was synthesized using Superscript III Reverse Transcriptase (Life Technologies). Quantitative PCR was performed using Fast SYBR Green Master Mix (Life Technologies). Gene expression was determined relative to *Gapdh* using the $\Delta\Delta$Ct method. Error bars represent S.E.M from at least 3 independent biological replicates. All quantitative PCR reactions were performed in a 7900HT Fast Real-Time PCR System (Life Technologies). Sequences of the PCR primers are listed in supplementary material Table S4.

Chromatin Immunoprecipitation

Cells were fixed in 1% formaldehyde for 15 minutes at room temperature and neutralized in 0.125 M glycine for 5 minutes followed by cell lysis (10 mM Tris-HCl pH 8, 10 mM NaCl, 0.2% NP-40) on ice for 10 minutes and nuclei lysis (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS) on ice for 10 minutes. Samples were diluted (20 mM Tris-HCl pH 8, 2 mM EDTA, 150 mM NaCl, 0.01% SDS, 1% Triton X-100) and then sonicated using a Misonix 4000 microtip probe (12 cycles, 15 seconds on/45 seconds off). Chromatin was immunoprecipitated using antibodies listed in supplementary material Table S2. DNA was assayed via quantitative PCR using primers listed in supplementary material Table S5.

Competing Interests

The authors declare no competing interests.

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