



Original research article

GATA6 phosphorylation by Erk1/2 propels exit from pluripotency and commitment to primitive endoderm



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ABSTRACT

The transcription factor GATA6 and the Fgf/Ras/MAPK signaling pathway are essential for the development of the primitive endoderm (PrE), one of the two lineages derived from the pluripotent inner cell mass (ICM) of mammalian blastocysts. A mutant mouse line in which *Gata6*-coding exons are replaced with H2BGFP (histone H2B Green Fluorescence Protein fusion protein) was developed to monitor *Gata6* promoter activity. In the *Gata6*-H2BGFP heterozygous blastocysts, the ICM cells that initially had uniform GFP fluorescence signal at E3.5 diverged into two populations by the 64-cell stage, either as the GFP-high PrE or the GFP-low epiblasts (Epi). However in the GATA6-null blastocysts, the originally moderate GFP expression subsided in all ICM cells, indicating that the GATA6 protein is required to maintain its own promoter activity during PrE lineage commitment. In embryonic stem cells, expressed GATA6 was shown to bind and activate the *Gata6* promoter in PrE differentiation. Mutations of a conserved serine residue (S264) for Erk1/2 phosphorylation in GATA6 protein drastically impacted its ability to activate its own promoter. We conclude that phosphorylation of GATA6 by Erk1/2 compels exit from pluripotent state, and the phosphorylation propels a GATA6 positive feedback regulatory circuit to compel PrE differentiation. Our findings resolve the longstanding question on the dual requirements of GATA6 and Ras/MAPK pathway for PrE commitment of the pluripotent ICM.

1. Introduction

The primitive endoderm (PrE) is one of the earliest lineages derived in mammalian embryos, and the genes and signaling pathways governing its differentiation have been extensively studied in quests to gain fundamental understanding of early development and pluripotency (Rossant et al., 2003; Morris et al., 2010; Yamanaka et al., 2010; Schrodde et al., 2013; Hermitte and Chazaud, 2014; Rossant and Tam, 2017). Prior to implantation, the cluster of cells referred to as the inner cell mass (ICM) enclosed by a layer of trophectoderm gives rise to two cell types: the PrE, which forms an epithelium that covers the other lineage, the epiblast (Epi) (Gardner, 1982; Bedzhov and Zernicka-Goetz, 2014; Chazaud and Yamanaka, 2016). Only a few genes are known to play essential roles in the differentiation and formation of PrE, based on findings of gene knockout embryos that fail to generate a PrE layer at the late blastocysts stages. The zinc finger transcription factor *Gata6* is essential for the formation of PrE lineage, since the *Gata6* knockout blastocysts lack PrE cells (Cai et al., 2008; Bessonard

et al., 2014; Schrodde et al., 2014). Forced expression of GATA6 is sufficient to propel the differentiation of embryonic stem (ES) cells towards the PrE lineage (Fujikura et al., 2002; Capo-Chichi et al., 2005). The endocytic adaptor protein Dab2 does not impact the differentiation into PrE lineage, but the PrE cells scatter and fail to form an epithelium in Dab2 null embryos (Yang et al., 2002, 2007; Moore et al., 2013). Similarly, $\beta 1$ integrin-null embryos contain no PrE epithelial structure but the differentiated PrE and Epi cells are segregated rather than form an embryonic cylinder with adjoining PrE and Epi layers (Moore et al., 2014).

The transcription factors, Nanog and Oct3/4 that are critical for pluripotent cell property, are also essential for ICM development and differentiation in blastocysts (Chambers et al., 2003; Mitsui et al., 2003; Le Bin et al., 2014). Oct3/4 impacts both PrE and Epi lineages in a dose dependent manner (Niwa et al., 2000), and Oct3/4 null embryos die at the peri-implantation stage because of ICM degeneration (Nichols et al., 1998; Niwa et al., 2000; Le Bin et al., 2014). The role of Oct3/4 in PrE development in mouse blastocyst is cell autonomous

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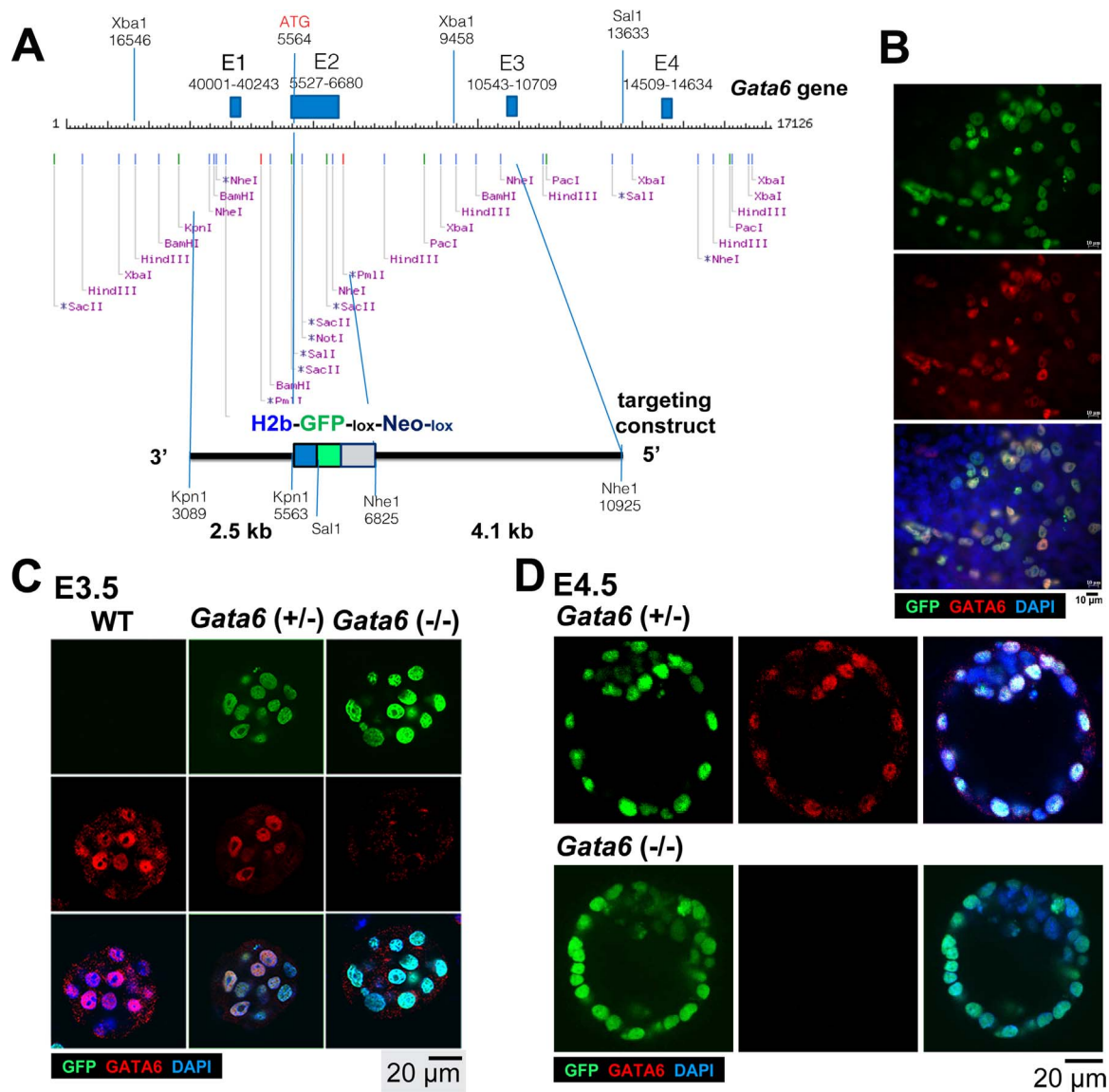


Fig. 1. GFP expression in H2BGFP knock-in blastocysts and implanted embryos. (A) Illustration of the targeting construct. The *Gata6*-GFP knockin line was produced following recombination in ES cells using a targeting construct with 2.5 kb 3' and 4.1 kb 5' homologous arms flanking a H2BGFP-Neo fragment. The expected mutant allele is the replacement of GATA6 coding sequence with H2BGFP protein at the translation start site (ATG), resulting a GFP reporter under the control of *Gata6* promoter. (B) Correlation of GFP signal and GATA6 protein in *Gata6*-H2BGFP knockin ES cells. The *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) ES cells were treated with retinoic acid (1 μM) for 4 days to induce primitive endoderm differentiation. The cells were stained with anti-GATA6 and counter stained with DAPI. The fluorescence signals of GFP, GATA6 immunostaining, and DAPI were captured and compared. (C) E3.5 wild type, *Gata6*^{H2BGFP/+} (*Gata6* (+/-)), and *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) embryos from matings of *Gata6*^{H2BGFP/+} parents were collected and identified by immunofluorescence. Positive GFP signals were observed in both *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) and *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) embryos, but were absent in wild type embryos. Positive GATA6 immunostaining was observed in wild type and *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) embryos, but was absent in *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) embryos. (D) Examples of GFP and GATA6 expression in *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) and *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) blastocysts at E4.5 stage are shown. The GFP signals and GATA6 immunostaining were compared.

(Frum et al., 2013). In contrast, Nanog is suggested to be required for PrE differentiation in a non-cell autonomous manner based on analysis of chimeric mutant embryos (Messerschmidt and Kemler, 2010). Additionally, Nanog is strictly essential for the maintenance of pluripotency and Epi cell fate determination, and Nanog null embryos fail to generate either Epi or PrE lineages (Chambers et al., 2003; Mitsui et al., 2003).

In addition to the transcription factors governing lineage gene expression and the proteins involved in cell adhesion and morphogenesis, the Fgf/Ras/MAPK signaling pathway also impacts pluripotency of embryonic cells and is essential for differentiation towards PrE lineage (Nichols et al., 2009; Yamanaka et al., 2010). Lack of PrE formation in knockout embryos of Fgf4 (Rappolee et al., 1994; Feldman et al., 1995; Goldin and Papaioannou, 2003; Kang et al.,

2013; Krawchuk et al., 2013), Fgfr2 (Yamanaka et al., 2010; Arman et al., 1998), or Grb2 (Cheng et al., 1998; Chazaud et al., 2006), components of the Ras/MAPK pathway, leads to the conclusion that the FGF/FGFR/Ras/MAPK signaling pathway is also required for the development of PrE (Chazaud and Yamanaka, 2016; Kuijk et al., 2012). Recent analyses also indicate that both Fgfr1 and Fgfr2 play roles in the embryonic cells for exit from the pluripotent state and commitment to PrE lineage, with dominant contribution from Fgfr1 in preimplantation development (Kang et al., 2017; Molotkov et al., 2017). Additional analyses indicate that Ras/MAPK signaling is upstream of GATA6, since ectopic expression of GATA6 is sufficient to bypass the requirement of Grb2 (Wang et al., 2011). It was suggested that FGF/Ras/MAPK pathway activation induces GATA6 expression to initiate PrE differentiation (Plusa et al., 2008; Hermitte and Chazaud, 2014).

Typical markers for PrE include GATA6, GATA4, and Dab2 that is a transcription target of GATA6 (Morrisey et al., 2000; Capo-Chichi et al., 2005); and markers for Epi cells are Nanog (Chambers et al., 2003; Mitsui et al., 2003) and Oct3/4 (Nichols et al., 1998). The pluripotent cells of the ICM are initially positive for both GATA6 and Nanog at an early stage (32–64 cells) (Saiz et al., 2016), but differentiate into GATA6-positive, Nanog-negative PrE, and GATA6-negative, Nanog-positive Epi populations as differentiation progresses to the 64–100 cell stage (Chazaud et al., 2006; Frankenberg et al., 2011). These observations lead to a model that mutual transcription suppression between GATA6 and Nanog/Oct3/4 determines the commitment of the ICM cells to either PrE or Epi lineages (Singh et al., 2007; Nakai-Futatsugi and Niwa, 2015; Schröter et al., 2015). The role of the Ras/MAPK pathway is speculated to break the balance of GATA6 and Nanog/Oct3/4 expression in selecting either PrE or Epi cell fate (Chazaud et al., 2006; Yamanaka et al., 2010; Kang et al., 2013). Presumably, phosphorylation by the activated serine/threonine kinase MAPK/Erk1/2 of one or more substrates ultimately triggers an increased Gata6 and repressed Nanog/Oct3/4 expression, respectively, leading to PrE commitment. However, the relevant substrate(s) in the regulation of the transcriptional circuits has not been recognized or determined.

We generated a GATA6 mutant mouse line by replacing the *Gata6* coding exons with green fluorescence protein (GFP) fusion with histone H2B expression construct, which was used to monitor *Gata6* promoter activity in both pre- and post implantation blastocysts. Our investigation of the *Gata6* heterozygote and null GFP reporter embryos led to several interesting findings on the regulation of *Gata6* promoter and

the role and mechanism of FGF/Ras/Erk1/2 signaling pathway in primitive endoderm development.

2. Results

2.1. Production of an *H2BGFP Gata6* knock-in mouse line

Previously we observed that *Gata6*-deficient mouse embryos lack PrE in implanted blastocysts at around E4.5, and the deformed E5.5 mutant embryos also are deprived of PrE-derived extraembryonic endoderms and degenerative (Cai et al., 2008). The essential role of GATA6 for PrE development was confirmed subsequently in blastocysts analyzed in culture (Bessonnard et al., 2014; Schrodde et al., 2014), and thus GATA6 appears to be the master transcription factor for PrE development. To monitor *Gata6* promoter activity in living embryos, we generated a GATA6 reporter mutant mouse line using homologous recombination in ES cells by substitution of a GFP coding sequence for a fragment of the *Gata6* gene starting at the translational start site and covering the following exon 2 (here, *Gata6* indicates the mouse gene, *Gata6*^{H2BGFP/+} the targeted allele, and GATA6 the protein) (Fig. 1A).

The GATA6 reporter line was first characterized rigorously in cells and tissues from *Gata6*^{H2BGFP/+} mice. As shown in an example of differentiating ES cells (Fig. 1B), the GFP signals from the generated *Gata6*^{H2BGFP/+} ES cells visualized by fluorescence microscopy closely correlated with GATA6 protein detected by immunofluorescence staining. Although, it appeared that the GFP signals tended to be slightly more prominent than signals from immunostaining of GATA6,

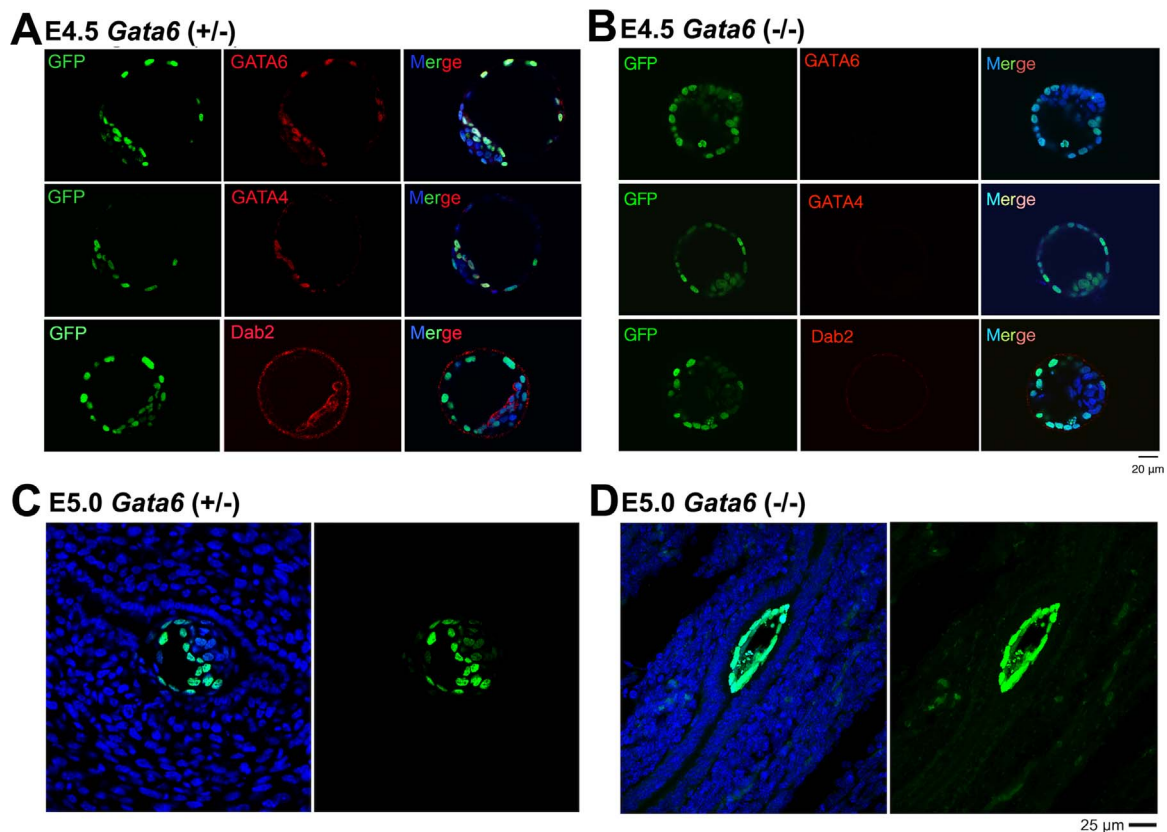


Fig. 2. GATA6 is required for primitive endoderm differentiation. (A) *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) heterozygous embryos were collected at E3.5 and matured in culture to around the E4.5 stage. The embryos were examined for GFP and analyzed by immunostaining for GATA4, GATA6, and Dab2 proteins. PrE cells are positive for GFP, Dab2, GATA4, and GATA6. Trophoblast cells are positive for GFP and GATA6 but are negative for GATA4. (B) Images of GFP and immunofluorescence of *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) blastocysts at the E4.5 stage are shown as representative examples. The embryos were examined for GFP signal and immunostaining for GATA4, GATA6, and Dab2 proteins. (C) Embryos in uterine tissues from a mating between *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) heterozygous mice were collected at E5.5 stage and analyzed following cryo-sectioning. The embryos on slides were for assessed for GFP fluorescence, and were evaluated for Gata6 positivity by immunostaining as well as PCR genotyped of cells collected from the slides. A representative *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) heterozygous embryo was shown for GFP fluorescence, and counterstained with DAPI. (D) A representative *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) homozygous mutant embryo is shown.

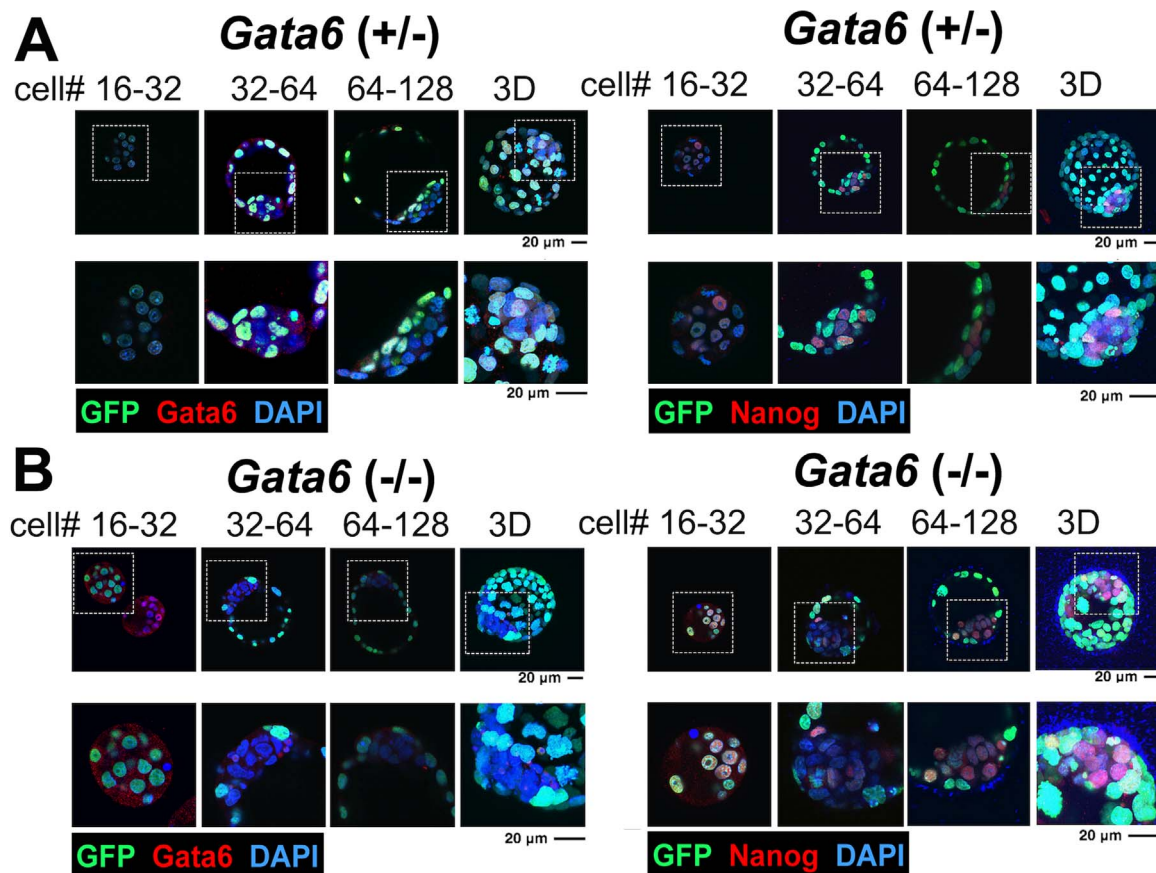


Fig. 3. GATA6 protein is required for its promoter activity in the ICM cells at E3.5 to E4.5 stages. (A) E3.5 to E4.5 *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) blastocysts were collected and identified by GFP immunofluorescence and immunostaining. The blastocysts were analyzed for GFP and immunostaining of GATA6 or Nanog, and representative examples of confocal images are shown at the 16–32, 32–64, and 64–128 cell stages, respectively. An area including the ICM is shown at a higher magnification in lower panels. Images of 3D reconstruction from the confocal sections are also shown. (B) Examples of GFP and immunofluorescence stainings of *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) embryos/blastocysts at the 16–32, 32–64, and 64–128 cell stage are shown.

where some cells exhibited strong GFP but had very faint GATA6 immunostaining (Fig. 1B). We have quantified both GFP and GATA6 immunostaining in multiple slides from *Gata6*^{H2BGFP/+} cells and embryos: the signals from individual cells co-localized in essentially most (99%) cells, however, a close but not perfect correlation in intensity was found. It was previously described from the study of another line of GATA6 mutant mice that a reduction of *Gata6* gene dosage caused a reduced number of PrE cells in *Gata6* (+/-) blastocysts (Schrode et al., 2014), though the heterozygous embryos appeared to develop normally afterward (Cai et al., 2008; Bessonard et al., 2014; Schrode et al., 2014). Also, discrepancies between reporter expression and Gata6 protein were found in another GATA6 reporter line using H2B-Venus transcriptional reporter allele (Freyer et al., 2015). Our analysis of the *Gata6*^{H2BGFP/+} mutants developed in our facility did not find notable differences between wildtype and heterozygous, and the *Gata6*^{H2BGFP/+} allele appear to be a suitable reporter of *Gata6* promoter activity.

2.2. GFP expression in the H2BGFP knock-in blastocysts

Mutant pre-implantation embryos were harvested from timed matings of *Gata6*^{H2BGFP/+} parents for analyses. At E3.5, the earliest stage harvested, GFP was present in nearly all the embryonic cells of either the heterozygous or homozygous embryos, and the GFP signal in each cell was relatively uniform (Fig. 1A). The GFP signal approximated the level of GATA6 protein detected by immunostaining in either E3.5 or E4.5 *Gata6*^{H2BGFP/+} embryos (Fig. 1C, D). In comparison, the GFP signal was much higher in *Gata6*^{H2BGFP/H2BGFP}, approximately doubled (1.8 ± 0.4 as the fluorescence per cell quantitated) that

found in *Gata6*^{H2BGFP/+} embryos at both E3.5 and E4.5 stages, indicating a gene dosage dependent expression. By E4.5, in *Gata6*^{H2BGFP/+} blastocysts, the GFP fluorescence was strong in the layer of PrE cells covering epiblast cells that were GFP-negative or very weak, whereas the GFP signal remained moderate in trophoctoderm cells (Fig. 1D). In *Gata6*^{H2BGFP/H2BGFP} blastocysts, the trophoctoderm cells were GFP positive, but all the presumptive ICM cells were GFP weak or negative, and no GFP-positive PrE layer was observed (Fig. 1D).

The observed reporter of GATA6 expression is consistent with the dynamics of GATA6 expression in blastocysts reported previously (Cai et al., 2008; Bessonard et al., 2014; Schrode et al., 2014).

2.3. Essential role of GATA6 for primitive endoderm differentiation

To confirm the earlier observation that GATA6 null blastocysts were unable to form PrE cells (Cai et al., 2008), the *Gata6*^{H2BGFP/H2BGFP} embryos before and after implantation were analyzed for the presence of PrE markers and structure, compared to *Gata6*^{H2BGFP/+} controls. The *Gata6*-positive (*Gata6*^{H2BGFP/+}) blastocysts at the E4.5 stage were found to harbor a PrE layer that was positive for GATA4 and Dab2 (Fig. 2A), while these markers were absent in the *Gata6*^{H2BGFP/H2BGFP} embryos (Fig. 2B). Sequential z-stack images of *Gata6*^{H2BGFP/+} embryos showed the GFP-positive PrE layer covering the GFP-low Epi cell aggregate (Supplementary Data Movie 1, 2). Analysis of consecutive z-stack images of *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) embryos from top to bottom indicated absence of PrE and diminutive GFP signal in the entire ICM (Supplementary Data Movie 3, 4).

Supplementary material related to this article can be found online

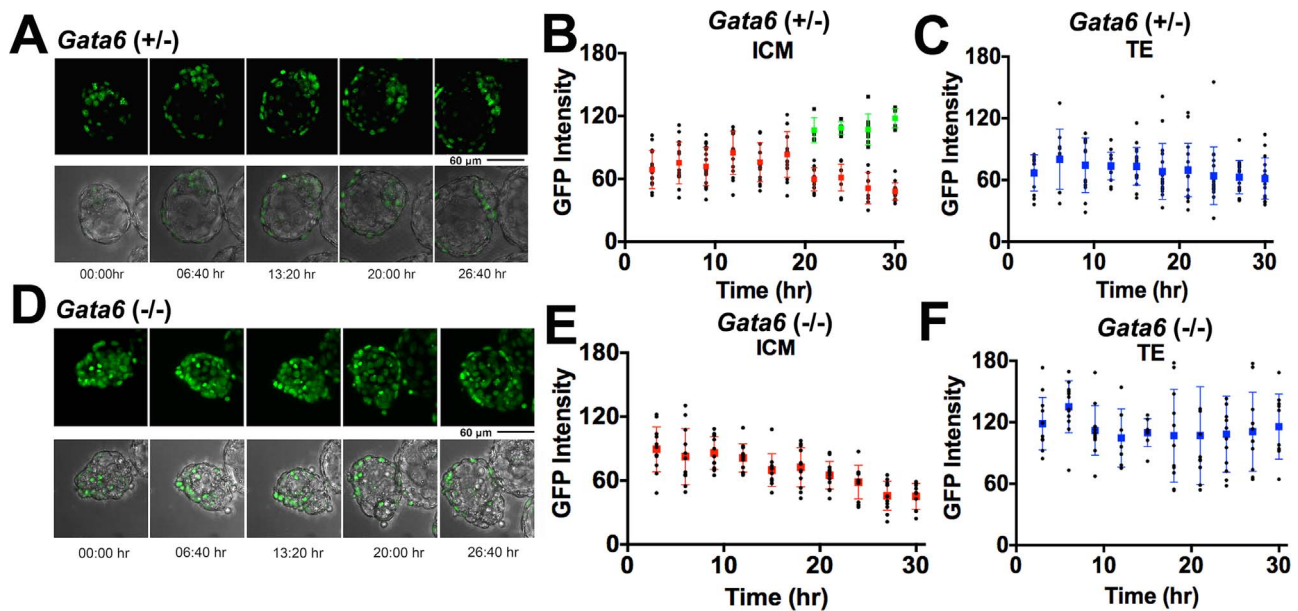


Fig. 4. Divergence GFP expression into two populations in the ICM cells of *Gata6* H2BGFP knock-in blastocysts. (A) An example shows a series of still images from a time-lapse movie of a *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) blastocyst from E3.75 (around 70 cells) to E4.0 (around 120 cells) stages. (B) The GFP intensity of individual ICM cells in the *Gata6*^{H2BGFP/+} blastocyst was analyzed along the time course and plotted. The cells that acquired a more intense GFP signal at the later time course (labeled in green color) adopted a PrE cell fate. (C) The GFP intensity of individual trophectoderm (TE) cells in the *Gata6*^{H2BGFP/+} blastocysts was monitored along the time course. (D) An example shows a series of confocal images from a time-lapse movie of a *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) blastocyst that matured from E3.75 (around 70 cells) to E4.0 (around 120 cells) stages. (E) The GFP intensity of individual ICM cells from the *Gata6*^{H2BGFP/H2BGFP} blastocyst was surveyed along a time course. The tracing of GFP levels on a panel of ICM cells was plotted. (F) The GFP intensity of the trophectoderm (TE) cells in the *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) blastocyst was assessed over a 30-h time course.

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By E5.0 stage, the newly implanted embryos exhibited a GATA6-positive PrE layer covering the GFP weak epiblast in *Gata6*^{H2BGFP/+} controls (Fig. 2C). Confocal sectioning through the embryo showed a well-formed GFP-positive PrE layer, whereas the Epi cells retained only trace levels of the GFP signal (Supplementary Data Movie 5). In the implanted *Gata6*^{H2BGFP/H2BGFP} embryos, the ICM was degenerating, and only a layer of strongly GFP-positive trophectoderm remained (Fig. 2D) (Supplementary Data Movie 6). Thus, as being verified in this new mutant allele generated, GATA6 is required for PrE differentiation and the formation of the embryonic epithelium in both pre- and post-implanted embryos.

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2.4. Requirement of GATA6 protein for maintenance of its promoter activity at the time of primitive endoderm differentiation at around E4.0 stage

Monitoring of GFP levels of heterozygous (*Gata6*^{H2BGFP/+}) (Fig. 3A) and homozygous (*Gata6*^{H2BGFP/H2BGFP}) (Fig. 3B) pre-implanted blastocysts at various stages revealed the dynamics of GFP expression. For *Gata6*^{H2BGFP/+} blastocysts at the 32–64-cell stage, the GFP signals in the ICM closely matched with GATA6 protein levels measured by immunostaining, and the GFP-positive cells partially overlapped with Nanog-positive cells (Fig. 3A). However in the *Gata6*^{H2BGFP/H2BGFP} blastocysts, GFP was positive initially in the 32-cell stage, but the signal became very weak in the entire ICM by 32–64 cell stage, and a PrE structure was absent in 64–128 cell stage, though the cells harbored varying levels of Nanog (Fig. 3B). The differences were especially obvious in confocal serial images of the blastocysts: the GATA6 null blastocysts expressed either no or very weak GFP in cells of the ICM despite the strong presence of GFP in trophectoderm cells (Fig. 3B). Thus, during progression of blastocysts to E4.5 stages at time PrE is formed, absence of GATA6 protein appears to impair the expression of GFP, and hence GATA6 protein is required for its promoter activity,

either directly or indirectly, in ICM but not in trophectoderm cells.

2.5. Divergence of *Gata6*-H2BGFP expression and segregation into two populations of ICM cells

We used time-lapse video microscopy to examine the dynamics of cell sorting and GFP expression in the *Gata6*-GFP knockin blastocysts in culture as the PrE differentiated and an epithelial structure organized. It is known that PrE differentiation occurs within the whole ICM but the differentiated cells sort to surface and form a PrE epithelium (Yamanaka et al., 2010). The cell sorting and organization of the PrE epithelium is thought to depend on Dab2-mediated establishment of apical polarity (Yang et al., 2002, 2007; Moore et al., 2013), and Dab2 is a transcription target of GATA6 (Morrisey et al., 2000; Capo-Chichi et al., 2005).

In *Gata6*^{H2BGFP/+} blastocysts starting at around the 68-cell stage prior to the formation of PrE, all the cells of the ICM moved dynamically and extensively throughout the ICM territory (Supplementary Data Movie 7). At a later (120-cell) stage when high-GFP PrE cells formed an epithelium, the relative position of the PrE cells became stabilized (Supplementary Data Movie 7). Cell division and apoptosis also occurred in blastocysts, and apoptotic cells were abundant especially in the *Gata6* (-/-) blastocysts at later stages (Supplementary Data Movie 8). In the analysis of images from 3 time lapse videos, 15 ± 5 apoptotic cells were identified in the *Gata6* (-/-) blastocysts, compared to 3 ± 2 apoptotic cells seen in those of *Gata6* (+/-). The sorting and formation of the PrE layer in the *Gata6*^{H2BGFP/+} blastocysts, and the extensive apoptosis and failure to form a PrE epithelium in *Gata6*^{H2BGFP/H2BGFP} embryos were evidenced as shown by a time-lapse video imaging (Supplementary Data Movie 7, 8).

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The progressive changes in the GFP signal to form PrE and Epi also were quantified from the time-lapse videos (Supplementary Data Movie 7, 8). All cells of the E4.0 *Gata6*^{H2BGFP/+} blastocysts initially possessed a moderate level of GFP signal (Fig. 4A). However by the 64-cell stage,

the signals diversified into two populations: the GFP signals in cells of the ICM either intensified or declined as the cells differentiated into PrE (marked as green color dots) or Epi (marked as black dots), respectively (Fig. 4B). In comparison, the GFP signals of the trophectoderm cells remained relatively constant (Fig. 4C) (Supplementary Data Movie 7). A reduction in cell motility accompanied PrE commitment as the cells assembled into an organized layer of GFP-positive PrE cells (Fig. 4A). In homozygous mutant blastocysts (*Gata6*^{H2BGFP/H2BGFP}), initially high GFP expression throughout the ICM subsided in all cells, and no PrE layer formed (Fig. 4D, E), though GFP intensity in trophectoderm cells persisted (Fig. 4A). These results suggest that the GATA6 is initially expressed in all ICM cells, but the protein is required to maintain and enhance its promoter activity in cells of the ICM and their PrE commitment, otherwise in the absence of GATA6, the originally moderate promoter activity declines and the entire ICM cell population becomes Epi.

2.6. GATA6 binding and activation of its own promoter in primitive endoderm differentiation

To verify this hypothesis that GATA6 is required for the maintenance of its own promoter, we tested ectopic expression of exogenous GATA6 on the intrinsic *Gata6* promoter activity in embryonic stem (ES) cells (Fig. 5A). Transfection/expression of GATA6 or GATA4 is sufficient to induce differentiation of pluripotent ES cells to the PrE lineage (Fujikura et al., 2002; Capo-Chichi et al., 2005), and we used this system to test the requirement of GATA proteins for *Gata6* promoter activity. Several lines of *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) and *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) ES cells were derived and expanded from blastocysts harvested from time matings of the mutant line. The undifferentiated pluripotent *Gata6*^{H2BGFP/+} and *Gata6*^{H2BGFP/H2BGFP} ES cells cultured in the presence of leukemia inhibitory factor (LIF) did not express GFP, and were positive for pluripotent factors Nanog and Oct3/4 but negative for the expression of differentiation markers including GATA6, GATA4, and Dab2, as detected by immunofluorescence. Upon a brief retinoic acid (RA) treatment to induce PrE differentiation, the *Gata6*^{H2BGFP/+} ES cells gained GFP expression,

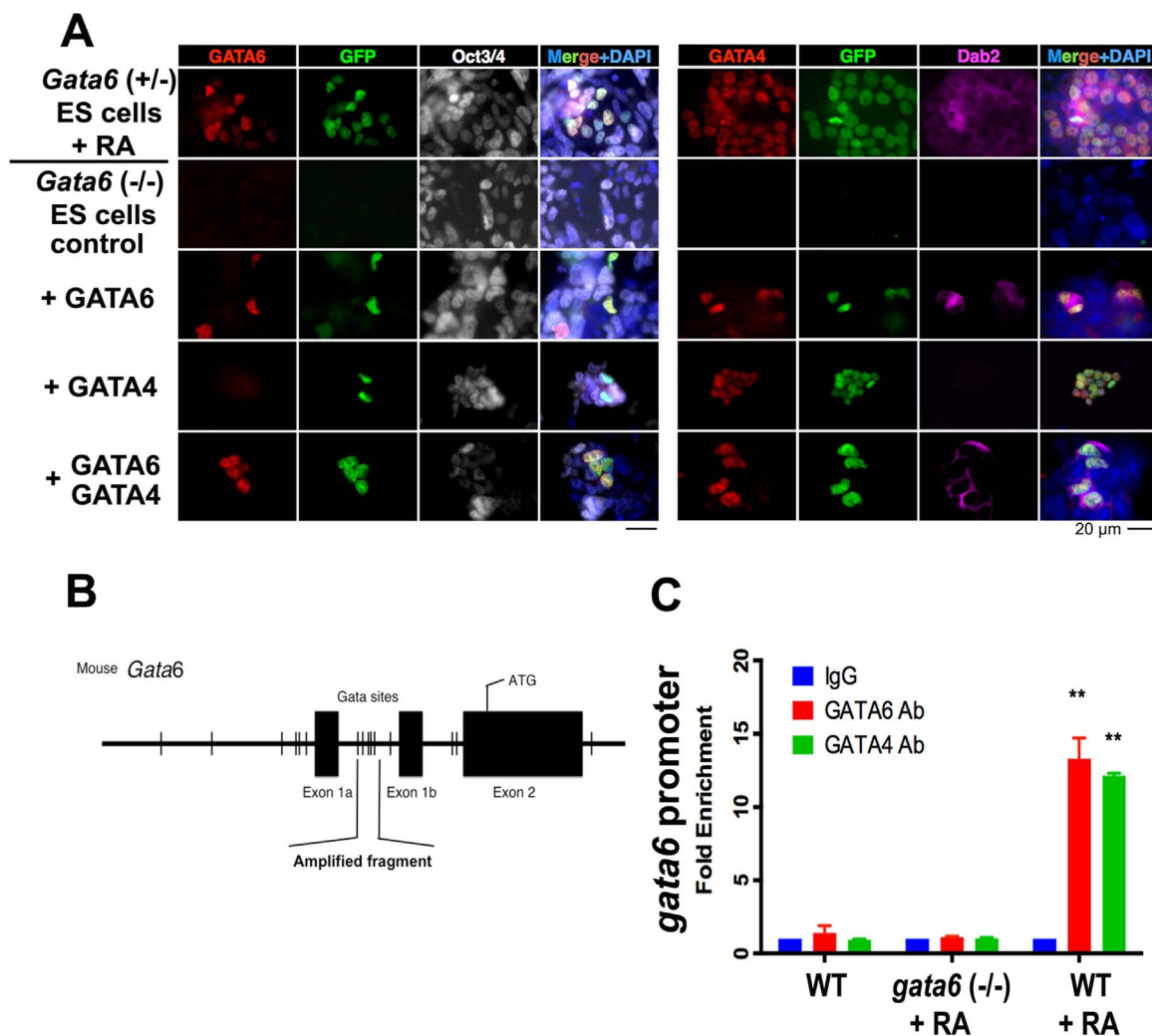


Fig. 5. GATA6 binding to and activation of its promoter in primitive endoderm differentiation. (A) *Gata6*^{H2BGFP/+} (*Gata6* (+/-)) and *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) ES cells were either treated with retinoic acid (RA) to induce PrE differentiation, or transfected with GATA6 and/or GATA4 expression plasmids. Two days following treatment, the cells were analyzed for GFP fluorescence, and for PrE markers including GATA6, GATA4, and Dab2 by immunofluorescence microscopy. (B) Illustration of *Gata6* promoter and the site for ChIP assay. A DNA fragment of putative *Gata6* promoter located between exon 1a and 1b was amplified in the ChIP assays. The sequence contains 5 consensus GATA factor-binding sites. (C) Association of GATA6 with *Gata6* promoter was analyzed by ChIP-qPCR assays. Briefly, nuclear extracts from wildtype (WT) or *Gata6* (-/-) ES cells with or without differentiation by retinoic acid (RA) treatment were used for immunoprecipitations using antibodies to GATA4 or GATA6. The immunoprecipitations were analyzed by PCR amplification of an approximately 200 base pair fragment to detect the enrichment of *Gata6* promoter sequences. Immunoprecipitation was performed in triplicate and the results are presented as mean fold enrichment over IgG control with standard deviation.

which closely correlated with immunostaining of GATA6 (Fig. 5A). The GFP signal accompanied the expression of GATA4 and Dab2 (Fig. 5A). Transfection and ectopic expression of GATA6 and/or GATA4 in the ES cells induces PrE differentiation (Fujikura et al., 2002; Capo-Chichi et al., 2005), which was validated with the induction of GFP expression in the *Gata6*^{H2BGFP/+} ES cells. Transfection of GATA6 and/or GATA4 expression plasmids in GATA6 null (*Gata6*^{H2BGFP/H2BGFP}) ES cells also initiated the expression of GFP, GATA4, and Dab2 (Fig. 5A), indicating either GATA6 and/or GATA4 is able to activate the *Gata6* promoter and propel PrE differentiation.

We considered the possibility that GATA6 binds directly to its own promoter to activate transcription. Consensus binding sequences for GATA factors, (A/T/C)GAT(A/T)(A), present and are conserved in both the human and murine *Gata6* promoter (Sakai et al., 1998; Brewer et al., 1999; Molkentin et al., 2000; Patient and McGhee, 2002; Caslini et al., 2006). Particularly, a previous ChIP-seq analysis indicates GATA6 binds to two sites corresponding to both *Gata6* promoters at exon1a and exon 1b (Wamaitha et al., 2015). To confirm the binding of

GATA6 to the promoter, we tested a fragment of *Gata6* sequence containing several consensus GATA factor-binding sites present in the second *Gata6* promoter, located between exon 1a and 1b, in immunoprecipitation (ChIP) assays (Fig. 5B). In RA-treated wildtype ES cells in which GATA6 expression was induced, ChIP assay determined that the *Gata6* promoter sequences were enriched in immunoprecipitation of GATA6-bound genomic DNA fragments (Fig. 5C). Similarly, GATA4 also bound the *Gata6* promoter (Fig. 5C). Compared to the background of undifferentiated or *Gata6* knockout ES cells, immunoprecipitation of GATA6 or GATA4 had a 13-fold or 12-fold enrichment, respectively, of the *Gata6* promoter (Fig. 5C). These results suggest that both GATA6 and GATA4 can bind the *Gata6* promoter and augment its transcription activity, creating a positive feedback regulatory loop during PrE differentiation.

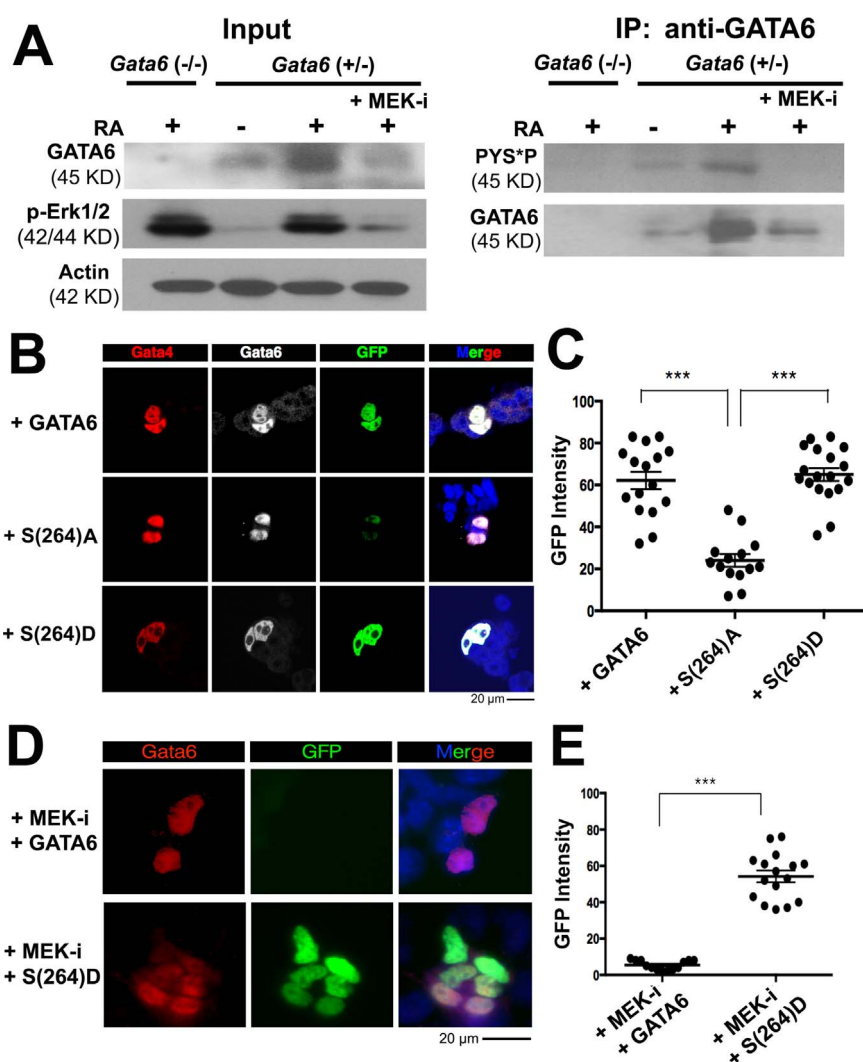


Fig. 6. Phosphorylation of GATA6 and its regulation of its own promoter activity. (A) ES cells of *Gata6* (+/-) and (-/-) genotypes were differentiated with retinoic acid (+ RA) for 5 days. PD0325901, a selective and non ATP-competitive MEK inhibitor (MEK-i), was added to the cells at day 4, at a concentration of 1 μ M. Whole cell lysates were used for immunoprecipitation using anti-GATA6. Both the lysate inputs and immunoprecipitations were analyzed by Western blot with anti-PYS(-p)P and anti-GATA6 antibodies. (B) Undifferentiated *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) ES cells were transfected with expression vectors of wildtype and mutant plasmids: GATA6, GATA6 (S264A), and GATA6 (S264D). At 48 h after transfection, the cells were quantified for GFP signals, and were also analyzed by immunofluorescence for GATA6 and GATA4. (C) GFP levels in individual cells were quantitated in each group. GFP signals stimulated by GATA6 (S264A) were statistically lower than those stimulated by wildtype or S264D Gata6 (***) $p < 0.001$ in Student's *T*-test). (D) Undifferentiated *Gata6*^{H2BGFP/H2BGFP} (*Gata6* (-/-)) ES cells were transfected with expression vectors of wildtype and mutant plasmids: GATA6 and GATA6 (S264D), in the presence or absence of PD0325901 (1 μ M). At 48 h after transfection, the cells were monitored for GFP signals, and were also analyzed by immunofluorescence microscopy for GATA6. (E) GFP levels in individual cells were quantitated in each group. GFP signals stimulated by wildtype GATA6 were statistically lower than those stimulated by S264D GATA6 (***) $p < 0.001$ in Student's *T*-test).

2.7. Enhanced activation of *Gata6* promoter by GATA6 phosphorylation

The GATA6 protein contains a consensus Erk phosphorylation site (PYS²⁶⁴P), and phosphorylation of the conserved serine residue is known to enhance the potency of *Gata6* transcriptional activation in human cancer cells and zebrafish cardiovascular development (Adachi et al., 2008; Kelly et al., 2014). We therefore investigated whether GATA6 is phosphorylated by Erk1/2 in differentiating ES cells and its impact on PrE differentiation. In *Gata6*-positive ES cells undergoing PrE differentiation following treatment with RA, GATA6 expression was induced, accompanying a striking increase in phospho-Erk1/2 level (Fig. 6A). Consistently, phosphorylation of GATA6 at a conserved consensus Erk1/2 site was dramatically increased, as detected by an anti-PYS(-p)P antibody in GATA6 immunoprecipitates (Fig. 6A). Inclusion of a MEK inhibitor (MEK-i) to suppress Ras/MAPK activation reduced GATA6 expression, and also completely blocked the phosphorylation of the remaining GATA6 protein (Fig. 6A). The phosphorylation of GATA6 by Erk1/2 was established by metabolic labeling with P³²-phosphate previously in a cell line (Adachi et al., 2008). Here, the immunoprecipitation and GATA6 phosphorylation experiments were confirmed with three different GATA6 antibodies and an antibody specific to a phosphorylated consensus Erk1/2 phosphorylation site, for which GATA6 has only one such site, the serine 264 in the mouse protein sequence (F-P-Y-S(264)-P-S-P).

We generated cDNA expression constructs for a non-phosphorylatable form of GATA6 (S264A) and a mutant mimicking the phosphorylated form of GATA6 (S264D), and transfected the plasmids into *Gata6*^{H2BGFP/H2BGFP} ES cells (Fig. 6B). Compared to wildtype GATA6 cDNA expression, transfection and expression of the non-phosphorylatable GATA6 (S264A) produced a much weaker GFP signal (Fig. 6B). In contrast, transfection of the phosphorylation-mimicking GATA6 (S264D) mutant induced a robust GFP signal despite the comparatively lower expression of the mutant protein (Fig. 6B). Expression of either wildtype or mutant GATA6 proteins induced GATA4 expression (Fig. 6B). Quantitation of the GFP signal in *Gata6*^{H2BGFP/H2BGFP} ES cells transfected with GATA6 wildtype or mutant cDNAs showed a statistically significant difference between the promoter activating activity of GATA6 (S264A) from that of wildtype or GATA6 (S264D) (Fig. 6C). Thus, phosphorylation of GATA6 at Serine 264 enhances its potency to stimulate *Gata6* promoter activity.

In another set of experiments, GFP induction by the expression of wildtype and mutant GATA6 was monitored in the presence of Ras/MAPK pathway inhibitors (MEK-i) (Fig. 6D). In the presence of MEK inhibitor, transfection and expression of wildtype or the non-phosphorylatable GATA6 (S264A) were unable to induce GFP in *Gata6*^{H2BGFP/H2BGFP} ES cells; however, expression of the phosphorylation mimicking GATA6 (S264D) was resistant to MEK inhibition to activate *gata6* promoter (Fig. 6D). Quantitation of GFP signals in the transfected cells (by immunostaining of GATA6) indicated a consistent suppression of the *Gata6* promoter following MEK inhibition, which was overcome by transfection of the phosphorylation mimicking GATA6 mutant (S264D) that was able to induce GFP expression not suppressed by MEK inhibitor (Fig. 6D). In several experiments, MEK inhibitors both PD0325901 or Selumetinib, were tested, and similar impact of MEK/Erk1/2 inhibition was observed.

Thus, phosphorylation of GATA6 by Erk1/2 at S264 is required for GATA6 to activate its promoter, and phosphorylated GATA6 (or phosphorylation mimicking mutant protein) is sufficient to activate the promoter in ES cells despite inhibition of the Ras/MAPK pathway.

3. Discussion

The current study of a mutant line with GFP-histone H2B reporter construct knockin at the *Gata6* loci attained three significant findings: an increase and diminishment of the initial uniform level of GATA6

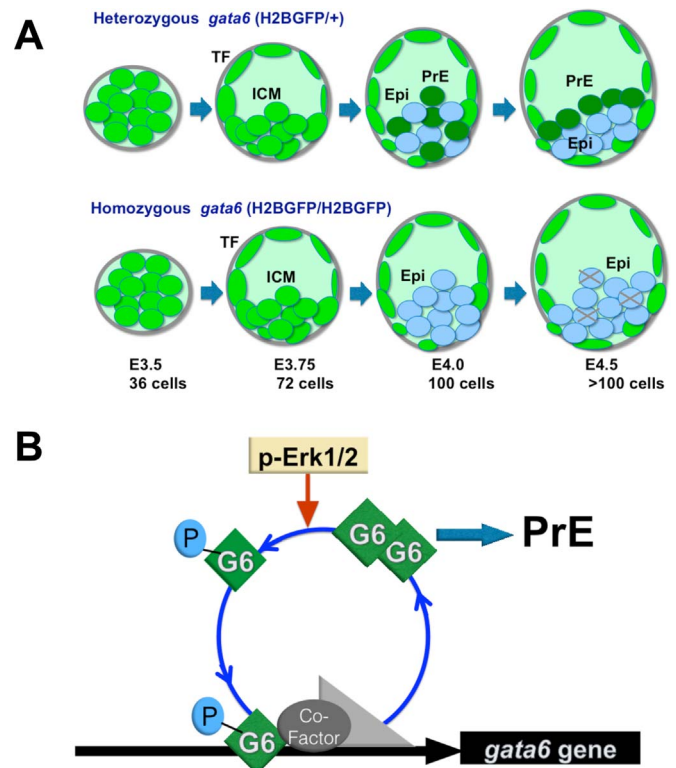


Fig. 7. (A) Illustration of the dynamic expression of GATA6 in blastocysts revealed by GATA6-GFP knockin reporters. In GATA6 positive blastocysts, all cells of the early (32-cell stage) blastula initially exhibit a moderate level of GATA6 expression. By the 64-cell stage, the signals diversified into two populations: the GFP signals in cells of the ICM either intensified or declined as the cells differentiated into PrE (marked as dark green color) or Epi (marked as light blue), respectively. In GATA6 null mutant blastocysts (*Gata6*^{H2BGFP/H2BGFP}), initially high GFP expression throughout the ICM subsided in all cells, and no PrE formed, though GFP intensity in trophoblast cells persisted. At later stages, extensive apoptosis occurred in the GATA6 null blastocysts. (B) Model for GATA6 phosphorylation and positive self-promotion of *Gata6* promoter in the commitment of primitive endoderm lineage. The current study produced several significant findings to support a model for PrE lineage commitment: 1. GATA6 binds to its own promoter to achieve a positive feedback in GATA6 expression. 2. Erk1/2 phosphorylation of GATA6 at serine 264 enhances its activity in transcriptional activation of its own promoter.

expression lead to the lineage commitment of ICM to PrE and Epi, respectively (Fig. 7A); GATA6 binds to its own promoter to achieve a positive feedback in PrE differentiation (Fig. 7B); and Erk1/2 phosphorylation of GATA6 at serine 264 enhances the potency of GATA6 in transcriptional activation of its own promoter (Fig. 7B). The findings suggest a plausible model for the lineage differentiation of PrE and Epi (Fig. 7). We found that all cells of the early (32-cell stage) blastula exhibit a moderate level of GATA6 expression. Thus, we reason that Fgf4 stimulation of a subset of the ICM cells leads to phosphorylation of GATA6 by activated Erk1/2, and the phosphorylated GATA6 gains an increased affinity to its promoter to initiate a positive feedback loop that augments transcriptional activity and GATA6 expression. Increased GATA6 level stimulates the expression of target genes, and these cells ultimately commit to the PrE lineage. In the remaining cells not receiving the FGF signal and possessing low Erk1/2 activity, the initial GATA6 expression subsides, possibly caused by suppression mediated by Nanog and Oct3/4, and subsequently the cells are compelled into the Epi lineage. Therefore, no PrE commitment can be achieved in the absence of either GATA6 or its phosphorylation by Ras/MAPK pathway activation to impel a GATA6 surge. In summary, our findings reveal a GATA6-positive feedback regulatory loop and provide a mechanistic explanation for the long sought impact of Ras/MAPK pathway in the development of PrE (Fig. 7).

Since both GATA6 and Nanog are present prior to the initiation of PrE differentiation in the blastocysts and the *Gata6* promoter is

moderately active in the absence of GATA6 protein as in the GATA6-null blastocysts, the stimulation of Fgf4/Ras/Erk pathway appears to trigger the break down of the balance between GATA6 and Nanog, and to initiate differentiation into GATA6 high/Nanog low PrE and GATA6 low/Nanog positive Epi cells. Our current experiments suggest that Erk1/2 phosphorylation of the basal GATA6 spurs a positive feedback that augments GATA6 level and subsequently leads to PrE differentiation. However, the results also suggest that an Erk1/2 substrate(s) other than GATA6 likely exists, and the phosphorylation triggers the maintenance of Nanog expression and suppression of *Gata6* promoter. Additionally, *Gata6* promoter activity seems to be constant during the time frame analyzed in TE cells. One obvious possible explanation is that the TE cells lack Fgfr2 and are not responsive to Fgf4 to activate the Fgf/Ras/Erk1/2 pathway. However, Fgfr1 and Fgfr2 are expressed, and their signaling plays a compensatory role in TE proliferation (Kang et al., 2017; Molotkov et al., 2017). Alternatively, an Erk1/2 substrate(s) responsible for suppressing or promoting GATA6 expression in PrE progenitor cells is not expressed in the TE cells.

The current intriguing finding of GATA6 phosphorylation by Erk1/2 provides a possible explanation for the well-researched requirement of transcription factor GATA6 and Fgf/Ras/Erk1/2 signal pathway in PrE commitment (Yamanaka et al., 2010; Cai et al., 2008; Bessonard et al., 2014; Schrode et al., 2014; Schröter et al., 2015). Likely there are multiple Erk1/2 substrates to be phosphorylated in the Fgf/Ras/Erk1/2-stimulated differentiation pluripotent ES cells to PrE; however, GATA6 is the key substrate as supported by the observation that the non-phosphorylatable form of GATA6 (S264A) has a much reduced ability to activate the *Gata6* promoter. In contrast, mutant GATA6 that mimics the phosphorylated protein, GATA6 (S264D), retains activity to activate the *Gata6* promoter even in the presence of MEK inhibitors, which suppress Erk1/2 activation. The phosphorylation of GATA6 by Erk1/2 at the S264 site appears to be conserved in zebrafish, mouse, and human. A previous study reported that Erk1/2-mediated phosphorylation of GATA6 enhances its binding to GATA6 on promoter and upregulates gene expression in human cancer cells (Adachi et al., 2008). Additionally, in zebrafish, Erk1/2 phosphorylation of GATA6 modulates gene expression in cardiovascular development (Kelly et al., 2014). Our current finding adds another example of GATA6 phosphorylation by Erk1/2, in the development of PrE lineage in blastocysts.

One unique aspect of the current finding is that the transcription target of GATA6 is the *Gata6* gene itself. Though the positive feedback of GATA6 regulation has not been observed previously, this seems reasonable as multiple GATA consensus binding sites are present at the *Gata6* promoter. Suggestively, a mutually reinforcing regulatory network of Nkx2.5 and GATA6 transcription factors has been proposed in the regulation of cardiac-specific gene expression (Molkentin et al., 2000). Additionally, in a ChIP-seq profiling of pluripotency factors binding to *Gata6* promoter in differentiating ES cells, exclusive binding of GATA6 to its own promoter region was detected in two shape peaks corresponding to the two promoters (Wamaitha et al., 2015). Although the previous study (Wamaitha et al., 2015) did not explore the observation further, the current results indicate the role of the binding of GATA6 to its own promoter as a positive feed forward regulatory loop.

These findings resolve several highly sought questions on signaling and gene regulation of early lineage pluripotency and embryonic stem cell differentiation that have been explored for the last 4 decades. Perhaps, the molecular mechanism proposed (Fig. 7) based on the current findings advance the study of early embryonic PrE development beyond descriptive observations and phenotypic analyses, and further analyses will resolve the questions on gene regulatory network and modulation by signaling pathway. The model integrating GATA6 and the Fgf/Ras/Erk1/2 signaling pathway will likely prompt further studies to verify the proposed molecular mechanism and complete additional details in the exit of ES cells from pluripotency and the development of PrE, one of the earliest embryonic lineage in mammals.

4. Material and methods

4.1. Targeting the *Gata6* allele and generation of the *Gata6*^{H2BGFP} reporter and mutant mice

In this manuscript, “*Gata6*” (Italic) refers to the mouse gene, and GATA6 the protein, according to guideline from HGNC (HUGO Nomenclature Committee) (<https://www.genenames.org/activities/human-and-mouse-orthologous-gene-nomenclature-humot>). A targeting vector was constructed by flanking a 1.4 kb fusion construct of histone 2B (H2B) and eGFP (enhanced Green Fluorescence Protein), and a lox flanked neomycin resistance (neo) construct with a 2.5 kb 5' arm and a 4.1 kb 3' arm of genomic DNA of *Gata6* gene (Fig. 1a). Expected targeting homologous recombination allele would result in replacement of *Gata6* exon 2 by the H2B-GFP-neo construct at the *Gata6* “ATG” translation start site. Following transfection of R1 murine ES cells and selections of neo resistant cells, PCR screening was performed to identify clones that had undergone correct homologous recombination. Selected ES cells were then injected into C57BL/6 host blastocysts and chimeras were generated. The neo gene, which was flanked by loxP sequences, was excised by breeding chimeras with the Sox2-Cre line. The resulting F1 mosaics were bred with C57BL/6 to establish the *Gata6*:H2BGFP strain lacking the neo gene and Sox2-Cre transgene. Genotyping was performed by PCR amplification of tail tissues or embryonic cells using the following oligonucleotides: 5' CCG GTG TGG AAC AGC TAT TTA 3'; 5' ACC TCC CTA TCC CAC TTC GT 3'; and 5' CTG ATC AAT TCC GTC TTC GC 3'. The wildtype allele amplified at 195 bp and the mutant, *Gata6*:H2BGFP, at 127 bp.

4.2. Generation of ES cells from mouse embryos and culture

Pre-implantation mouse embryos were flushed from uteri at 3.5 days post coitus. The blastocysts were cultured individually in wells of a 24-well plate on a feeder layer of gamma-irradiated fibroblasts, in ES cell media. The embryonic outgrowths were trypsinized and triturated, then re-plated into a new tissue culture well. ES cell clones visible after 7–10 days were serially propagated, PCR genotyped, and cryopreserved.

All ES cell lines were maintained in a pluripotent state by culturing in ES cell medium supplemented with 1000 U/ml of recombinant LIF (ESGRO, Chemicon International) in a humidified incubator with 5% CO₂. The ES cell culture medium consisted of DMEM supplemented with 15% (v/v) fetal bovine serum, 2 mM L-glutamine, non-essential amino acids, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 0.1 mM β-mercaptoethanol. The ES cells were routinely cultured on a feeder layer of gamma-irradiated fibroblasts, or on gelatin-coated tissue culture grade plastic well prior to experiments.

For immunofluorescence microscopy, live ES cells were fixed with 4% PFA at room temperature for 20 min, following by incubation in 0.1% Triton X-100 for 5 min. Standard procedures for immunofluorescence microscopy were used. Multiple secondary antibodies conjugated with the appropriate Alexa fluorochrome were used for simultaneous imaging of multiple antigens. DAPI (4'–6-diamidino-2-phenylindole) solution was used as a generic nuclear counterstain and applied at the end of the procedure and slides were mounted in Prolong Gold Antifade.

4.3. Mouse blastocyst *in vitro* culture and immunofluorescence staining, and time lapse video microscopy

E3.5 day blastocysts were flushed from uterine horns, collected into one 60-mm culture dish, and incubated in 300 μl of KSOM medium (EMD-Millipore) in a 37 °C, 5% CO₂ incubator. For immunofluorescence, the blastocysts were fixed in 4% paraformaldehyde plus 5% sucrose and 1% Triton X-100 for 1 h at room temperature, washed twice for 5 min in 100 μl PBS by transferring to fresh wells. Then the

blastocysts were blocked overnight in 5% BSA at 4 °C. The following day, blastocysts were incubated with the appropriate primary antibodies for 1 h at room temperature, washed, and blocked overnight in 2% BSA at 4 °C. Following incubation with secondary antibodies for 1 h at room temperature, the blastocysts were washed twice, incubated for 30 min with DAPI, washed again, and finally transferred to and stored at 4 °C in PBS in a glass bottom culture dish (MatTek, Ashland, MA) until imaged. The primary antibodies and concentration were as follows: 1:300 dilution, rabbit anti-Nanog (Abcam, ab80892, Cambridge, MA); 1:300 dilution, rabbit polyclonal anti-GATA4 (Santa Cruz, sc9053); 1:1000 dilution, rabbit polyclonal anti-GATA6 developed previously (10); rabbit anti-GATA6 (H92 or C20, Santa Cruz) and goat anti-GATA6 antigen affinity-purified polyclonal antibody (R & D systems, AF1700); 1:300 dilution, mouse monoclonal anti-Oct3/4 (Santa Cruz, sc-5279); and 1:1000 dilution, mouse monoclonal anti-Dab2 (BD, 610464). The secondary antibodies were Alexa fluor-conjugated (Alexa488, Alexa555, Alexa647) secondary antibodies (Molecular Probes, Thermo Fisher Scientific, Waltham, MA). The images shown are representatives of 3–6 independent experiments performed.

For live imaging, embryos/blastocysts were cultured in glass-bottomed dishes (MatTek) in a thermo-regulated chamber. KSOM medium was used to maintain embryo viability and HEPES solution was added into the culture medium to maintain pH. Phase contrast and GFP fluorescence data were acquired from living embryos using an inverted Leica SP5 confocal microscope operated by LAS AF software with a 20 × (N/A 0.7) objective every 20 min for 7–10 z planes. The optical section of 17 μm was identical to the z step size. Movies of 3D time-lapse sequences were compiled and annotated using Volocity® 3D Image Analysis Software from Perkin Elmer (Thermo Fisher Scientific). Volocity software was used to quantitate the GFP intensity and track cell movement via individual GFP-positive nuclei. The movies shown were selected representation from at the least 3 independent embryo preparations.

4.4. Plasmid constructions

The wild type mouse GATA6 expression vector driven by CMV promoter, GATA6-pcDNA1 plasmid, was purchased from Addgene (plasmid #51929). GATA6 site-mutated plasmids were prepared by using Q5® Site-Directed Mutagenesis Kit (New England BioLab #E0554S). Site-directed mutants of mouse GATA6 plasmid, GATA6 (S264A), and GATA6 (S264D), were generated by the overlap extension method following the manufacturer's protocol. Primer sequences are listed as follows: GATA6 (S264A), Forward: 5' CTTTC CCTAC GCGCC CAGCC CGC 3'; Reverse: 5' CGTGC AGAGG CGTGG GCC 3'. GATA6 (S264D), Forward: 5' CTTTC CCTAC GATCC CAGCC CGCC ATGG 3'; Reverse: 5' CGTGC AGAGG CGTGG GCC 3'. The produced expression constructs were sequenced to verify the targeted mutations.

4.5. ES cells transfections and use of MEK inhibitors

ES cells were cultured in 6-well plates at 5×10^5 cells/well (or normal seeding density) and incubated overnight in ES cell media. The next day, transfections were performed with Lipofectamine 2000 (LF2000) reagent using 8–12 μl LF-2000 diluted in 400 μl Opti-MEM, and 4 μg DNA in each well. The combined transfection mixtures were incubated for 20 min at room temperature and added into ES cells for 4 h before adding ES cell culture medium. Following transfection, the cells were monitored for the presence of GFP signals compared with vector-transfected controls, and GFP intensity was quantified using ImageJ software at 2 and 4 days.

For some experiments, selective and non-ATP-competitive MEK inhibitors, PD0325901 at 1 μM or Selumetinib (AZD6244) at 1–50 μM, was added to the cells for up to 5 days. The inhibitors did not show noticeable toxicity as judged by cell number and morphology at the

dosage and duration used, and inhibition by PD0325901 or Selumetinib produced similar results.

4.6. ChIP-qPCR assay

ChIP assay was performed using the SimpleChIP® Enzymatic Chromatin IP kit (Agarose Beads) (Cell Signaling #9002). The general procedure for ChIP assay was followed and is similar to that reported previously (Caslini et al., 2006). Chromatin was immunoprecipitated by incubating with antibodies to GATA6 (R & D systems, AF1700) or GATA4 (Santa Cruz Biotechnology, sc-9053), at 4 °C overnight. Mouse IgG antibodies were used as a negative control. For quantitative ChIP-qPCR, total inputs served as internal controls. EpiTect ChIP qPCR Primer Assay kit (QIAGEN, Cat# GPM1033446(+))01A) was used to for the amplification of *Gata6* gene promoter of a 1 kb fragment starting at position –371.

4.7. Western blotting and antibodies

For Western blot analysis of cell lysates, primary antibodies used include: mouse monoclonal anti-Dab2 (BD Transduction Labs #610465), mouse monoclonal anti-Oct3/4 (Santa Cruz Biotechnology, sc-170 5279), mouse monoclonal anti-GATA4 (Santa Cruz Biotechnology, sc-9053), mouse monoclonal anti-GFP (Abcam, ab32146), and rabbit polyclonal anti-GATA6 (Cai et al., 2008). The above antibodies were also used for immunofluorescence microscopy and immunohistochemistry. An affinity purified rabbit antibody recognizing the phosphorylated motif by Erk1/2, anti-PYS(-p)P (Cell signaling #2325), was used to determine GATA6 phosphorylation by Erk1/2 in Western blot. The secondary antibodies were horseradish peroxidase (HRP) conjugated goat or mouse anti-rabbit or anti-mouse (BioRad; Jackson Immunolab; Zymed). SuperSignal West Extended Duration Substrate (PIERCE) was used for chemoluminescence detection of protein bands.

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Author contributions

YM and XXX developed concepts and planned the experiments. RM produced mutant mice and embryos. YM, RM, WT, ERS, and JT contributed to experiments and data analyses. CC performed promoter and DNA sequence analyses. YM and XXX analyzed the results and prepared initial draft of manuscript. All authors, especially RM and ERS, contributed to editing of the manuscript.

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