

# The chromatin modifier *Satb1* regulates cell fate through Fgf signalling in the early mouse embryo

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## ABSTRACT

The separation of embryonic from extra-embryonic tissues within the inner cell mass to generate the epiblast (EPI), which will form the new organism, from the primitive endoderm (PE), which will form the yolk sac, is a crucial developmental decision. Here, we identify a chromatin modifier, *Satb1*, with a distinct role in this decision. *Satb1* is differentially expressed within 16-cell-stage embryos, with higher expression levels in the inner cell mass progenitor cells. Depleting *Satb1* increases the number of EPI cells at the expense of PE. This phenotype can be rescued by simultaneous depletion of both *Satb1* and *Satb2*, owing to their antagonistic effect on the pluripotency regulator *Nanog*. Consequently, increasing *Satb1* expression leads to differentiation into PE and a decrease in EPI, as a result of the modulation of expression of several pluripotency- and differentiation-related genes by *Satb1*. Finally, we show that *Satb1* is a downstream target of the Fgf signalling pathway, linking chromatin modification and Fgf signalling. Together, these results identify a role for *Satb1* in the lineage choice between pluripotency and differentiation and further our understanding of early embryonic lineage segregation.

**KEY WORDS:** *Satb1*, Epiblast, Primitive endoderm, Cell lineage specification, Preimplantation, Mouse

## INTRODUCTION

The early mammalian embryo must correctly specify three distinct cell lineages: the epiblast (EPI), which gives rise to the embryo proper, and the two extraembryonic lineages, the trophoblast (TE) and the primitive endoderm (PE), which go on to form crucial supportive structures, the placenta and the yolk sac, respectively. By the 16-cell stage, the mouse embryo has a population of outside and inside cells that follow different fates. The outside cells will give rise to the TE, whereas the inside cells will form the pluripotent inner cell mass (ICM) of the blastocyst. The PE and the EPI are both derived from the ICM of the early blastocyst. Previous research has shown that in the early blastocyst the ICM contains a mixed population of PE and EPI progenitors in a mosaic ‘salt-and-pepper’ distribution, which sort themselves into distinct layers by the time the blastocyst is ready to implant [embryonic day (E) 4.5] through active cell movements (Chazaud et al., 2006; Kurimoto et al., 2006; Meilhac et al., 2009; Plusa et al., 2008). Even though they are a

mixed population early on, the individual cells in the early blastocyst are distinct enough that they go on to form either PE or EPI, but rarely both (Morris et al., 2010). It was shown that EPI precursors expressing the pluripotency marker *Nanog* secrete Fgf4 ligand in the ICM, which can initiate a signalling cascade in Gata6-positive PE precursors that have the Fgfr2 receptor highly expressed on their membranes (Frankenberg et al., 2011; Kurimoto et al., 2006; Morris et al., 2013; Ohnishi et al., 2014). This Fgf signalling is crucial for preventing *Nanog* from inhibiting Gata6 and committing cells to a PE cell fate (Frankenberg et al., 2011; Kang et al., 2013; Krawchuk et al., 2013; Schrode et al., 2014). Indeed, when Fgf signalling is inhibited, all ICM cells are directed towards a *Nanog*-positive EPI cell fate without forming any PE, whereas overexpression results in the opposite phenotype, with all cells being converted into Gata6- and Sox17-positive PE (Chazaud et al., 2006; Feldman et al., 1995; Frankenberg et al., 2011; Nichols et al., 2009; Yamanaka et al., 2010). Although the role of Fgf signalling has been well described in the embryo, much still remains unknown about how the cell-fate choice between PE and EPI occurs. Our aim was to contribute to the identification of new regulators of this lineage decision process.

When we mined a pre-existing data set for genes differentially expressed between the first precursors of ICM (inside cells) and TE (outside cells) at the 16-cell stage (Graham et al., 2014), our attention was drawn to *Satb1*, a chromatin modifier, which was three times more highly expressed in inside cells compared with outside cells, potentially indicating a role within the ICM. Although the role of *Satb1* in the early mouse embryo is unknown, it has been shown to regulate pluripotency in mouse embryonic stem cells (mESCs; Savarese et al., 2009), to regulate self-renewal and pluripotency in both haematopoietic (Will et al., 2013) and trophoblast (Asanoma et al., 2012) stem cells and to promote the differentiation of haematopoietic stem cells (Satoh et al., 2013). Here, we wished to test the hypothesis that *Satb1* contributes to lineage specification within the early mouse embryo.

## RESULTS

### Temporal and spatial expression of *Satb1* in preimplantation development

To investigate the potential role of *Satb1* in early mouse embryos, we first used qRT-PCR to analyse its expression throughout preimplantation development. This revealed high levels of maternal *Satb1* mRNA at the zygote and two-cell stages, before the zygotic genome is activated, a reduction in *Satb1* at the four-cell stage before expression increased at the eight-cell stage and was fairly stable until the blastocyst stage (Fig. 1A). The presence of maternal mRNA and the stable levels of expression after the eight-cell stage prompted us to investigate *Satb1* protein levels by immunofluorescence. We found that the overall expression of protein was highly similar to that of the mRNA, with maternal protein present in the zygote and at the two-cell stage and a drop in

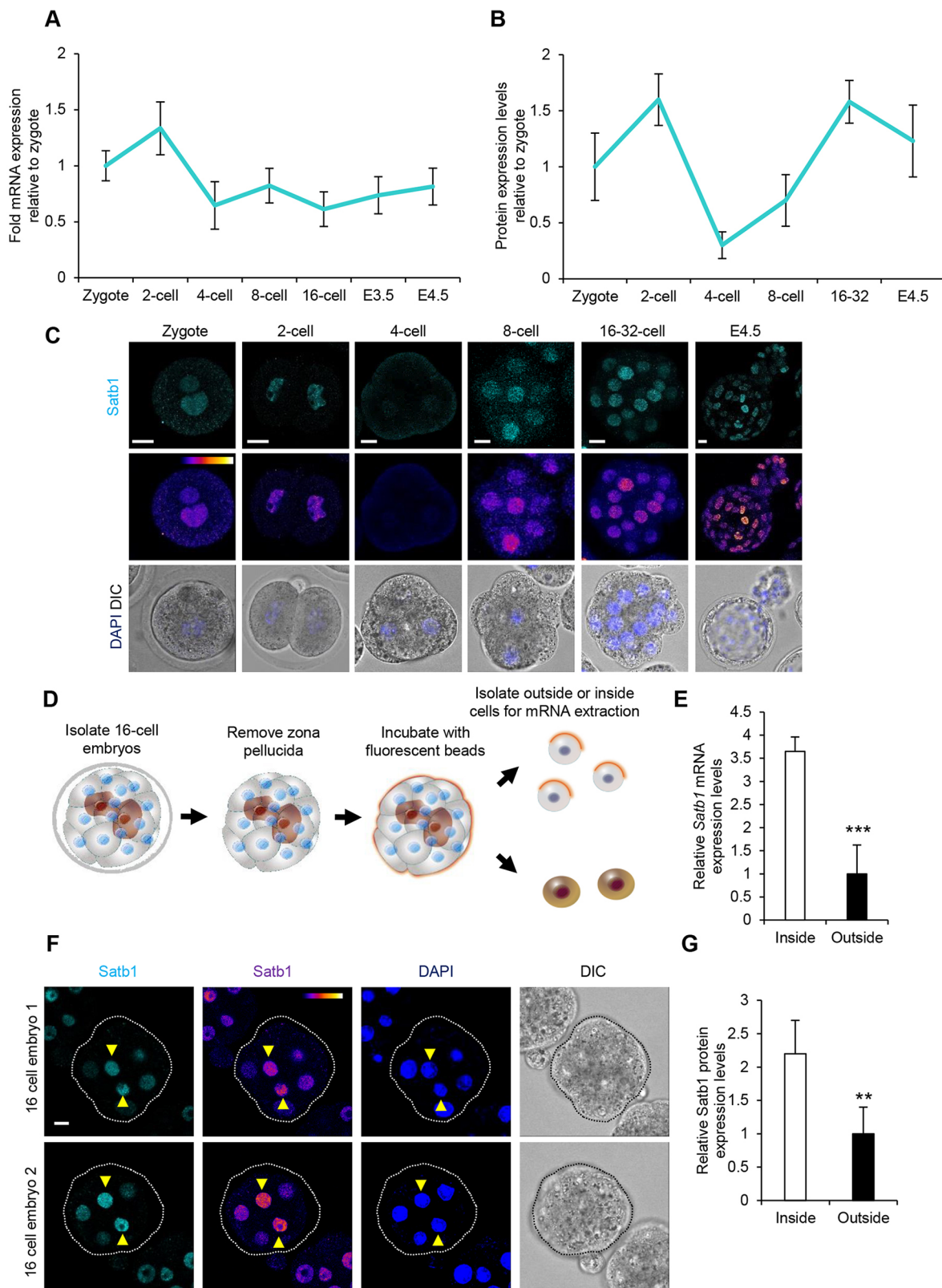
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**Fig. 1. *Satb1* expression throughout preimplantation development.** (A) qRT-PCR of embryos at zygote ( $n=42$ ), two-cell ( $n=43$ ), four-cell ( $n=39$ ), eight-cell ( $n=41$ ), 16-cell ( $n=41$ ), E3.5 ( $n=54$ ) and E4.5 ( $n=56$ ) to investigate *Satb1* mRNA levels. (B) Quantification of relative fluorescent intensity of *Satb1* staining throughout preimplantation development. Representative images are presented in C. (C) Immunofluorescence of *Satb1* in zygote ( $n=14$ ), two-cell ( $n=11$ ), four-cell ( $n=12$ ), eight-cell ( $n=15$ ), 16- to 32-cell ( $n=13$ ) and E4.5 ( $n=16$ ) embryos. (D) Scheme of isolation of inside and outside cells at the 16-cell stage for qRT-PCR shown in E. (E) qRT-PCR of inside cells ( $n=35$ ) and outside cells ( $n=41$ ) from 16-cell stage embryos to investigate *Satb1* mRNA levels. (F) Immunofluorescence of *Satb1* in 16-cell embryos ( $n=13$ ). Embryo boundary is outlined in white or black. Inside cells are indicated by yellow arrowheads. (G) Quantification of relative fluorescent intensity of *Satb1* staining. \*\* $P<0.01$ , \*\*\* $P<0.001$ . Representative images are shown in F. Scale bars: 10  $\mu$ m.

expression by the four-cell stage (Fig. 1B,C). Protein levels increased at the eight-cell (in a relatively homogenous fashion; Fig. S1A,B) and 16-cell stages, with *Satb1* protein still present until the blastocyst stage in both the TE and ICM (Fig. 1B,C).

We first identified *Satb1* as a gene of interest when examining our earlier mRNA sequencing results (Graham et al., 2014) that revealed it to be three times more highly expressed in inside cells compared with outside cells at the 16-cell stage. To confirm this expression pattern, we determined *Satb1* mRNA levels in inside and outside cells using qRT-PCR. To isolate the individual populations of inside or outside cells, we labelled 16-cell stage embryos by briefly incubating them in a suspension of 0.2  $\mu$ m fluorescent beads and then segregating inside and outside cells by gentle pipetting, as has been done previously (Graham et al., 2014). Separated individual outside (fluorescent) and inside (non-fluorescent) cells were pooled together for mRNA extraction (Fig. 1D). In total, 35 inside cells and 41 outside cells (over three experiments) were collected. Inside cells were found to have over 3.5 times more *Satb1* mRNA than outside cells (Fig. 1E;  $P<0.001$ ).

Given that *Satb1* protein expression peaked at the 16-cell stage, we next investigated whether the differential expression of *Satb1* mRNA at the 16-cell stage is recapitulated at the protein level. Fluorescence intensity measurements of *Satb1* staining for outside cells (those that had at least one domain in contact with the outside of the embryo) were compared with the intensity of inside cells (cells that were entirely surrounded by other cells) relative to 4',6-diamidino-2-phenylindole (DAPI). Intensity measurements were done on the layer-normalized sections using the ImageJ measure function. We found that inside cells had more than twofold more *Satb1* protein than the outside cells (Fig. 1F,G). These results indicate that at both protein and mRNA levels, *Satb1* is differentially expressed at the 16-cell stage.

### Depletion of *Satb1* increases number of pluripotent cells

To determine whether *Satb1* might play any role in the preimplantation embryo, we next decreased its expression using a combination of three *Satb1*-specific small interfering RNAs (siRNAs). We first confirmed that these siRNAs reduced *Satb1* at both the mRNA and protein level despite the prevalence of maternal protein and mRNA (Fig. 2A,B) and that the reduction in *Satb1* protein persisted until the blastocyst stage (Fig. S1C,D). To test the effect of *Satb1* knockdown, we injected zygotes with *Satb1* siRNA and cultured embryos until the blastocyst stage to compare the cell lineage allocation to embryos injected with a control siRNA (Fig. 2C). We found that *Satb1* RNA interference (RNAi) blastocysts had a severely reduced number of PE cells as assessed by Sox17 expression (Fig. 2D,E, Fig. S2). The total number of cells (average of 105 in control and 103 in *Satb1* siRNA blastocysts) as well as the number of TE cells (*Cdx2*<sup>+</sup> cells; average of 86 in control and 83 in *Satb1* siRNA) did not change after *Satb1* RNAi (Fig. 2D,E). Importantly, we found that the 38% reduction in PE cells was met with a 47% increase in EPI cells as assessed by the expression of Nanog and Sox17 (Fig. 2D,E), suggesting that reduced levels of *Satb1* bias the ICM to produce more EPI rather than PE. To confirm this result, we next injected each *Satb1* siRNA individually. We observed the same developmental defect using individual siRNAs as noted with the combination of *Satb1* siRNAs, with a reduction in PE cells and an increase in EPI cells (Fig. S1E,F). This phenotype was also found to be proportional to the efficacy of *Satb1* knockdown (Fig. S1G), indicating that the bias in cell fate observed upon *Satb1* depletion is specific to decreased *Satb1* and not attributable to off-target effects. We validated these findings by

assessing the expression of two additional PE markers, *Gata6* and *Pdgfra*, as well as an additional EPI marker, *Sox2*, after *Satb1* RNAi, and found a similar bias, with *Satb1*-reduced embryos having an increase in EPI and a decrease in PE by the blastocyst stage (Fig. S3). We next investigated the timing of the effect of *Satb1* RNAi in the embryo. We found that at the 16-cell stage (Fig. S4A,B) and at the initiation of the blastocyst, the 32-cell stage (Fig. S4C,D), there was no effect on the distribution or expression pattern of *Gata6* or *Nanog* after *Satb1* RNAi. However, by the early blastocyst stage we noted a significant reduction in the number of cells expressing *Gata6* after *Satb1* RNAi (Fig. S4E,F). These data suggest that although *Satb1* has no effect on the initiation of PE specification, it does have a specific role in PE lineage commitment.

The reduction in PE and increase in EPI after *Satb1* RNAi suggested that *Satb1* could have a role in the cell-fate choice within the ICM. To verify this result, we next determined whether individual blastomeres with reduced *Satb1* could have a preferential fate. To this end, we injected one blastomere of two-cell stage embryos with *Satb1* or control siRNA, together with the membrane-bound phosphoprotein *Gap43*-GFP (Benowitz and Routtenberg, 1987) mRNA, which can serve as a marker of cell lineage by labelling the membranes of injected cells (Fig. 2F). The embryos were cultured for 72 h, until the late blastocyst stage, and the contribution to TE, EPI and PE was scored by assessing molecular markers for each lineage and cell position within the embryo. In comparison with control-injected embryos, we found that *Satb1* siRNA-injected blastomeres contributed significantly more to the EPI (Fig. 2G,H;  $P<0.001$ ). Consequently, *Satb1* siRNA-injected blastomeres also contributed significantly fewer cells to the PE (Fig. 2G,H;  $P<0.001$ ; cells contributed to the PE: 4.96 in control embryos, 2.28 in *Satb1* RNAi embryos). In agreement with previous results, injection of *Satb1* siRNA into half of the embryo had no effect on the relative total contribution of injected cells or the contribution to the TE when compared with control-injected embryos (Fig. 2G,H). These results indicate that clonal depletion of *Satb1* biases cell-fate choice in the embryo: cells with lower *Satb1* will preferentially give rise to the EPI as opposed to the PE.

### Increasing *Satb1* decreases the number of pluripotent cells

Given that reducing *Satb1* directs cells towards the pluripotent lineage, we hypothesized that *Satb1* might have a role in promoting a PE lineage. To investigate the expression pattern of *Satb1* in presumptive PE and EPI cells, we analysed *Satb1* expression, together with *Gata6* (a marker of PE progenitors), in the blastocyst ICM. We found that *Satb1* expression was significantly higher in PE precursors as opposed to EPI precursors (Fig. 3A,B;  $P<0.001$ ), as would be expected of a gene with a role in PE specification. To investigate whether overexpressing *Satb1* might have the opposite effect to its reduction, we reverse transcribed mRNA from a *Satb1* cDNA, injected it into zygotes (400 ng/ $\mu$ l) and let embryos develop until morula stage, when we analysed them by qRT-PCR. We found that the injection of *Satb1* mRNA resulted in a more than twofold increase in *Satb1* mRNA levels (Fig. 3C;  $P<0.05$ ), indicating that overexpressing *Satb1* is effective. To test whether overexpressing *Satb1* mRNA was able to rescue the phenotype seen after *Satb1* siRNA, we depleted *Satb1* siRNA in the zygote and then overexpressed *Satb1* mRNA in both blastomeres of the two-cell stage embryo (Fig. S5A). Overexpression of *Satb1* was able to return the number of PE and EPI cells to levels similar to controls, providing evidence for the specificity of the siRNA phenotype (Fig. S5B,C).

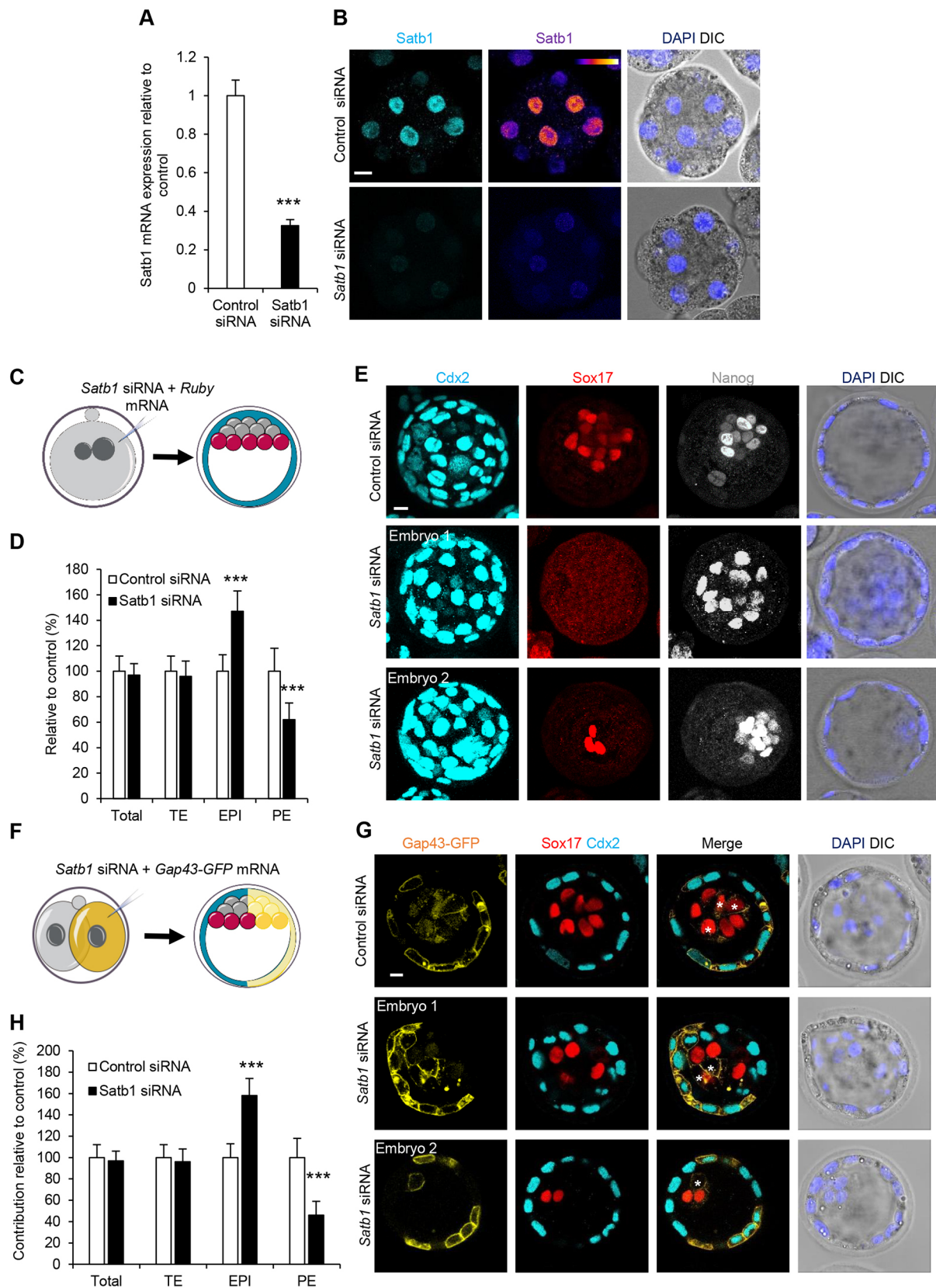


Fig. 2. See next page for legend.

To test the consequences of increasing *Satb1* mRNA on lineage allocation, we first injected *Satb1* mRNA into zygotes and allowed them to develop until the blastocyst stage and compared the

contribution of mRNA-injected embryos to TE, EPI and PE (Fig. 3D). Overexpression of *Satb1* resulted in a significant increase in the number of PE cells ( $P < 0.01$ ) and a significant decrease in the

**Fig. 2. Reducing *Satb1* biases ICM cell fate towards EPI over PE.** (A) qRT-PCR of embryos injected with control siRNA ( $n=52$  embryos, three biological repeats) and *Satb1* siRNA ( $n=61$  embryos, three biological repeats) to investigate *Satb1* mRNA levels. Embryos were injected at zygote and isolated at the eight-cell stage. Embryos injected with *Satb1* siRNA show a reduction in *Satb1* mRNA by the eight-cell stage. (B) Immunofluorescence of *Satb1* in eight-cell embryos after being injected with control ( $n=11$ ) or *Satb1* siRNA ( $n=15$ ). (C) Scheme of *Satb1* siRNA experiment shown in D and E. Zygotes were injected with *Satb1* siRNA or control siRNA and cultured until E4.5. (D) Contribution of control ( $n=29$ ) and *Satb1* ( $n=36$ ) siRNA-injected embryos to EPI, PE and TE. Representative images of the experiment are shown in E, (E) Confocal images of control and *Satb1* siRNA-injected embryos. *Nanog*, (EPI), *Sox17* (PE) and *Cdx2* (TE) were used as lineage markers (related to Fig. S2). (F) Scheme of clonal *Satb1* siRNA experiment shown in G and H. One blastomere of two-cell stage embryos was injected with *Satb1* siRNA, or control siRNA, and *Gap43-GFP* mRNA. Embryos were cultured to the late blastocyst stage, and the contribution of the injected cells' progeny to each lineage was analysed. (G) Confocal images of control ( $n=21$ , average of 5.2 *Sox17*-positive/*Gap43-GFP*-negative and an average of 4.96 *Sox17*-positive/*Gap43-GFP*-positive blastomeres per embryo) and *Satb1* ( $n=29$ , average of 7.84 *Sox17*-positive/*Gap43-GFP*-negative and an average of 2.28 *Sox17*-positive/*Gap43-GFP*-positive blastomeres per embryo) siRNA-injected embryos. *Sox17* (PE) and *Cdx2* (TE) were used as lineage markers. Asterisks indicate ICM cells contributed from injected blastomeres. (H) Contribution of *Satb1* siRNA-injected cells from experiment shown in G to TE, PE and EPI, relative to control siRNA-injected cells. \*\*\* $P<0.001$ . Scale bars: 10  $\mu\text{m}$ .

number of EPI cells ( $P<0.01$ ) when compared with controls (Fig. 3E,F), the opposite effect of what we found when knocking down *Satb1*. These results indicate that modulating the levels of *Satb1* has a specific effect on the differentiation of the ICM into PE or EPI: an increase in *Satb1* levels pushes ICM cells preferentially to form PE instead of EPI.

To verify this result, we also determined the lineage contribution when increasing *Satb1* clonally. To this end, we injected 400 ng/ $\mu\text{l}$  of *Satb1* mRNA together with *Gap43-GFP* mRNA into one blastomere of a two-cell embryo, cultured the embryos to the blastocyst stage and assessed lineage contribution using molecular markers as well as cell position (Fig. 3G). We found that *Satb1* mRNA injection resulted in a significant decrease in contribution to the EPI (Fig. 3H,I;  $P<0.05$ ; cells contributed to the EPI: 5.16 in control embryos, 3.61 in *Satb1* RNAi embryos) as well as a significant increase in PE contribution relative to control (Fig. 3H,I). These results were not attributable to a reduction in total or TE cell contributions, as both *Satb1* mRNA and control mRNA embryos were similar in their TE and total number of cells (Fig. 3H,I). Therefore, clonal overexpression of *Satb1* biases ICM cells to form PE and not EPI. Collectively, these results, together with the clonal siRNA results, indicate that modulating the amount of *Satb1* in the embryo has a specific effect on cell-fate choice within the ICM.

#### Simultaneous depletion of *Satb1* and *Satb2* rescues *Satb1* depletion

*Satb2* is closely related to *Satb1*, and it has been shown that knocking down both *Satb1* and *Satb2* simultaneously in mESCs can rescue the impaired differentiation noted in *Satb1*<sup>-/-</sup> mESCs (Savarese et al., 2009). We therefore decided to investigate whether *Satb2* RNAi could also rescue the *Satb1* siRNA phenotype in the embryo. We first tested the effectiveness of *Satb2* siRNA using qRT-PCR. To this end, *Satb1* siRNA, *Satb2* siRNA or a combination of both was injected into zygotes at a final total concentration of 12  $\mu\text{M}$  and embryos were collected at the 16-cell stage for mRNA extraction. We found that the knockdown of *Satb2* siRNA did not affect *Satb1* mRNA levels but was effective in reducing *Satb2* mRNA by 63% when compared with control

(Fig. 4A;  $P<0.01$ ). Interestingly, *Satb1* RNAi resulted in a more than twofold increase in *Satb2* mRNA while reducing *Satb1* mRNA by almost 70% (Fig. 4A;  $P<0.001$ ). The opposite result was found when *Satb1* was overexpressed, with a 50% reduction in *Satb2* mRNA along with a twofold increase in *Satb1* mRNA (Fig. S6;  $P<0.05$ ). These results show that RNAi for both of these closely related genes is specific to each gene and that *Satb1* might be a negative regulator of *Satb2*. Additionally, double knockdown of *Satb1* and *Satb2* reduced the levels of both mRNAs to ~40% of the control values (Fig. 4A;  $P<0.01$ ), indicating that the siRNAs can work simultaneously without interfering with one another when injected into the same embryos.

We next determined the effect of *Satb2* siRNA on lineage specification and whether or not it could rescue the *Satb1* siRNA phenotype. To this end, *Satb1* siRNA, *Satb2* siRNA or a combination of both were injected into zygotes at a final total concentration of 12  $\mu\text{M}$  and embryos were allowed to develop until E4.5, when their lineage specification was evaluated (Fig. 4B). We found that *Satb2* siRNA by itself had no effect on preimplantation development, with a similar number of TE, PE and EPI cells present compared with controls (Fig. 4C,D). However, double knockdown of *Satb1* and *Satb2* siRNA was able to rescue the *Satb1* siRNA phenotype significantly, leading to the number of EPI and PE cells being more similar to control embryos (Fig. 4C,D). These results indicate that *Satb2* and *Satb1* have antagonistic effects on cell-fate choice within the ICM.

#### *Satb1* modulates the expression of cell-fate regulators

As our results indicate that modulating the levels of *Satb1*, and to a lesser degree *Satb2*, affects the cell-fate choice within the ICM, we next wished to determine the changes in gene expression as a result of changing the levels of *Satb1* and *Satb2*, concentrating on key cell-fate determinants at this stage. To this end, we injected *Satb1* siRNA, *Satb1* mRNA, *Satb2* siRNA or *Satb2* mRNA into zygotes and allowed them to develop until the morula stage (about the 32-cell stage) before mRNA extraction (Fig. 5A,B). We found that *Satb1* RNAi resulted in a significant increase in the key EPI regulators *Nanog*, *Oct4* and *Sox2* (Fig. 5A; 3.9-, 2.5- and 1.98-fold, respectively; *Nanog* and *Oct4*:  $P<0.001$ ; *Sox2*:  $P<0.01$ ). It also resulted in a significant decrease in the differentiation markers of the TE, such as *Cdx2* ( $P<0.05$ ) and *Id2* ( $P<0.001$ ), and also PE marker genes, such as *Gata6* ( $P<0.01$ ) and *Sox17* ( $P<0.01$ ). As expected, injection of *Satb1* mRNA had the opposite effect, with a decrease in *Nanog* and increases in *Id2*, *Gata6* and *Sox17* expression (Fig. 5A; *Nanog*:  $P<0.05$ ; *Id2*, *Gata6* and *Sox17*:  $P<0.001$ ). *Satb2* RNAi also resulted in decreased expression of *Nanog* (Fig. 5B;  $P<0.05$ ) but did not alter the expression of any of the other genes examined here (Fig. 5B), perhaps accounting for the lack of a phenotype noted after *Satb2* RNAi. Interestingly, overexpression of *Satb2* resulted in an almost twofold increase in *Nanog*, *Oct4* and *Sox2* without affecting the other lineage markers (Fig. 5B; *Nanog* and *Sox2*:  $P<0.05$ ; *Oct4*:  $P<0.01$ ), indicating a potential role in promoting an EPI lineage. In agreement with this, we found *Satb2* to be co-expressed with *Nanog* in the early blastocyst and, consequently, to be significantly more highly expressed in EPI cells as opposed to PE cells by the late blastocyst (Fig. S7A,A',B;  $P<0.001$ ). Overall, these results indicate that *Satb1* modulates the expression of cell-fate regulators during preimplantation development and that *Satb1* and *Satb2* have antagonistic effects on *Nanog* expression within the early embryo.

#### Fgf signalling regulates *Satb1*

As the above results indicated that *Satb1* is involved in the specification of PE and EPI in the ICM, we next wished to

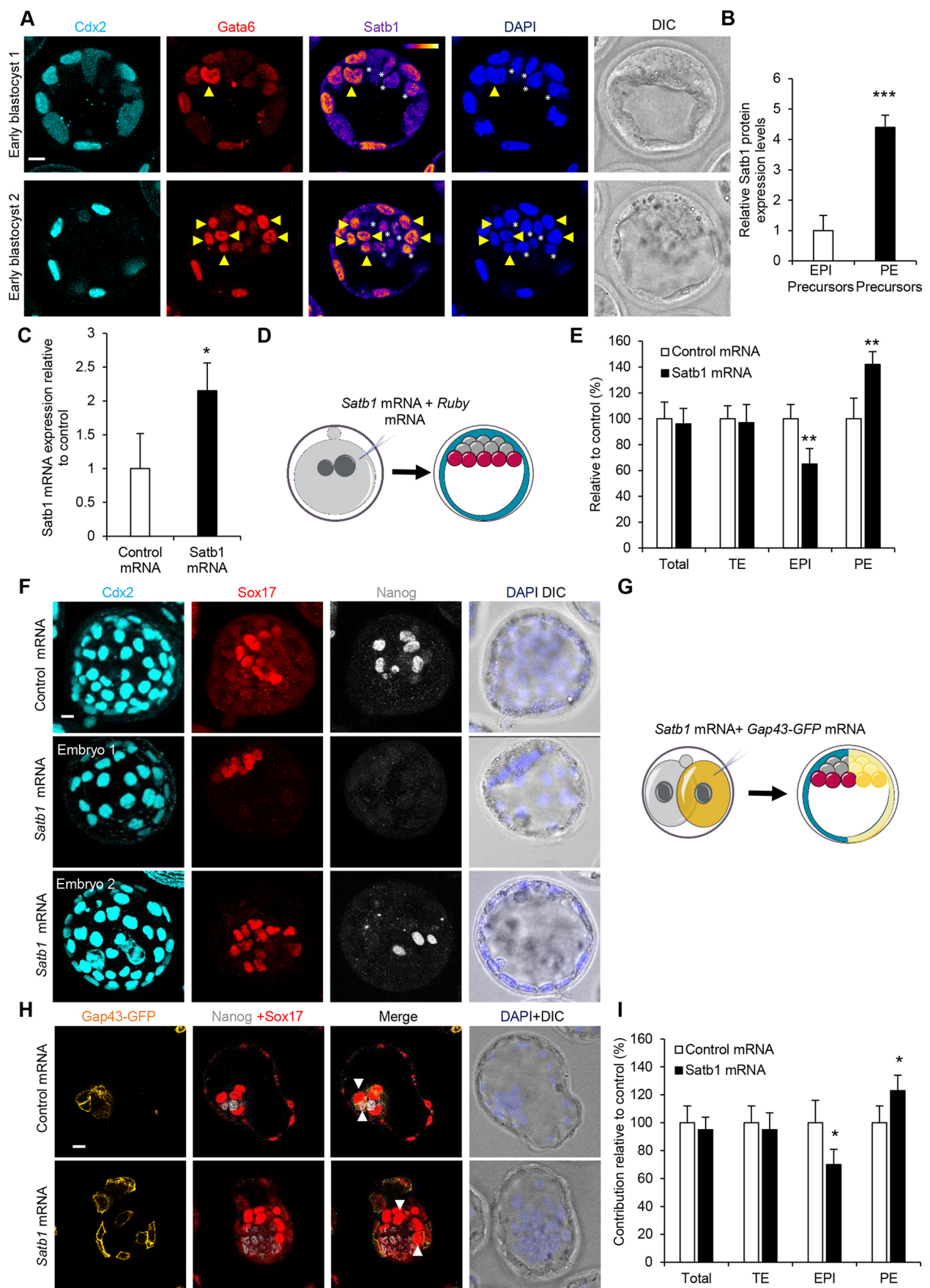


Fig. 3. See next page for legend.

**Fig. 3. Effect of *Satb1* overexpression on preimplantation development.**

(A) Confocal images of *Satb1* staining in early blastocysts ( $n=19$ ). *Gata6* (PE) and *Cdx2* (TE) were used as lineage markers. Yellow arrowheads indicate PE precursors. White asterisks indicate EPI precursors. (B) Quantification of relative fluorescent intensity of *Satb1* staining from A. (C) qRT-PCR of embryos injected with control mRNA ( $n=42$  embryos) or *Satb1* mRNA ( $n=54$  embryos) to investigate *Satb1* mRNA levels. (D) Scheme of *Satb1* overexpression experiment shown in E and F. Zygotes were injected with *Satb1* or control mRNA and cultured until E4.5. (E) Contribution of cells injected with *Satb1* mRNA ( $n=23$ ) to TE, PE and EPI, relative to cells injected with control mRNA ( $n=25$ ). Representative images of the experiment are shown in F. (F) Confocal images of control and *Satb1* mRNA-injected embryos. Nanog (EPI), Sox17 (PE) and *Cdx2* (TE) were used as lineage markers. (G) Scheme of clonal *Satb1* mRNA experiment shown in H and I. One blastomere of two-cell stage embryos was injected with *Satb1* mRNA and *Gap43-GFP* mRNA. Embryos were cultured to the late blastocyst stage, and the contribution of the injected cell's progeny to each lineage was analysed. (H) Confocal images of embryos injected with control ( $n=19$ , average of 5.32 Nanog-positive/*Gap43-GFP*-negative and an average of 5.16 Nanog-positive/*Gap43-GFP*-positive blastomeres per embryo) and *Satb1* mRNA ( $n=26$ , average of 7.27 Nanog-positive/*Gap43-GFP*-negative and an average of 3.61 Nanog-positive/*Gap43-GFP*-positive blastomeres per embryo). Sox17 (PE) and Nanog (EPI) were used as lineage markers. White arrowheads indicate ICM cells contributed from injected blastomeres. (I) Contribution of *Satb1* mRNA-injected cells from experiment shown in H to TE, PE and EPI, relative to control mRNA-injected cells. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ . Scale bars: 10  $\mu$ m.

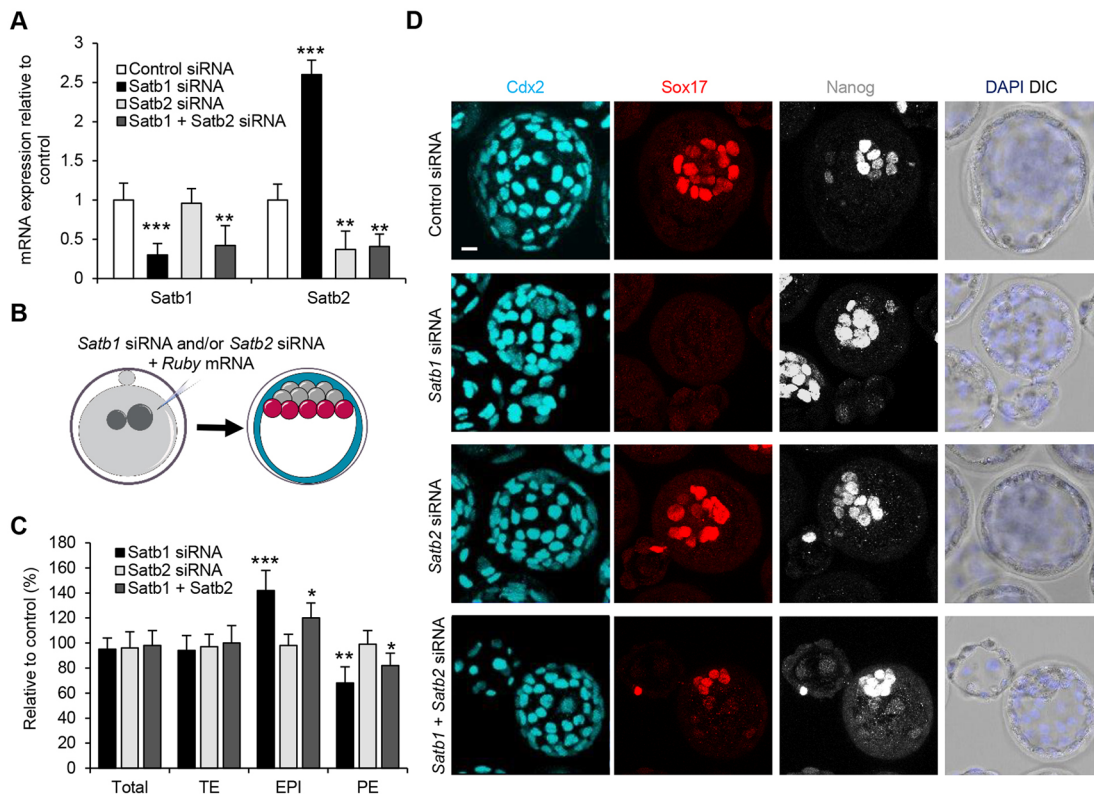
determine the upstream regulator of *Satb1*. It was shown that the inhibition of Fgf signalling influenced the dependence of mESCs on *Satb1* (Savarese et al., 2009). This is particularly interesting because Fgf signalling is crucial to PE fate specification (Frankenberg et al., 2011; Kang et al., 2013; Morris et al., 2013; Yamanaka et al., 2010).

To determine whether inhibition of the Fgf signalling pathway affects *Satb1* expression in the early mouse embryo, we determined the effects of two different Fgf signalling pathway inhibitors on *Satb1* expression. We used an Fgf receptor inhibitor (Morris et al., 2013; Nichols et al., 2009; Yamanaka et al., 2010) and a Mek inhibitor (Nichols et al., 2009; Schrode et al., 2014; Yamanaka et al., 2010) because they are well documented to block PE formation in the embryo. We treated two-cell stage embryos with the Fgf receptor inhibitor at a concentration of 100 nM (Morris et al., 2012) and the Mek inhibitor at a concentration of 0.5  $\mu$ M (Yamanaka et al., 2010) and then allowed the embryos to develop until the eight-cell stage, when they were fixed and immunostained for *Satb1* (Fig. 6A). We found that embryos treated with either inhibitor showed a significant decrease in *Satb1* protein by the eight-cell stage (Fig. 6B,C;  $P<0.001$ ).

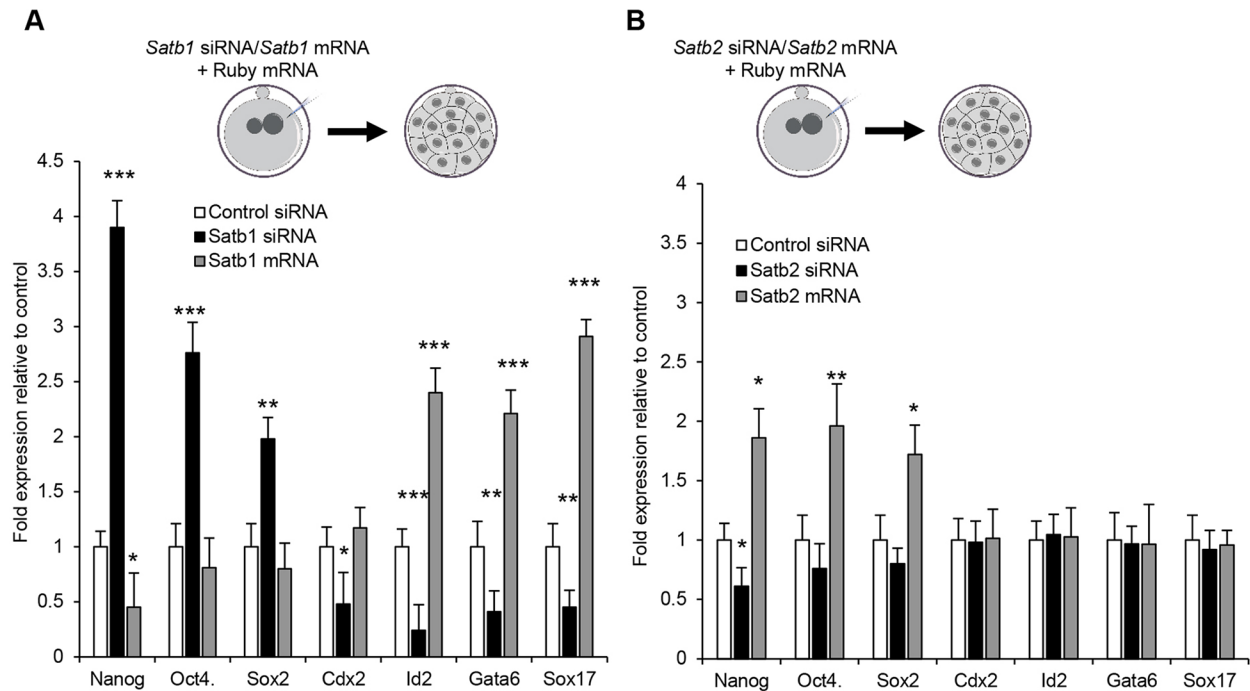
To examine whether the reduction in *Satb1* levels after Fgf inhibition could be rescued by the addition of exogenous *Satb1* mRNA, we injected *Satb1* mRNA into embryos at the two-cell stage and then treated them with inhibitors until the eight-cell stage (Fig. 6A). In these embryos, *Satb1* was returned to similar levels as in controls (Fig. 6B,D). These results suggest that Fgf signalling is involved in the regulation of *Satb1* in the preimplantation mouse embryo.

**DISCUSSION**

The specification of three distinct cell lineages in the mouse embryo occurs during two cell-fate decisions. The first cell-fate decision physically separates the population of ICM and TE cells,



**Fig. 4. Rescue of *Satb1* siRNA phenotype by *Satb2* siRNA.** (A) qRT-PCR of embryos injected with control ( $n=39$  embryos), *Satb1* ( $n=52$  embryos), *Satb2* ( $n=64$  embryos) or *Satb1*+*Satb2* ( $n=52$  embryos) siRNA to investigate *Satb1* mRNA levels. (B) Scheme of *Satb1* and *Satb2* siRNA experiment shown in C and D. Zygotes were injected with *Satb1* siRNA and/or *Satb2* siRNA and cultured until E4.5. (C) Contribution of cells injected with *Satb1* ( $n=16$ ), *Satb2* ( $n=29$ ) and *Satb1*+*Satb2* ( $n=32$ ) siRNA to TE, PE and EPI, relative to control [control numbers were normalized to 100, to allow comparison with experimental siRNA-injected embryos ( $n=13$ ) (not shown)]. Representative images of the experiment are shown in D. (D) Confocal images of embryos injected with control, *Satb1*, *Satb2* and *Satb1*+*Satb2* siRNA. Nanog (EPI), Sox17 (PE) and *Cdx2* (TE) were used as lineage markers. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ . Scale bar: 10  $\mu$ m.



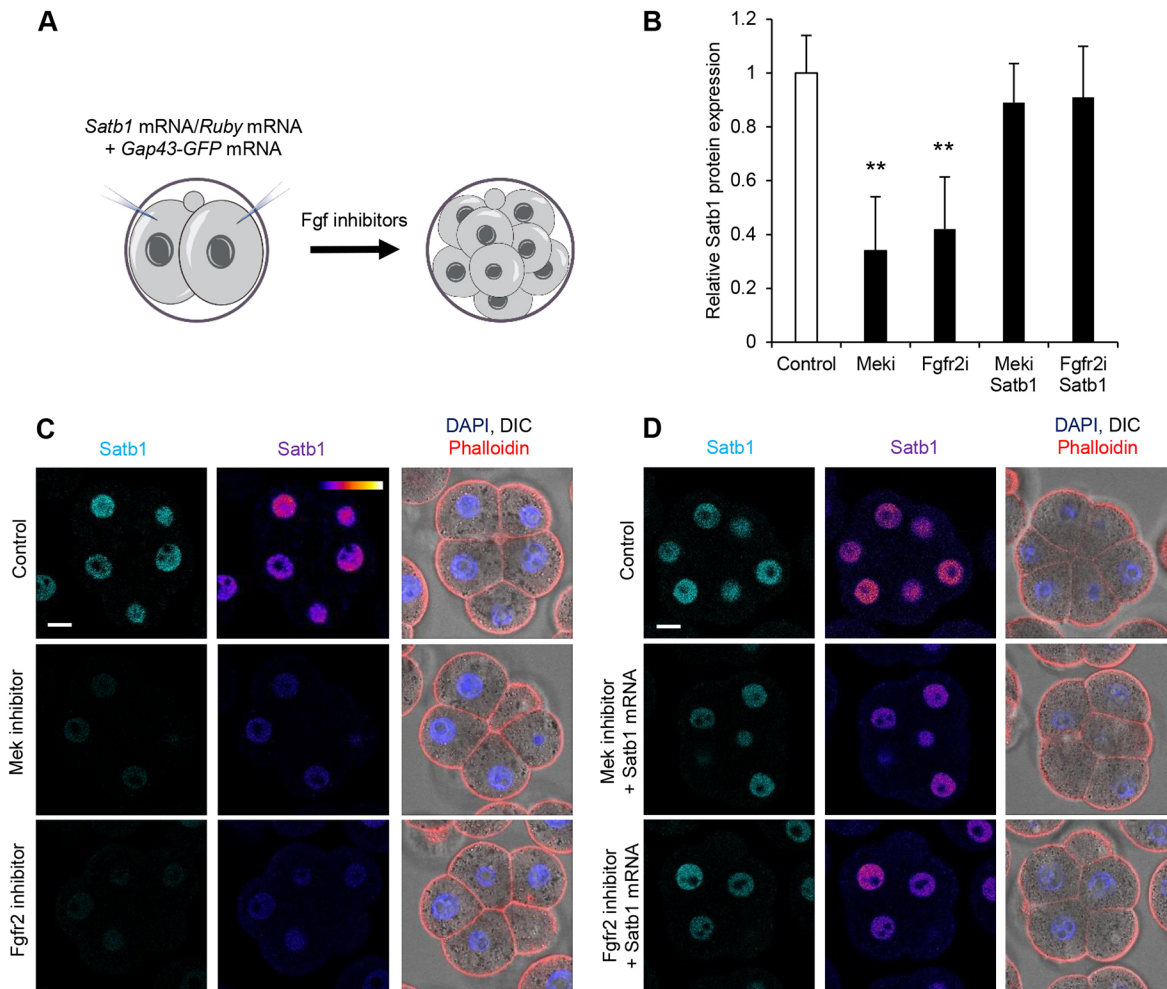
**Fig. 5. Gene expression changes after modulation of *Satb1* and *Satb2*.** (A) qRT-PCR of various genes in embryos injected with control siRNA ( $n=59$  embryos), *Satb1* siRNA ( $n=62$  embryos) or *Satb1* mRNA ( $n=71$  embryos). (B) qRT-PCR of various genes in embryos injected with control siRNA ( $n=59$  embryos), *Satb2* siRNA ( $n=73$  embryos) or *Satb2* mRNA ( $n=58$  embryos). In all instances, zygotes were injected and cultured until morula. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .

whereas the second cell-fate decision further specifies the ICM into the PE and the EPI. It is critical that all three lineages are correctly specified in order to form a blastocyst capable of implanting into the uterine wall and developing further. Here, aiming to identify new regulators that control cell-fate choice in preimplantation development, we discovered the chromatin modifier *Satb1* as an important player. *Satb1* was first identified in thymocytes, where it is known to regulate gene expression by organizing the structure of higher-order chromatin into loop domains and by acting as a ‘landing platform’ for chromatin-remodelling enzymes (Cai et al., 2006; Yasui et al., 2002). In mESCs, *Satb1* was shown to regulate pluripotency by directly repressing *Nanog*; *Satb1* knockout mESCs maintained *Nanog* expression even when placed into differentiation medium (Savarese et al., 2009). However, the role of *Satb1* in the preimplantation embryo remains unknown. Here, we find that expression of *Satb1* is specifically upregulated, at both mRNA and protein levels, in the inner cells of 16-cell stage embryos, when the ICM is first specified, indicating a potential role within the specification of these cells. We further find that *Satb1* is specifically upregulated within the PE precursors, signifying its potential importance to the specification of the PE. We confirm this hypothesis by downregulating *Satb1*, which we show leads to a reduction in the number of PE cells and an increase in the number of EPI cells by the blastocyst stage. In agreement with this, overexpression of *Satb1* has an opposite effect on lineage specification and promotes a PE lineage within the ICM. Our clonal knockdown and overexpression experiments further support these findings as we find that blastomeres with reduced *Satb1* preferentially give rise to EPI and those with increased *Satb1* preferentially give rise to PE. We find that *Satb1* does not have an effect on the 16- to 32-cell stage embryo when PE specification is initiated. Rather, it has a role in the commitment of cells within the blastocyst to the PE lineage. We also find that this change in

cell fate is attributable to modulation of the expression of a series of lineage-specific genes downstream of Fgf signalling.

We find that although modulating *Satb1* expression clearly affects cell-fate commitment in the preimplantation mouse embryo, it rarely results in a complete ablation of either the PE or EPI lineages. This is important when viewed in context of the *Satb1* knockout mice, which survive during embryogenesis but die by 3 weeks of age (Alvarez et al., 2000). If *Satb1* is important for the regulation of a balance between pluripotency and differentiation as has been shown here, how can this be reconciled with the lack of a preimplantation phenotype in knockout mice? It has previously been shown that the minimal requirement for successful development is three to four pluripotent cells by the time of implantation (Morris et al., 2012; Soriano and Jaenisch, 1986). If *Satb1*<sup>-/-</sup> embryos did have a phenotype, based on the results from this study, they would most probably have an ICM with high numbers of EPI cells and low numbers of PE cells. Similar phenotypes have been found in *Fgf4*<sup>+/-</sup> and *Gata6*<sup>+/-</sup> embryos, and in both cases, embryos were able to recover by E4.5 (Bessonnard et al., 2014; Krawchuk et al., 2013). Therefore, it is possible that although *Satb1* helps to organize and specify the correct number of PE and EPI cells within the ICM, it might not be absolutely essential for embryo survival and can be compensated for, in agreement with the highly regulative nature of mammalian development.

*Satb1* is closely related to another family member, *Satb2*, leading us to investigate whether *Satb2* might also have a function in preimplantation development. We find that although downregulating *Satb2* by itself has no effect on development, depletion of both genes at once is partly able to rescue the *Satb1* RNAi phenotype. This is in agreement with the results in mESCs, because knockdown of both *Satb1* and *Satb2* rescued the disruption in differentiation noted in *Satb1*<sup>-/-</sup> mESCs (Savarese et al., 2009). Our results indicate that this is likely to be because knocking down *Satb2* reduces *Nanog* mRNA, the opposite effect to reducing *Satb1*.



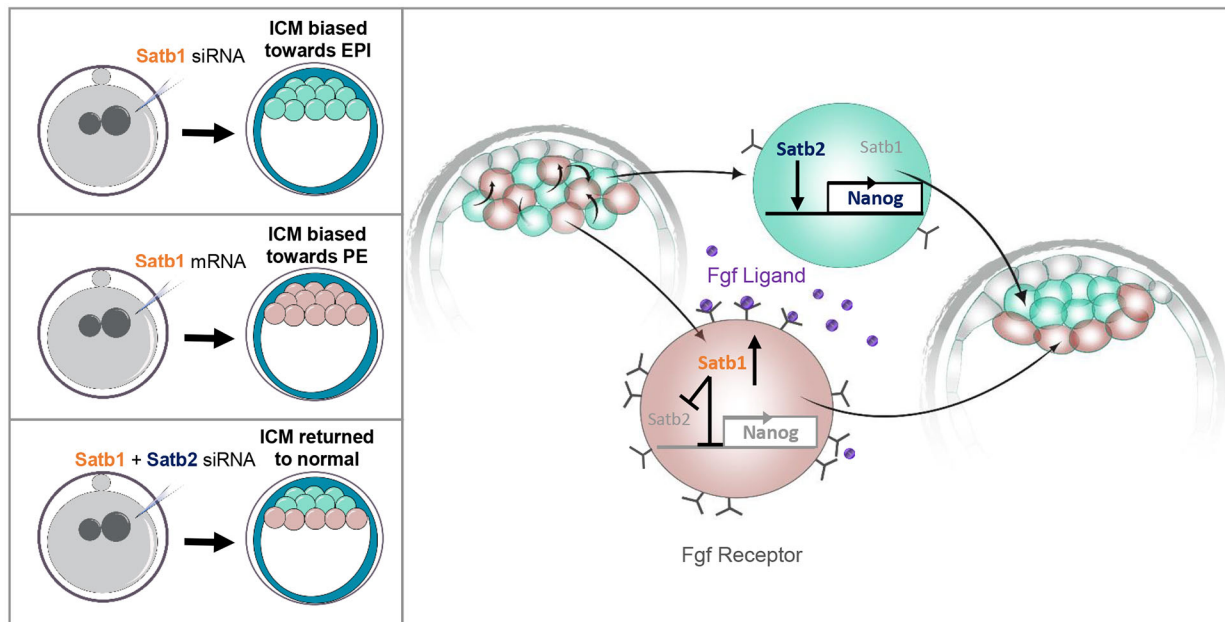
**Fig. 6. The effect of Fgf signalling on *Satb1* expression.** (A) Scheme of Fgf inhibition experiments in B–D. Two-cell stage embryos were either injected with *Satb1* mRNA or *Ruby* mRNA and treated with a Fgf signalling inhibitor or left untreated before isolation at the eight-cell stage for analysis. (B) Quantification of relative fluorescent intensity of *Satb1* staining from C and D. (C) Confocal images of control embryos ( $n=18$ ) and embryos treated with Mek inhibitor ( $n=20$ ), or Fgfr2 inhibitor ( $n=22$ ). (D) Confocal images of control embryos ( $n=18$ ), embryos treated with Mek inhibitor and injected with *Satb1* mRNA ( $n=17$ ) and embryos treated with Fgfr2 inhibitor treated and injected with *Satb1* mRNA ( $n=15$ ). \*\* $P<0.01$ . Scale bars: 10  $\mu$ m.

In agreement with this, overexpression of *Satb2* is able to increase *Nanog* expression, providing evidence that *Satb2* is a positive regulator of *Nanog*. *Satb1* and *Satb2* therefore have antagonistic effects on *Nanog* expression. Thus, we hypothesize that when *Satb1* alone is reduced it releases its repression on both *Nanog* and *Satb2*, and this is enough to bias cell-fate choice towards the EPI. This bias is strengthened by the fact that *Satb1* also acts as a positive regulator of PE differentiation factors *Sox17* and *Gata6*. Knocking down both *Satb1* and *Satb2* removes both the repression and activation of *Nanog* expression, with the net effect of normalizing *Nanog* expression levels. The results we present here showing the effect of *Satb2* on *Nanog* might help to explain why it has been impossible to derive *Satb2*<sup>-/-</sup> mESCs (Savarese et al., 2009), because without appropriate expression level of *Nanog*, it would be impossible to derive functional ESC clones.

The effect of *Satb2* on *Nanog* expression raises the question of why reducing *Satb2* levels in the embryo did not affect development to the same degree as modulation of *Satb1*. One explanation is that although *Satb2* siRNA was able to reduce *Nanog* levels, more than 65% of *Nanog* mRNA was still present after RNAi. Although *Nanog* is a crucial factor in determining cell fate, in the highly regulative mouse embryo a 35% reduction in *Nanog* levels might

not be sufficient to drive cell-fate changes. Additionally, our results suggest that although *Satb2* might affect only *Nanog* expression in embryos, *Satb1* has effects on the expression of multiple genes, including *Cdx2*, *Gata6*, *Id2* and *Sox17*. The same pattern, albeit with the opposite effects on expression, was noted for overexpression of *Satb1* and *Satb2*. The combined effects of *Satb1*, on numerous genes, are sufficient to drive cell-fate changes within the ICM. Modulating *Satb2*, however, only moderately affects *Nanog*. This can also explain why the double knockdown of *Satb1* and *Satb2* results in only a partial rescue of the *Satb1* siRNA phenotype, because although *Nanog* expression levels might be saved, the effects on the other genes regulated by *Satb1* are not.

Finally, our results indicate that *Satb1* expression is controlled by Fgf signalling, as we find that inhibition of Fgf signalling inhibits *Satb1* expression, which can be restored by the addition of exogenous *Satb1* mRNA. We also attempted to rescue the perturbation in cell fate that is noted after Fgf signalling inhibition (Nichols et al., 2009; Schrodde et al., 2014; Yamanaka et al., 2010) by overexpressing *Satb1* mRNA but were unable to restore the expression of PE markers by the blastocyst stage (data not shown). We predict that this is because the Fgf signalling pathway has a wide variety of targets in the mouse embryo, and so rescuing one



**Fig. 7. Model for the role of *Satb1* in ICM lineage segregation.** A ‘salt-and-pepper’ distribution of *Fgfr2* by the 64-cell stage means that different cells have different responses to *Fgf4* signalling. Cells with lower levels of *Fgfr2* are less susceptible to *Fgf4* signalling and do not upregulate *Satb1*. *Nanog* is therefore more highly expressed, promoting the formation of EPI. When *Satb1* is knocked down using a specific siRNA there is an increase in *Nanog* and therefore an increase in the number of EPI cells present in the embryo. Conversely, cells that are more susceptible to *Fgf4* signalling have higher levels of *Satb1*. Higher expression of *Satb1* leads to the inhibition of *Nanog* and *Satb2*. This in turn leads these cells to differentiate into PE. Overexpression of *Satb1* with an mRNA is likewise able to bias cell fate towards the PE lineage. Reducing both *Satb1* and *Satb2* simultaneously is able to restore the balance in PE and EPI by removing both an activator and repressor of *Nanog*.

downstream pathway is not sufficient to overcome the multiple effects of inhibiting Fgf signalling. In agreement, repression of Fgf signalling results in a similar but much stronger phenotype compared with downregulation of *Satb1*, with all ICM cells expressing EPI lineage markers (Frankenberg et al., 2011; Yamanaka et al., 2010). This indicates that *Satb1* might be one of the links between Fgf signalling and its downstream targets in early mouse embryos. In agreement with this, Fgf signalling and *Satb1* both promote stem cell maintenance and proliferation and inhibit differentiation of trophoblast stem cells (Asanoma et al., 2012; Tanaka et al., 1998).

Taken together, our results suggest that *Satb1* might act as a chromatin modifier, modulating gene expression downstream of Fgf signalling, pointing to a crucial missing step of chromatin remodelling that can serve to establish the progenitors of the two distinct lineages within the ICM. We speculate that *Satb1* could potentially act in two manners to direct ICM cell fate. Firstly, as *Satb1* has been found, through co-immunoprecipitation experiments, to bind directly to the 5′ flanking sequence of *Nanog* in mESCs (Savarese et al., 2009), we predict that *Satb1* would also bind directly upstream of *Nanog* to repress its transcription in mouse embryos. Secondly, as we find that *Satb1* can regulate the expression of numerous genes in the mouse embryo, we predict that this is a function of its ability to act as a ‘landing platform’ that is able to recruit chromatin-remodelling enzymes to activate or repress gene expression, as has been shown previously (Yasui et al., 2002).

In conclusion, it can be hypothesized that cells within the ICM have different levels of Fgf receptor, *Fgfr2*, on their membranes, as has been previously shown (Guo et al., 2010; Krupa et al., 2014; Kurimoto et al., 2006; Morris et al., 2013; Ohnishi et al., 2014). Cells with more receptor are more susceptible to Fgf ligand (which is secreted by the inside cells at this stage) and thus have higher levels of *Satb1* (Fig. 7). Higher expression of *Satb1* would lead to

the inhibition of the pluripotency factors *Nanog* and *Satb2*, which in turn would lead them to initiate differentiation into the PE lineage (Fig. 7). Cells that are less susceptible to *Fgf4* signalling will have reduced *Satb1* which, in turn, leads to more *Nanog* expression and therefore biases cell fate towards the pluripotent EPI lineage. Additionally, loss of both *Satb1* and *Satb2* removes both an activator and repressor of *Nanog*, resulting in the formation of a normal ICM (Fig. 7). This hypothesis, based on the results we present here, helps to further our understanding of the mechanism that leads to resolution of the ‘salt-and-pepper’ distribution of *Gata6*- and *Nanog*-expressing progenitors within the ICM.

## MATERIALS AND METHODS

### Collection of mouse embryos

Embryo recovery was done on superovulated F1 (C57Bl/6xCBA) females between 4 and 6 weeks old, as has been described previously (Piotrowska et al., 2001). Following collection or experimental manipulation, embryos were cultured in drops of potassium-supplemented simplex optimised medium (KSOM; Millipore) supplemented with 4 mg/ml bovine serum albumin under paraffin oil at 37.5°C in air enriched with 5% CO<sub>2</sub>.

### Collection of individual cells

Individual cells for qRT-PCR were collected at the 16-cell stage (78 h after human chorionic gonadotrophin), as has been done previously (Graham et al., 2014). Embryos were isolated directly at the 16-cell stage and incubated in M2 with a fluorescently labelled 0.2 mm microsphere suspension (Polysciences, Inc.) diluted to 1:50 for 30 s. Outside (strongly fluorescent) and inside (non-fluorescent) cells were collected, grouped and placed into Arcturus Biosciences PicoPure RNA isolation kit extraction buffer for mRNA isolation.

### Treatment with chemical inhibitors

To inhibit Fgf signalling, embryos were treated from the zygote to eight-cell stage with an Fgf receptor inhibitor at a concentration of 100 nM

(Stemgent; PD173074) or a Mek inhibitor (Stemgent; PD0325901) at a concentration of 0.5  $\mu$ M, both in KSOM. Inhibitors were dissolved in dimethyl sulphoxide (DMSO; final concentration of DMSO was 0.005%). Control embryos were incubated in the equivalent DMSO concentration but in the absence of the inhibitor. Following treatment with inhibitors, embryos were fixed and processed for immunostaining.

### Microinjection of siRNAs and mRNAs

All microinjections were done with siRNAs for *Satb1* and *Satb2* as well as AllStars Negative Control siRNA purchased from Qiagen. For siRNA sequences, see Supplementary Materials and Methods. *Satb1* and *Satb2* cDNA (Dharmacon) was cloned into pRN3P as described previously (Zernicka-Goetz et al., 1997). *In vitro* transcription was undertaken on linearized cDNA using the mMessage mMachine T3 kit (Ambion) according to the manufacturer's instructions. Microinjection of embryos with siRNA (always at a final concentration of 12  $\mu$ M) or mRNA [together with *Ruby* mRNA (200 ng/ $\mu$ l) or *Gap43-GFP* mRNA (400 ng/ $\mu$ l) as markers of injection] was carried out in M2 covered in oil on a glass depression slide using a Femtojet micro-injection system (Eppendorf). Embryos were cultured in KSOM under paraffin oil at 37.5°C in air enriched with 5% CO<sub>2</sub>.

### Immunofluorescence

Immunofluorescence was carried out as described previously (Jedrusik et al., 2008). Multichannel imaging was acquired on a Leica SP5 inverted confocal microscope using Leica LAS AF software and a 20 $\times$  or 40 $\times$  oil immersion objective. Confocal z-stacks were exported to ImageJ for image processing, intensity measurements and cell counting. For details of the immunofluorescence protocol and intensity measurements as well as antibody details, see supplementary Materials and Methods.

### qRT-PCR

qRT-PCR was carried out as previously described (Goolam et al., 2016). *Gapdh* or *H2afz* was used as the endogenous control. *H2A.Z* was used when different stages of preimplantation development were compared. Three biological repeats were undertaken for every qRT-PCR. For primer details, see supplementary Materials and Methods.

### Statistical analyses

Unless otherwise specified, Student's unpaired *t*-tests were used to test significance (\**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001). All error bars represent s.e.m.

### Acknowledgements

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

All experiments were performed by M.G. in M.Z.-G.'s laboratory. Data were analysed and interpreted by M.G. and M.Z.-G. The manuscript was written by M.G. and M.Z.-G.

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### Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.144139.supplemental>

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## Supplementary Materials and Methods

### siRNA sequences

The sequences of the siRNAs used are as follows: Satb1 siRNA 1–AAGGTGGTACAAACATTTCAA, Satb1 siRNA 2–CAGGAAATGAAGCGTGCTAAA, Satb1 siRNA 3 – CCCGAAGTACACCATCATCAA, Satb2 siRNA 1 – CCGAAGGACTAGACTGTGAA, Satb2 siRNA 2–ATGGCCCATCTGATAAACCAA, Satb2 siRNA 3–CAGGGATTATTGTCAGAGATA.

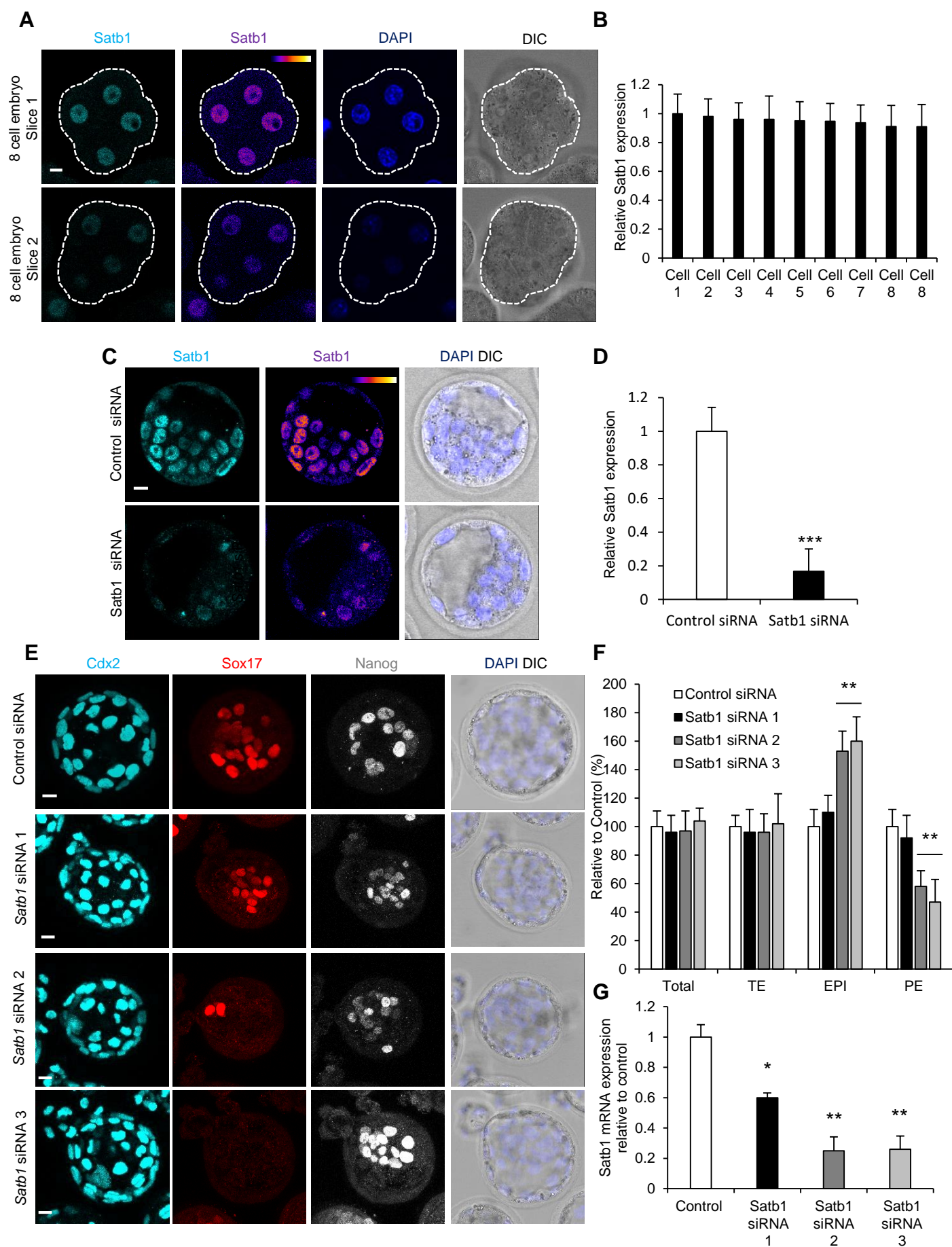
### Immunofluorescence protocol and intensity measurements and antibody details

ICM cells were identified through sequential scanning through embryo z-stacks by their position as well as through the use of lineage markers. Nanog expressing cells in the ICM that did not express PE markers were identified as EPI. Inside and outside cells were identified by careful scanning through the z-stack. Only in cases when outer (with nuclei that were not surrounded by other cells and adjacent to the outside of the embryo) and inner (with nuclei that were entirely surrounded by other cells) cells could be unambiguously identified where they used for analyses. Fluorescence intensity was quantified by normalising to DAPI and layer-normalising using the built-in IMAGEJ function. Intensity measurements were done on the normalised sections using the IMAGEJ measure function. For antibody details see Supplementary Materials and Methods. Primary antibodies used: goat anti-Sox17 (1:200; R&D Systems, AF1924), goat anti-Pdgfra (1:200; Santa Cruz, sc-31178), goat anti-Gata6 (1:200; R&D Systems, AF1700), rabbit anti-Nanog (1:200; Abcam, AB80892), rabbit anti-Sox2 (1:200; Abcam, ab59776), rabbit anti-Sox2 (1:200; Millipore, AB5603) mouse anti-Cdx2 (1:200; Biogenex, AM392), rabbit anti-Satb1 (1:50; Abcam, AB49061), rabbit anti-Satb2 (1:200; Abcam, AB34735), rat anti-Nanog (1:200; Ebiosciences, 14-5761-80). Secondary antibodies used: Alexa Fluor 647 donkey anti-mouse IgG, Alexa Fluor 568 donkey anti-goat IgG, Alexa Fluor 568 donkey anti-rabbit IgG, Alexa Fluor 647 donkey anti-rabbit IgG, Alexa Fluor 647 donkey anti-goat IgG, Alexa Fluor 647 phalloidin.

### Primer details

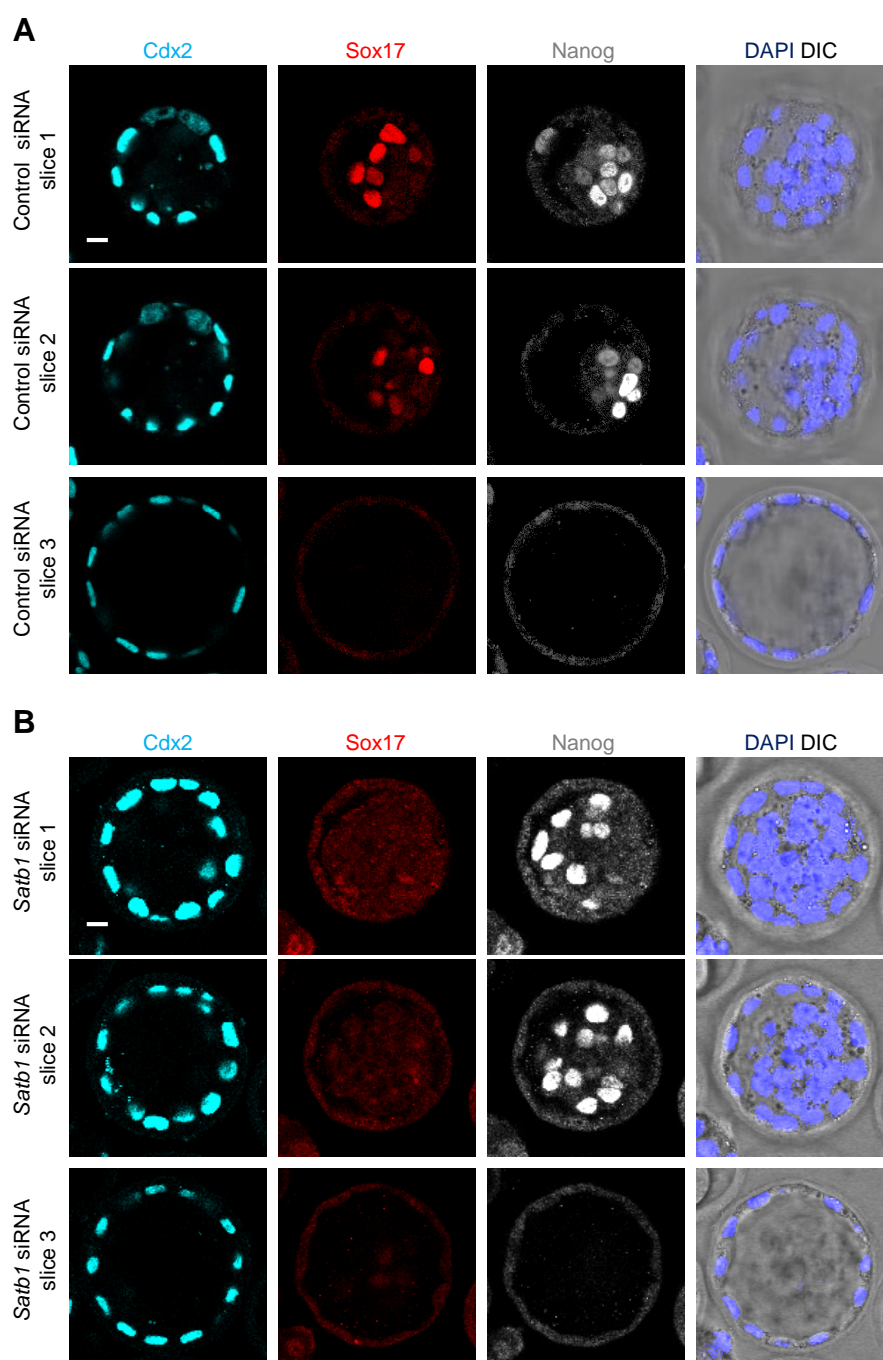
The following primers were used all written 5'-3': Gapdh Forward, AGAGACGGCCGCATCTTC, Reverse, CCCAATACGGCCAAATCCGT'; Histone H2A.Z Forward, CGTCAGAGAGACGCTTACCG, Reverse, AAGCCTCCAACCTTGCTCAAA; Satb1 Forward, AGTGCCCCCTTTCACAGAG, Reverse, TGCTGCTGAGACATTTGCAT; Satb2

Forward, ATGAACCCCAATGTGAGCAT, Reverse, GTTGTCGGTGTGAGGTTTT; Cdx2  
 Forward, AACCTGTGCGAGTGGATG, Reverse, TCTGTGTACACCACCCGGTA; Nanog  
 Forward, GGTTGAAGACTAGCAATGGTCTGA, Reverse, TGCAATGGATGCTGGGATACT;  
 Oct3 Forward, TTGGGCTAGAGAAGGATGTGGTT, Reverse,  
 GGAAAAGGGACTGAGTAGAG TGTGG; Sox2 primer set 1 Forward,  
 GCGGAGTGGAACTTTTGTCC, Reverse, CGGGAAGCGTGTACTTATCCTT; Sox2 primer  
 set 2 Forward, GCGGAGTGGAACTTTTGTCC Reverse,  
 GGGAAGCGTGTACTTATCCTTCT; Sox17 Forward, GATGCGGGATACGCCAGTG,  
 Reverse, CCACCACCTCGCCTTTCAC; Id2 Forward, ATGAAAGCCTTCAGTCCGGTG,  
 Reverse, AGCAGACTCATCGGGTCGT; Gata6 Forward, TTGCTCCGGTAACAGCAGTG,  
 Reverse, GTGGTCGCTTGTGTAGAAGGA



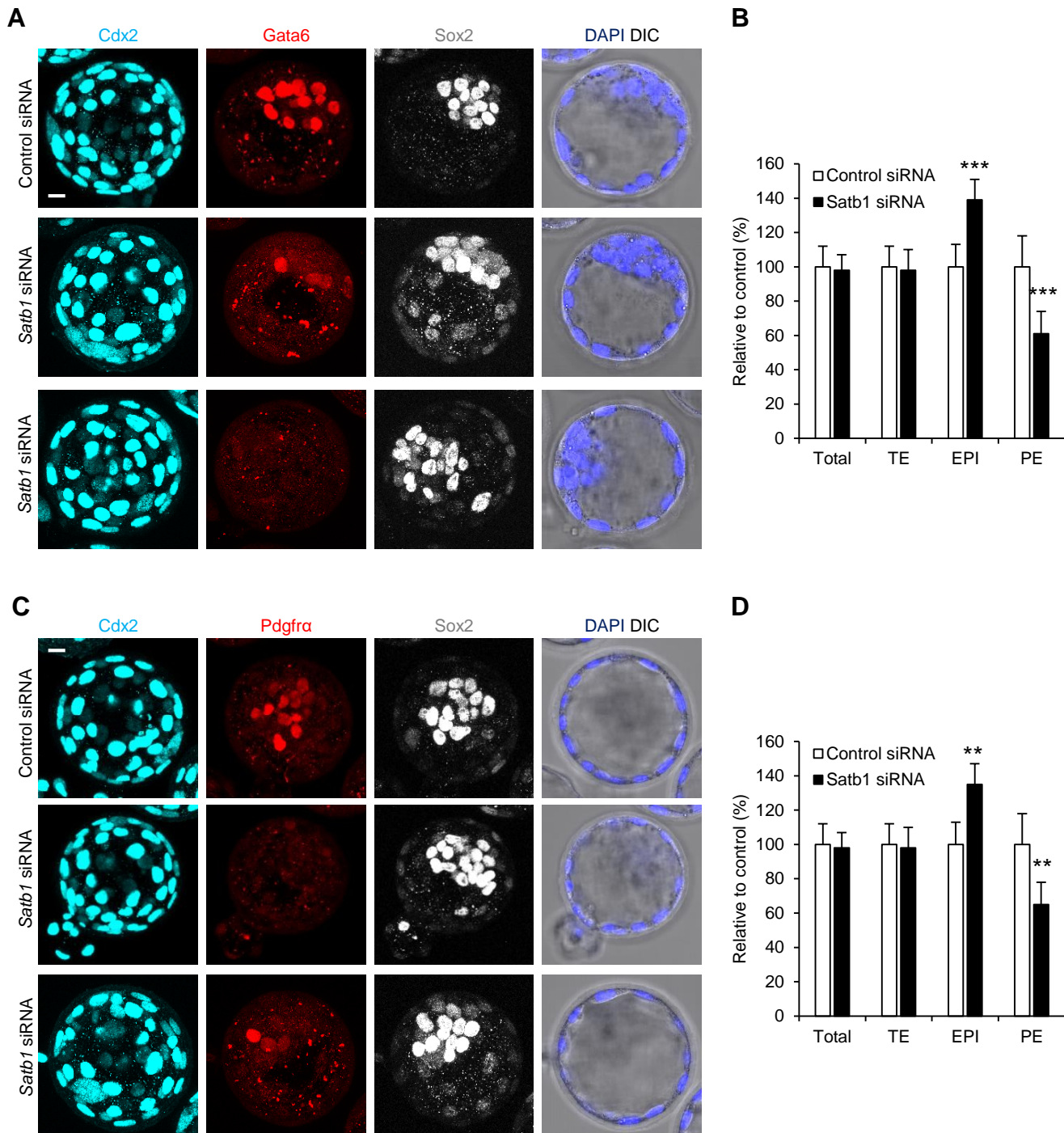
**Figure S1. Confirmation of *Satb1* siRNA persistence and specificity and *Satb1* embryo staining.** A) Immunofluorescence of *Satb1* in 8-cell embryos (n=25). Embryo boundary is outlined in white. B) Quantification of immunofluorescence represented in A. Fluorescence quantified and normalized to the nucleus with the strongest staining per individual embryo. C) Immunofluorescence of *Satb1* in early blastocysts after being injected with control (n=14) or *Satb1* siRNA (n=18). D) Quantification of relative fluorescent intensity of *Satb1* staining from C. E) Confocal images of control and *Satb1* siRNA 1, *Satb1* siRNA 2 and *Satb1* siRNA 3 injected embryos. Nanog, (EPI), Sox17 (PE) and Cdx2 (TE) were used as lineage markers. Quantification of this experiment shown in F. F) Contribution of control (n=17) and *Satb1* siRNA 1 (n=19), 2 (n=21), and 3 (n=23) injected embryos to EPI, PE, and TE. G) qRT-PCR of control (n=47 embryos, three biological repeats), *Satb1* siRNA 1 (n=58 embryos, three biological repeats), *Satb1* siRNA 2 (n=53 embryos, three biological repeats), *Satb1* siRNA 3 (n=49 embryos, three biological repeats) injected embryos to investigate *Satb1* mRNA levels.

Student's t-test was used to test significance \*=  $p < 0.05$ , \*\*=  $p < 0.01$ , \*\*\*=  $p < 0.001$ . Error bars represent s.e.m. Scale bars, 10  $\mu\text{m}$ .



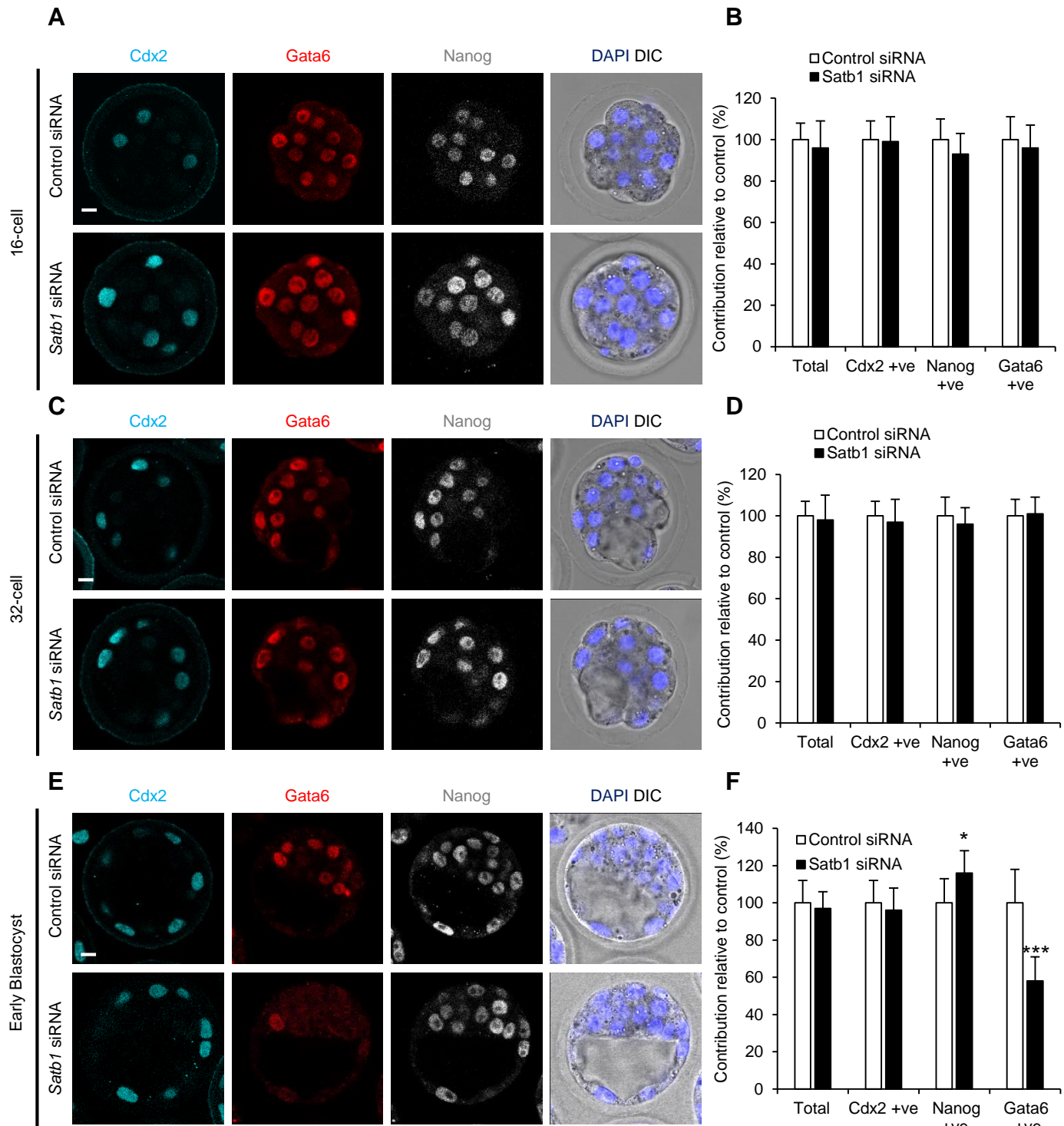
**Figure S2. Z-stack slices of confocal images from Figure 2E.** A) Slices of the confocal z-stack of the control siRNA injected embryo presented in Fig 2 E. Nanog, (EPI), Sox17 (PE) and Cdx2 (TE) were used as lineage markers B) Slices of the confocal Z-stack of the *Satb1* siRNA injected embryo presented in Fig 2 E (Embryo 1). Nanog, (EPI), Sox17 (PE) and Cdx2 (TE) were used as lineage markers.

Scale bars, 10  $\mu$ m.

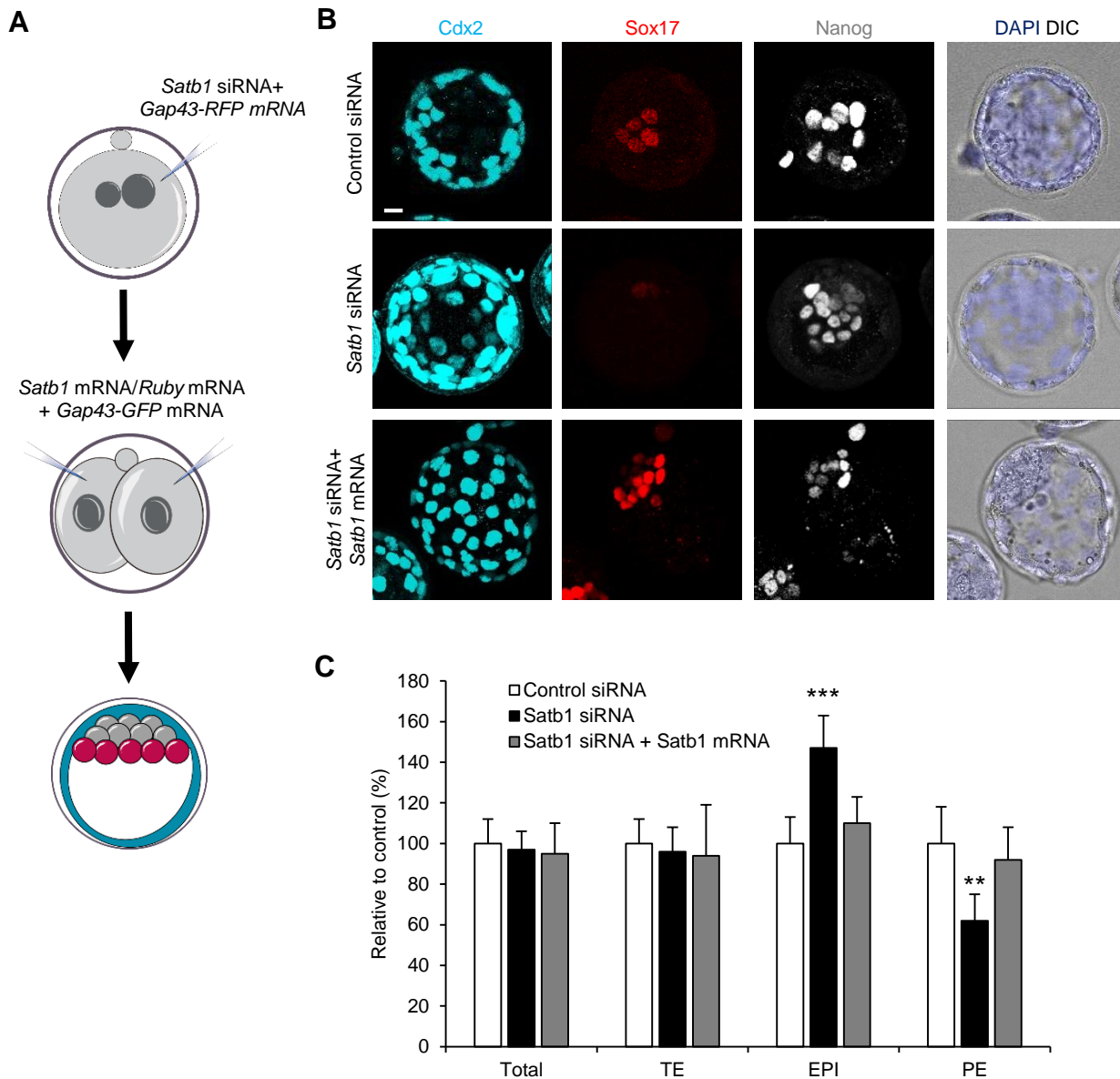


**Figure S3. *Satb1* siRNA phenotype assessed by Sox2, Gata6 and Pdgfra.** A) Confocal images of control (n=31) and *Satb1* siRNA (n=30) injected embryos. Sox2, (EPI), Gata6 (PE) and Cdx2 (TE) were used as lineage markers. B) Contribution of control and *Satb1* siRNA injected embryos represented in A to EPI, PE, and TE. C) Confocal images of control (n=27) and *Satb1* siRNA (n=29) injected embryos. Sox2, (EPI), Pdgfra (PE) and Cdx2 (TE) were used as lineage markers. D) Contribution of control and *Satb1* siRNA injected embryos represented in C to EPI, PE, and TE.

Student's t-test was used to test significance \*=  $p < 0.05$ , \*\*=  $p < 0.01$ . Error bars represent s.e.m. Scale bars, 10  $\mu$ m.

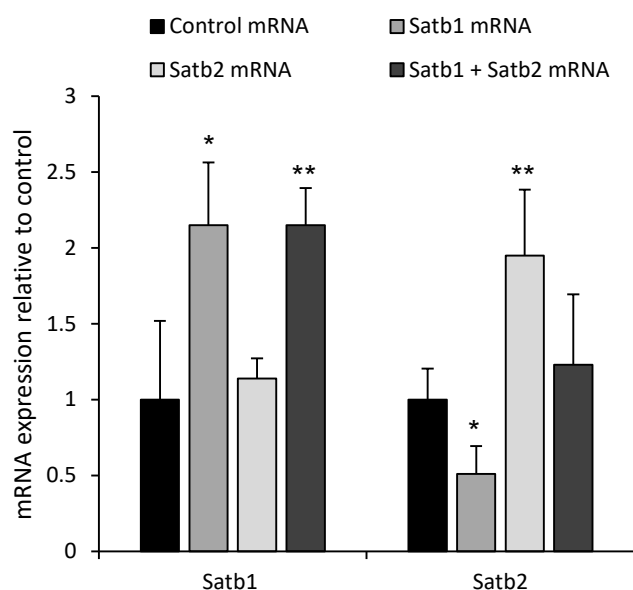


**Figure S4. Timing of effect of *Satb1* RNAi.** A) Confocal images of control (n=15) and *Satb1* siRNA (n=21) injected embryos at the 16-cell stage showing the localisation and distribution of Cdx2, Gata6 and Nanog. B) Relative number of blastomeres that are positive for Cdx2, Nanog and Gata6 from control and *Satb1* siRNA injected embryos represented in A. C) Confocal images of control (n=17) and *Satb1* siRNA (n=19) injected embryos at the 32-cell stage showing the localisation and distribution of Cdx2, Gata6 and Nanog. D) Relative number of blastomeres that are positive for Cdx2, Nanog and Gata6 from control and *Satb1* siRNA injected embryos represented in C. E) Confocal images of control (n=19) and *Satb1* siRNA (n=28) injected embryos at the early blastocyst stage showing the localisation and distribution of Cdx2, Gata6 and Nanog. F) Relative number of blastomeres that are positive for Cdx2, Nanog and Gata6 from control and *Satb1* siRNA injected embryos represented in E. Student's t-test was used to test significance \* = p<0.005, \*\*\* = p<0.001. Error bars represent s.e.m. Scale bars, 10  $\mu$ m.

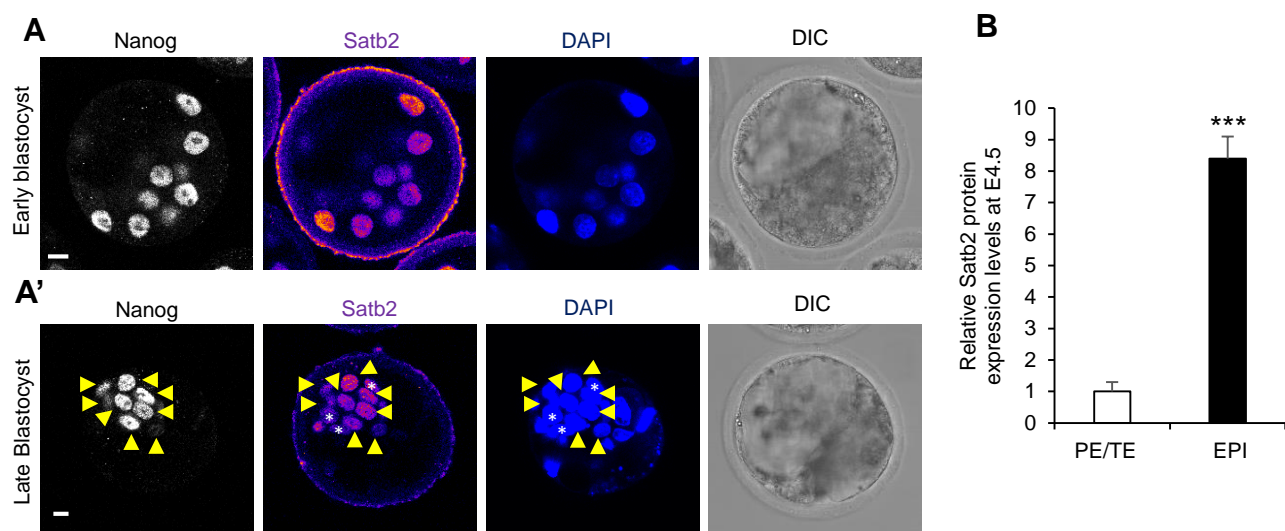


**Figure S5. Rescue of *Satb1* siRNA phenotype by *Satb1* mRNA.** A) Scheme of *Satb1* rescue experiment shown in B and C. Zygotes were injected with *Satb1* siRNA or control siRNA. At the 2-cell stage both blastomeres were then injected with either *Satb1* mRNA or *Ruby* mRNA as a control and then cultured until E4.5. B) Confocal images of control siRNA (n=12), *Satb1* siRNA (n=21) and *Satb1* siRNA + *Satb1* mRNA (n=26) injected embryos. Nanog (EPI), Sox17 (PE) and Cdx2 (TE) were used as lineage markers. C) Contribution of *Satb1* siRNA and *Satb1* siRNA + *Satb1* mRNA injected embryos to TE, PE and EPI, relative to control siRNA injected cells from experiment shown in B.

Student's t-test was used to test significance \*\*=  $p < 0.01$ , \*\*\*=  $p < 0.001$ . Error bars represent s.e.m. Scale bar, 10  $\mu$ m.



**Figure S6. Effect of Satb1 and Satb2 overexpression on Satb1 and Satb2 mRNA levels.** qRT-PCR of control (n=63 embryos, three biological repeats), Satb1 (n=71 embryos, three biological repeats), Satb2 (n=58 embryos, three biological repeats), Satb1 + Satb2 (n=47 embryos, three biological repeats) mRNA injected embryos to investigate Satb1 and Satb2 mRNA levels. Student's t-test was used to test significance \*\*=  $p < 0.01$ , \*\*\*=  $p < 0.001$ . Error bars represent s.e.m. Scale bar, 10  $\mu$ m.



**Figure S7. Satb1 expression pattern in blastocysts.** A) Confocal images of Satb2 and Nanog staining in early blastocysts (n=12). A') Confocal images of Satb2 and Nanog staining in late blastocysts (n=16). Yellow arrows indicate EPI cells positive for Satb2. White asterisks indicate PE cells positive for Satb2. B) Quantification of relative fluorescent intensity of Satb2 staining in EPI cells compared to PE/TE cells in late blastocysts as shown in A'. EPI cells were identified by the expression of Nanog. Scale bars, 10  $\mu$ m.