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ABSTRACT

Receptor tyrosine kinase signaling (RTK) are key regulators of the formation of the primitive endoderm (PrE) and the epiblast (Epi) from the inner cell mass of the mouse preimplantation embryo. Among them, FGF signaling is critical for PrE cell specification while PDGF signaling is critical for the survival of committed PrE cells. Here, we investigated possible functional redundancies between FGF, PDGF and KIT signaling and showed that only PDGF signaling is involved in PrE cell survival. In addition, we analyzed the effectors downstream of PDGFRα. Our results suggest that the role of PDGF signaling in PrE cell survival is mediated through PI3K-mTOR and independently from p53. Lastly, we uncovered a role for PI3K-mTOR signaling in the survival of Epi cells. Taken together, we propose that survival of ICM cell lineages relies on the regulation of PI3K-mTOR signaling through the regulation of multiple signaling pathways.

INTRODUCTION

Eutherian mammalian embryos require close interactions with the mother uterine environment to properly develop. To do so, the first days of embryonic development, referred as the preimplantation period, are devoted to the specification and segregation of extraembryonic tissues, the trophectoderm (TE) and the primitive endoderm (PrE), from the pluripotent epiblast (Epi) that will form the embryo proper [1]. TE gives rise to the fetal portion of the placenta, a transient organ dedicated to fetal-mother interactions. PrE participates to the yolk sac membrane that ensures nutrients supply and is critical for embryo patterning. By the time of implantation, the blastocyst embryo comprises these three distinct cell lineages, which are spatially segregated and are defined by a set of specific markers, including key lineage transcription factors. The specification of these first cell lineages has been extensively studied in mouse and is thought to occur in two steps [1-3]. First, TE and inner cell mass (ICM) cells emerge followed by the subsequent specification of Epi and PrE within the ICM. The latter requires both intrinsic and extrinsic cues which involve the initiation of Epi and PrE transcriptional programs in all blastomeres and, in particular, the expression of the master regulators *Nanog* (Epi-specific) and *Gata6* (PrE-specific) [4]. Subsequently, Epi and PrE markers are progressively restricted in a salt-and-pepper pattern, so that the mouse ICM is composed of the juxtaposition of committed Epi and PrE cells [5, 6]. Committed cells are eventually reorganized by a cell sorting process so that the PrE lies in contact to the blastocoel cavity and surrounds the Epi [4]. This step of spatial organization into two distinct compartments involves complex processes including actin-dependent cell movements,

positional information sensing by sorted PrE cells and epithelialization [5, 7, 8]. Programed cell death or apoptosis is also involved at these stages and has been proposed to remove mispositioned cells [5, 8-10].

Several receptor tyrosine kinase signaling pathways are thought to orchestrate Epi/PrE formation including FGF and PDGF signaling. FGF4/FGFR1/FGFR2 are critical for PrE specification [11-14]. Indeed, genetic and pharmacological modulation of FGF activity directly impacts on cell lineage decision so that gain of FGF activity favor PrE commitment while a loss of FGF activity favors Epi cell fate [15-18]. Current model posits that the first emerging Epi cells secrete FGF4 ligand and instruct uncommitted neighboring cells [18]. FGFR1 plays a major role in PrE cell specification as demonstrated by recent studies of the phenotype of *Fgfr1* knockouts [13, 14]. *Fgfr2* inactivation does not affect PrE formation but exacerbates the phenotype of *Fgfr1* mutants [13, 14]. PDGF signaling plays also critical roles in PrE biology. *Pdgfra* expression is restricted to PrE cells and its inactivation reduces the number of PrE cells [19, 20]. Further analysis revealed that PDGF signaling regulates PrE survival at the time when committed PrE cells undergo cell sorting [19, 20]. PDGF signaling regulates the activity of several downstream effectors including PI3K and MAPK [21, 22]. It is not clear which of these transduction pathways are involved in PrE cell survival. *Kit*-deficient embryos develop to term and newborn mutants die of severe anemia [23, 24]. However, KIT signaling could also be involved in early mammalian development since cKIT is expressed during the preimplantation period [23, 24]. To our knowledge, this question has never been addressed.

Here, we provide genetic evidences that KIT, FGFR2 and PDGFRα do not exert any major functional redundancy. In addition, we investigated which downstream effectors relays PDGFR signaling. We demonstrated a major role of PI3K-mTOR in this process, which acts independently from p53. Lastly, we uncovered an additional role of PI3K-mTOR in regulating survival of Epi cells. Taken together, our study unravels an unsuspected complexity in the signaling pathways orchestrating ICM cell lineages.

MATERIALS AND METHODS

Mouse husbandry

All experiments were conducted according to the French and European regulations on care and protection of laboratory animals (EC Directive 86/609, French Law 2001-486 issued on June 6, 2001) and were approved by the Institut Pasteur ethics committee (n° 2012-0011). Mouse strains were CD1 (Charles River laboratories, France), *Trp53*$^{+/-}$ [25] (JAX stock # 002101), *Pdgfra* $^{+/H2B-GFP}$ [26] (JAX stock #007669), *ZP3-Cre* $^{+/Tg}$ [27], *Fgfr2* $^{+/CKO}$ [28] (JAX stock #007569) and *Kit* $^{+/wLacZ}$ [23].

Embryo recovery and culture

Embryos were recovered at E3.75 (salt-and-pepper ICM) or E4.5 (monolayer of PrE cells) by flushing oviducts or *uteri* in M2 (Millipore), washed twice and cultured inside their intact zona pellucida in 400 µL KSOM (Millipore) in Nunc 4-well plates until the stages of interest at 37 °C, 8% CO2. For treatments, we used mTOR inhibitors Rapamycin (Sigma-Aldrich) and INK128 (Selleckchem), JAK inhibitor I (JAKi) (CalbioChem) and LIF (Millipore). All treatments were performed in parallel using the same batch of embryos more than three independent experiments.

Immunostaining

Embryos were fixed in 4% PFA overnight at 4°C and then washed three times in PBT (PBS, 0.1% Triton X-100). Embryos were incubated in blocking solution (PBT, 10% FBS) during 1 hour and then incubated with primary antibodies overnight at 4°C. We

used anti-GATA6 (1/100, AF1700, R&D Systems), anti-CDX2 (1/100, MU392A-UC, Biogenex), anti-NANOG (1/100, 8822, Cell Signaling; 1/100 14-5761, eBioscience), anti-SOX17 (1/100, AF1924, R&D Systems), anti-GATA4 (1/100, sc-1237, Santa Cruz), anti-pSTAT3 (1/100, Cell Signaling). pSTAT3 was performed according to [29]. After several washes in PBT, embryos were incubated with secondary antibodies coupled with Alexa 488nm, 546nm, 647nm (1/300, Invitrogen and Hoeschst (1.6µM, Sigma Aldrich) 2 hours at room temperature and then washed three times during 10 minutes before analyzing them with SP5 (Leica) or LSM800 (Zeiss) confocal microscope. Embryos were eventually recovered and genotyped by classical or nested PCR [30]. For *Fgfr2* genotyping, we performed a first PCR with *Fgfr2*-F0, -R2 and -R5 primers and second PCR with *FgfR*2-F1 and -R2 or *FgfR2*-F1 and -R4. *Pdgfra* genotyping was performed as previously described [19]. The recombinant allele $Pdgfra^{H2B\text{-}GFP}$ Kit^{wLacZ} was genotyped using *Pdgfra* genotyping primers. *Trp53* genotyping was performed using a classical PCR with *Trp53*-556, -557 and -558 primers.

Images were analyzed and processed using Icy, Fiji and Photoshop CS5 softwares. We manually counted the number of cells positive for GATA6/SOX17/GATA4/NANOG.

Primers for genotyping

FgfR2 : F0 : TGTCTGCCTGTCTCCCGTGCT; F1 : ATAGGAGCAACAGGCGG; R2 : TGCAAGAGGCGACCAGTCAG; R4 : CAGTGGTTAAAGAGTGCTTGCT; R5 : GTATCTGGGGGCTGGAGAACT.

Trp53: 556 : CCATGCAGGAGCTATTACACA; 557 : AGCGTGGTGGTACCTTATGAG; 558 : GCTATCAGGACATAGCGTTGG.

ES cell culture and differentiation

Kit $^{+/LacZ}$ and *Kit* $^{LacZ/LacZ}$ ES cells were obtained from ([31, 32]). ESC were routinely cultured and differentiated as previously described in [19] and processed for immunostaining. Data were acquired using a microscope Zeiss Apotome 2 upright equipped with an Axiocam MRm camera. E14TG2a ES cells were grown on FCS and LIF on gelatin-coated dishes and incubated with DMSO, Rapamycin, INK128 or JAKi for 16 hours.

Western blot

Treated ES cells were lyzed into extraction buffer (10 mM Tris HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP40, 10% glycerol, 30 mM NaP, 50 mM NaFluoride) completed with protease inhibitor (Complete, EDTA free, Roche) and phosphatases inhibitors (phosStop easy pack, Roche) with 2.5 UI of benzonase nuclease (Sigma). Lysates were incubated on ice for 30 min and non-soluble fraction was separated by centrifugation. Protein denaturation was performed in Laemmli buffer at 95°C for 5 min. After separation on polyacrylamide gel (Biorad), proteins were transferred on Immun-Blot PVDF membrane (Biorad) and incubated overnight with the following antibodies: anti-Phospho-S6 Ribosomal Protein ser235/236 (1/1000, 4856, Cell Signaling), anti-Phospho-STAT3 (Tyr705) (1/2000, 9145, Cell Signaling), anti-RPS6 (1/1000, 5G10, Cell signaling) and anti-STAT3 (79D7) (1/2000, 4904T, Cell Signaling). Membranes were incubated 1h30 at room temperature with an anti-rabbit IgG HRP-linked secondary antibody (1/10000, NA934V, Amersham) and signals were visualized using Pierce ECL2

(80196, Thermo Scientific) (Fig.S1).

Statistical analysis

Graphs and statistical tests were performed using Prism (Graphpad).

RESULTS

KIT signaling is not involved in extraembryonic endoderm formation

Genetic loss of function of *Pdgfra* led to an increase of cell death at the time of PrE cell sorting which is manifested by a reduction of PrE cell number [19, 20]. Similarly, pharmacological inhibition of PDGF signaling using Gleevec also reduced the number of PrE cells [20]. However, Gleevec inhibits additional RTK including c-KIT [33, 34] which is expressed at the blastocyst stage in the ICM [23] opening the possibility that c-KIT signaling could be also involved. Thus, we tested the possibility that KIT signaling could be also involved in PrE cell survival and maturation. However, *Kit* and *Pdgfra* are genetically linked, being both located on chromosome 5 and separated by around 400kb. We thus generated double heterozygous $Pdgfra^{H2B\text{-}GFP}$ Kit^{+}, $Pdgfra^{+}$ Kit^{wLacZ} males and crossed them with wild-type females. 1 out of 129 progenies carried the recombinant haplotype $Pdgfra^{H2B\text{-}GFP}$ Kit^{wLacZ}, allowing us to establish a mouse colony. We then analyzed the effect of removing one or two *Kit* functional alleles on *Pdgfra* mutant phenotype (Fig. 1 and Fig. S2). *Pdgfra* mutant E4.5 embryos exhibited a PrE cell number reduction of 2.9-fold with one Kit^{+} allele (Fig. 1A) and 1.6-fold with no *Kit* functional allele (Fig. 1B) compared to *Pdgfra* wild-type littermates. This is consistent with the PrE cell number reduction we found in previous studies for *Pdgfra* mutants (1.6-fold in E4.5 embryos [19], and 3.4-fold in E3.5 cultured 24h *in vitro* [20]). The difference between the PrE cell number reduction could be due to the mixed genetic backgrounds. Indeed, Kit^{wLacZ} and $Pdgfra^{H2B\text{-}GFP}$ alleles were originally maintained on 129/Sv and B6.129S4 backgrounds respectively, while $Pdgfra^{+/H2B\text{-}GFP}$, $Kit^{+/+}$ and $Pdgfra^{+/H2B\text{-}GFP}$, $Kit^{+/wLacZ}$

mice were produced from separate crosses involving different degree of introgression onto CD1 outbred genetic background. We also noticed a slight increase of Epi cell number in double *Pdgfra* and *Kit* mutants but not significant compared wild-type (p-value: 0.08) and double heterozygous (p-value: 0.24) (Fig. 1B). This observation could reflect a moderate role in Epi/PrE specification and/or in Epi progenitor cell survival.

We previously showed that *Kit* is not required for the establishment and the maintenance of extraembryonic endoderm XEN cells [19]. However, it is not clear whether KIT signaling could be involved in *in vitro* cellular models of PrE formation. To test this possibility, we differentiated *Kit* mutant ES cells into extraembryonic endoderm cell lineages either by retinoic acid treatment or GATA4/6 overexpression and immunostained with PrE markers (GATA4, GATA6, SOX17) (Fig. S3). We did not observe obvious differences compared to heterozygous ES cells. From these experiments, we conclude that *Kit* is likely not involved in extraembryonic endoderm formation and does not interact genetically with *Pdgfra* in that lineage.

No functional redundancy between PDGFRα, KIT and FGFR2

FGF signaling is critical for ICM cell lineage specification [6, 11-18]. *Fgf4* inactivation impairs PrE formation and so all ICM cells adopt an Epi fate [11, 12]. Several FGF receptors are expressed in preimplantation embryo and an inverse correlation between *Fgfr2* and *Fgf4* expression within the ICM at E3.5 is thought to presage PrE/Epi specification [35, 36]. Gene expression analysis recently revealed that *Fgfr1* and *Fgfr2* expression are initiated at different timing in early or mid-blastocyst embryos [13]. Recent studies demonstrated an essential role of FGFR1 in the

specification of PrE cell lineage while FGFR2 might exert an accessory role in PrE-biased ICM cells [13, 14].

We thought to analyze the phenotype of *Fgfr2* mutant embryos at preimplantation stages. We first analyzed the composition of embryos recovered at E3.5 and cultured 24h *in vitro* (Fig. S4). Wild-type, heterozygous and zygotic mutant embryos exhibited a similar composition of PrE (WT: 15.5±0.6 cells; zHET: 15.6±0.8 cells; zHOM: 12.8±1.1 cells), Epi (WT: 11.9±0.8 cells; zHET: 12.1±0.6 cells; zHOM: 11.3±0.7 cells) and TE (WT: 67.5±4.3 cells; zHET: 73.9±2.7 cells; zHOM: 75.5±3.7 cells) cells (Fig. S4A). The absence of overt phenotype could be due to the presence of maternal stores accumulated during oogenesis. Thus, we next analyzed the phenotype of maternal and zygotic E3.75 mutants at the time when PrE and Epi cells have been specified and are distributed in a salt-and-pepper pattern. No difference was observed regarding cell number of PrE (mzHET: 7.9±1.4 cells; mzHOM: 7.0±1.4 cells), Epi (mzHET: 13.3±1.4 cells; mzHOM: 12.7±1.1 cells) and TE (mzHET: 56.1±4.1 cells; mzHOM: 56.7±3.0 cells) (Fig. S4B).

Since *Fgfr2* is expressed in PrE-biased ICM cells [13], we hypothesized that it could cooperate with PDGF signaling. We thus tested a possible genetic interaction between PDGF, KIT and FGF signaling and analyzed the composition of each cell lineages at E4.5 (Fig. 2). First, TE cell number was not significantly modified. Concerning Epi cells, we noticed a significant increase in $Fgfr2^{+/+}$ $Pdgfra,Kit^{-/-}$ compared to $Fgfr2^{+/+}$ $Pdgfra,Kit^{+/+}$ but not when compared to $Fgfr2^{+/-}$ $Pdgfra,Kit^{-/-}$ and $Fgfr2^{-/-}$ $Pdgfra,Kit^{-/-}$ opening the possibility that KIT signaling could play a moderate role in Epi/PrE cell specification and/or Epi cells as suggested previously (Fig. 1B). Concerning PrE cell number, we observed a strong reduction in $Pdgfra,Kit^{-/-}$ mutants with two, one or no

Fgfr2 functional allele (3.1-fold between *Fgfr2*$^{+/+}$ *Pdgfra,Kit*$^{+/+}$ and *Fgfr2*$^{+/+}$ *Pdgfra,Kit*$^{-/-}$; 2.2-fold between *Fgfr2*$^{+/-}$ *Pdgfra,Kit*$^{+/+}$ and *Fgfr2*$^{+/-}$ *Pdgfra,Kit*$^{-/-}$; 2.1-fold between *Fgfr2*$^{-/-}$ *Pdgfra,Kit*$^{+/+}$ and *Fgfr2*$^{-/-}$ *Pdgfra,Kit*$^{-/-}$). However, there was no significant difference between *Fgfr2*$^{+/+}$ *Pdgfra,Kit*$^{-/-}$, *Fgfr2*$^{+/-}$ *Pdgfra,Kit*$^{-/-}$ and *Fgfr2*$^{-/-}$ *Pdgfra,Kit*$^{-/-}$. We thus conclude that there is no strong genetic interaction between *Fgfr2*, *Kit* and *Pdgfra* during mouse preimplantation development.

PDGF signaling exerts its survival activity independently of p53

During skeletal development, PDGF signaling regulates cell proliferation and cell survival through p53 [37]. Interestingly, nuclear p53 is detected in preimplantation embryos around E3.5 [38] and p53 regulates *Nanog* expression in mESC [39] suggesting that p53 might regulate ICM cell lineage decisions. We first analyzed the consequences of removing *Trp53* on early cell lineage decisions (Fig. S5). Embryos were recovered at E4.5 from crosses between *Trp53*$^{+/-}$ females with *Trp53*$^{-/-}$ males and immunostained with Epi (NANOG) and PrE (SOX17) markers (Fig. S5A). Zygotic mutants exhibited similar Epi, PrE and TE cell numbers compared to heterozygous littermates (23.3±2.1 Epi cells in zHET *vs* 22.1±2.0 in zHOM; 36.5±3.2 PrE cells in zHET *vs* 30.4±3.9 in zHOM; 73.2±4.2 TE cells in zHET *vs* 81.4±3.4 in zHOM). To avoid the possibility that a role of p53 could be masked by the presence of maternal stores, we also analyzed the cellular composition of E4.5 embryos recovered from *Trp53*$^{-/-}$ females bred with *Trp53*$^{+/-}$ males (Fig.S5B). We observed a similar lineage composition between maternal and zygotic mutant and maternal and zygotic heterozygous (16.7±2.0 Epi cells in mzHET *vs* 19.6±2.4 in mzHOM; 19.2±2.7 PrE cells in mzHET *vs* 18.4±2.1 in mzHOM; 72.9±4.2 TE cells in

mzHET *vs* 70.0±7.0 in mzHOM). Based on these observations, we conclude that p53 is dispensable for early cell lineage decisions.

We next tested whether p53 genetic removal could rescue the PrE defects in *Pdgfra* mutant embryos (Fig. 3). *Pdgfra*$^{H2B\text{-}GFP/\ H2B\text{-}GFP}$ E4.5 embryos exhibited a similar specific reduction of PrE cells but not Epi cells with one or no functional *Trp53* allele (2.1-fold between *Pdgfra*$^{+,\ H2B\text{-}GFP}$ *Trp53*$^{+/-}$ and *Pdgfra*$^{H2B\text{-}GFP,\ H2B\text{-}GFP}$ *Trp53*$^{+/-}$; 2.3-fold between *Pdgfra*$^{+,\ H2B\text{-}GFP}$ *Trp53*$^{-/-}$ and *Pdgfra*$^{H2B\text{-}GFP,\ H2B\text{-}GFP}$ *Trp53*$^{-/-}$). Our data demonstrate that loss of PDGF signaling affects PrE cell survival independently of p53.

PI3K and mTOR mediate ICM cell survival

PDGF signaling is involved in various cell processes and is mediated by multiple downstream signal transducers including MAPK and PI3K [21, 22]. Interestingly, PDGF signaling has been shown to act through PI3K to regulate cell survival during skeletal development [37].

To evaluate a putative role of PI3K signaling, we first analyzed the effect of the inhibition of PI3K activity on early embryonic cell survival. Embryos were cultured at E3.5 before PrE specification in presence of LY294002 for 24h and were subsequently analyzed. We used a range of concentrations for which the phosphorylation of the ribosomal protein S6 (p-RPS6), a target of PI3K/mTOR pathway, was decreased in ES cells as assayed by western-blot (Fig. S1). We observed that increasing concentrations of LY294002 affects the survival of both Epi and PrE but not TE cell lineage (Fig. 4A and data not shown).

We next investigated which signaling could be regulated by PI3K in Epi and PrE

cells. One such candidate was mTOR since PI3K/AKT/mTOR signaling is involved in the regulation of apoptosis and cell proliferation [40]. To test this hypothesis, E3.5 embryos were cultured for 24h in presence of either Rapamycin, an inhibitor of mTORC1 [41], or INK128, an inhibitor of both mTORC1and mTORC2 [42]. We validated that both inhibitors treatment inhibited RPS6 phosphorylation in ES cells (Fig. S1). p-RPS6 signal reduction was drastic in INK128-treated cells and moderate in Rapamycin-treated cells. In the embryos, we did not observe any effect of Rapamycin treatment ranging from 0.5 to 10μM on Epi and PrE cell numbers (Fig.4B), indicating that mTORC1 is dispensable in ICM lineages at that period. In contrast, inhibition of both mTORC1 and mTORC2 with INK128 resulted in marked reduction of both Epi and PrE cell numbers (Fig. 4C).

Thus, we conclude that the survival of both ICM cell lineages requires the activity of PI3K and mTOR.

JAK/STAT and mTOR signaling cooperate in the regulation PrE cell survival

It was previously proposed that LIF signaling acts upstream of JAK/STAT to regulate PrE cell expansion [29]. Interestingly, p-STAT3 was detected in PrE sorted cells at late blastocyst stage [29] suggesting that JAK/STAT signaling could be involved in PrE cell survival at this stage. We hypothesized that exogenous activation of LIF signaling might rescue PrE defects resulting from loss of PDGF signaling. We first observed that 8h LIF treatment promotes an upregulation of p-STAT3 in wild-type ICM cells at E3.75 (Fig. S6A), indicating that incubation with exogenous LIF triggers hyperactivation of STAT signaling in ICM cell lineages. Then, we cultured E3.5 embryos

from *Pdgfra*$^{+/H2B\text{-}GFP}$ intercrosses in presence of LIF and analyzed the number of Epi and PrE cells 24h later (Fig. 5A). We observed that the specific reduction of PrE cell number in *Pdgfra* mutant embryos (by 4.9-fold compared to wild-type) was not rescued when mutant embryos were cultured in presence of LIF (by 2.6-fold compared to LIF treated-wild type embryos). In addition, LIF was not sufficient to rescue the PrE defects consecutive to the pharmacological inhibition of PDGF signaling by Gleevec (Fig. S6B). Thus, modulating LIF activity does not rescue PDGFRα function. We next cultured E3.5 embryos for 24h in presence of a JAK inhibitor at concentrations ranging from 0.5 to 5μM for which p-STAT3 signals were decreased in treated-ES cells (Fig. 5B, Fig. S1). We did not observe any impact on Epi and PrE cell numbers. Altogether, our data indicate that LIF/JAK/STAT signaling has no major role in the survival of ICM cell lineages by the time of Epi and PrE sorting.

PDGF signaling is able to regulate STAT3 activity through JAK kinases [43]. To test a possible functional cooperation between JAK/STAT and mTOR, we therefore analyzed the consequences of combined inhibition of mTORC1 and JAK/STAT. Interestingly, we observed a significant 1.5-fold reduction of PrE cell number when both inhibitors were used while Epi cell number was not affected (Fig. 5C). Based on these observations, we propose that JAK/STAT and mTORC1 signaling pathways cooperate specifically in PrE to promote cell survival. Lastly, we also combined both JAK/STAT and mTORC1/2 inhibition and did not observe any enhanced effect compared to the effect of mTORC1/2 inhibition alone on both Epi and PrE cell survival (Fig. 5D).

DISCUSSION

Distinct functions of RTK signaling during early mammalian embryogenesis

Previous studies using both *Pdgfra* genetic invalidation and pharmacological inhibition using Gleevec which inhibits tyrosine kinase activity of several proteins including PDGFR and KIT have demonstrated that PDGF signaling is critical for PrE cell survival. However, since KIT is detected in the early embryo [23] and in ES and XEN cells [44], we thought to ascertain possible functional redundancy between these RTKs. Our results provide strong evidence that KIT signaling does not play major role in the establishment of early embryonic lineages and that FGFR2, KIT and PDGFRα do not exert redundant functions. Our data are in agreement with our previous finding of an absence of strong genetic interactions between *Fgf4* and *Pdgfra* [20]. However, a recent study provided contrasting observations showing modest but not significant reduction of PrE compartment in *Pdgfra*-deficient embryos, which was exacerbated in the absence of *Fgfr2* [45]. A possible explanation for this discrepancy is the use of different experimental settings between the two studies including: the genetic background (129Sv4 or mixed C57BL6/129Sv4 *versus* mixed 129Sv/C57BL6/CD1 genetic background), the origin of the embryos (superovulation *versus* natural mating), the embryonic stage with eventually *ex vivo* culture (E3.5 cultured for 48h versus E4.5).

We thus reinforce the idea that ICM cell lineage diversification relies on the dynamic sequential signaling pathways which involves at least two major RTKs : FGF (specification) and PDGF (survival/expansion).

p53 is not involved in early embryonic lineage decisions

PDGF signaling exerts its activity through the regulation of various signaling transducers. During skeletal development, PDGF signaling regulates cell survival through p53. This regulation does not seem to be involved in the PrE cell lineage as exemplified by our demonstration that loss of *p53* does not rescue the defects resulting from *Pdgfra* inactivation. Several reports have proposed that p53 acts as an important regulator of early embryonic cell fate during early embryonic development. Nuclear p53 is detected in E3.5 [38] and *ex vivo* stress culture conditions promotes cell death within the blastocyst through regulation of p53 activity [46]. In addition, p53 regulates *Nanog* expression in mESC and its induction leads to differentiation through direct repression and activation of pluripotency- and differentiation-associated genes respectively [39, 47]. However, our data show that p53 does not play a major role during *in vivo* early mammalian embryogenesis.

A major role of PI3K and mTOR in ICM cell survival

In this study, we identified a major role of PI3K-mTOR signaling in the survival of both PrE and Epi cell lineages. Consistent with our data, inactivation of either *Pik3cb,* coding for p110β catalytic subunit of PI3K [48] or *mTor* [49, 50] leads to an embryonic lethality around the time of implantation. Interestingly, we showed that inhibition of both mTOR complexes, mTORC1 and mTORC2, phenocopied the effect of PI3K inhibition but not the inhibition of mTORC1 alone. This suggests that either mTORC2 is the major effector of PI3K in ICM cell lineages or mTORC1 and mTORC2 can compensate for each other. However, gene inactivation studies have suggested that mTORC1 but not

mTORC2 is required during early embryogenesis. Indeed, genetic loss of RAPTOR (mTORC1 specific protein) leads to early post-implantation lethality, while RICTOR (mTORC2 specific protein) leads to embryonic lethality at later stages, around E10.5 [51]. In contrast, pharmacological inhibition of both mTOR complexes but not of mTORC1 alone induced a paused pluripotent state in mouse preimplantation embryos [52]. In this context, phenotypical analysis of compound embryos should give important information about the respective contribution of mTORC1 and mTORC2 in ICM cell lineages.

We therefore propose a model whereby the role of PDGF signaling in PrE cell survival is mediated through PI3K anb both mTOR complexes (Fig. 6). Our data confirm and extend the recent finding by Molotkov et al., using a mutant allele of *Pdgfra* coding for a receptor unable to bind PI3K, suggesting that PDGF signaling is acting through PI3K in PrE cells [45]. In this study, we also unravel a key role of PI3K-mTOR signaling in Epi cell survival during sorting consistent with the fact that PI3K signaling has been shown to regulate *in vitro* the self-renewal and cell proliferation of ES cells [40]. Supplementation with IGF-1 or Insulin, which signal through PI3K, has been shown to improve the development of cultured preimplantation embryos leading to increased blastocyst formation rate and ICM cell number [53, 54]. However, in previous studies, treatments were performed for prolonged duration and when and how IGF-1 and Insulin signaling were acting has not been determined. Recently, a critical role for IGF-1 receptor has been demonstrated for the survival of trophectoderm cells through its interaction with E-cadherin [55]. Today, it is thus still unclear how PI3K activity is regulated *in vivo* within the epiblast.

Dissecting downstream effectors of cellular signaling is hampered by two major issues. First, they are regulated by various upstream inputs, including RTKs, which make difficult to ascertain the role of specific signal inputs. Second, several crosstalks might operate between distinct regulatory networks. Here, we focused on the inter-relationship between JAK/STAT and PI3K/mTOR [56]. Indeed, phosphorylated STAT3 is enriched in the PrE and regulation of JAK/STAT activity through LIF signaling stimulates PrE expansion [29]. Our data suggest that JAK/STAT and mTORC1 cooperate within the PrE but regulating LIF/JAK/STAT is not sufficient to rescue the PrE defect in *Pdgfra* mutant embryos. Taken together, we propose that PrE cell survival is regulated by several inputs including LIF and PDGF signaling pathway that control the activity of distinct but connected regulatory networks including JAK/STAT and PI3K/mTOR (Fig. 6).

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COMPETING INTERESTS

The authors declare no competing interest.

AUTHORS CONTRIBUTIONS

S.B., J.A., Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; S.V.-P., S.C., Acquisition of data; M.C.-T., Conception and design, Analysis and interpretation of data, Drafting or revising the article.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE LEGENDS

Figure 1: Impact of *Pdgfra* and *Kit*-combined deletion on blastocyst composition. NANOG (grey), GATA4 (red) and GFP (green) immunolocalization in E4.5 embryos showed no impact of *Kit* heterozygous (**A**) and homozygous deletion (**B**) in *Pdgfra* mutant embryos. Pictures correspond to a projection of 5 confocal optical slices. Scale bars: 20 μm. Error bars indicate SEM. n, number of embryos analyzed. Genotypes are indicated as (A): WT ($Pdgfra^{+/+}$, $Kit^{+/+}$), HET ($Pdgfra^{+/H2B\text{-}GFP}$, $Kit^{+/+}$ or $Pdgfra^{+/H2B\text{-}GFP}$, $Kit^{+/wLacZ}$), HOM ($Pdgfra^{H2B\text{-}GFP/H2B\text{-}GFP}$, $Kit^{+/wLacZ}$) and (B): WT ($Pdgfra^{+/+}$, $Kit^{+/+}$), HET ($Pdgfra^{+/H2B\text{-}GFP}$, $Kit^{+/wLacZ}$), HOM ($Pdgfra^{H2B\text{-}GFP/H2B\text{-}GFP}$, $Kit^{wLacZ/wLacZ}$). Statistical Mann–Whitney tests are indicated when significant (*, $p < 0.05$; **, $p < 0.01$).

Figure 2: Impact of *Pdgfra*, *Kit* and *Fgfr2*-combined deletion on blastocyst composition. NANOG (grey), GATA6 (red) and GFP (green) immunolocalization in E4.5 embryos showed no impact of *Kit* and *Fgfr2* deletion on *Pdgfra* phenotype. Pictures correspond to a projection of 5 confocal optical slices. Scale bar: 20 μm. Error bars indicate SEM. n, number of embryos analyzed. *Fgfr2* genotypes are indicated as: WT ($Fgfr2^{+/+}$), HET ($Fgfr2^{+/KO}$), HOM ($Fgfr2^{KO/KO}$) and *Pdgfra;Kit* genotypes as: WT ($Pdgfra^{+/+}$, $Kit^{+/+}$), HET ($Pdgfra^{+/H2B\text{-}GFP}$, $Kit^{+/wLacZ}$), HOM ($Pdgfra^{H2B\text{-}GFP/H2B\text{-}GFP}$, $Kit^{wLacZ/wLacZ}$). Statistical Mann–Whitney tests are indicated when significant (*, $p < 0.05$; **, $p < 0.01$).

Figure 3: *Trp53* deletion does not rescue *Pdgfra* mutant phenotype. NANOG (grey),

GATA4 (red) and GFP (green) immunolocalization in E4.5 embryos showed no impact of *Trp53* deletion on the number of PrE cells in *Pdgfra*-deficient embryos. Pictures correspond to a projection of 5 confocal optical slices. Scale bar: 20 μm. Error bars indicate SEM. n, number of embryos analyzed. *Pdgfra;Kit* genotypes are indicated as: HET ($Pdgfra^{+/H2B\text{-}GFP}$, $Kit^{+/wLacZ}$), HOM ($Pdgfra^{H2B\text{-}GFP/H2B\text{-}GFP}$, $Kit^{wLacZ/wLacZ}$) and *Trp53* genotypes as: HET ($Trp53^{+/-}$), HOM ($Trp53^{-/-}$). Statistical Mann–Whitney tests are indicated when significant (*, $p < 0.05$; **, $p < 0.01$).

Figure 4: Role of TOR signaling on ICM cell survival. Wild-type CD1 embryos were cultured from E3.75 to E4.5 in presence or absence of LY29402 **(A)**, Rapamycin **(B)** or INK128 **(C)** and number of NANOG-positive (grey) and GATA4-positive (red) cells were quantified. Pictures correspond to a projection of 5 confocal optical slices. Scale bars: 20 μm. Error bars indicate SEM. n, number of embryos analyzed. Statistical Mann–Whitney tests are indicated when significant (**, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

Figure 5: Analysis of JAK-STAT and TOR signaling on PrE cell survival. (A) Culture of *Pdgfra*-deficient embryos from E3.75 to E4.5 in presence of LIF. **(B-D)** Wild-type CD1 embryos were cultured from E3.75 to E4.5 in presence or absence of JAKi (B), combined with Rapamycin (C) or INK128 (D). Number of NANOG-positive (grey) and GATA4-positive (red) cells were quantified. Pictures correspond to a projection of 5 confocal optical slices. Scale bars: 20 μm. Error bars indicate SEM. n, number of embryos analyzed. Statistical Mann–Whitney tests are indicated when significant (*, p <

0.05 ; **, $p < 0.01$; ****, $p < 0.0001$).

Figure 6: Model of LIF and JAK-STAT interactions on ICM cell survival in late-stage blastocyst. Cell survival in Epi cells is mediated by PI3K-mTOR signaling. PrE cell survival required both JAK-STAT and PI3K-mTOR signaling which are regulated by PDGF and LIF external signaling inputs.

GRAPHICAL ABSTRACT LEGEND

Survival of Primitive Endoderm (PrE) cells in the blastocyst embryo is mediated by PI3K/mTOR signaling pathway independently from p53. This activity is mainly driven by Platelet-derived Growth Factor receptor (PDGFR) but not KIT or FGFR2 receptors. Lastly, LIF/JAK-STAT signaling cooperates with PDGF to regulate PrE cell survival.

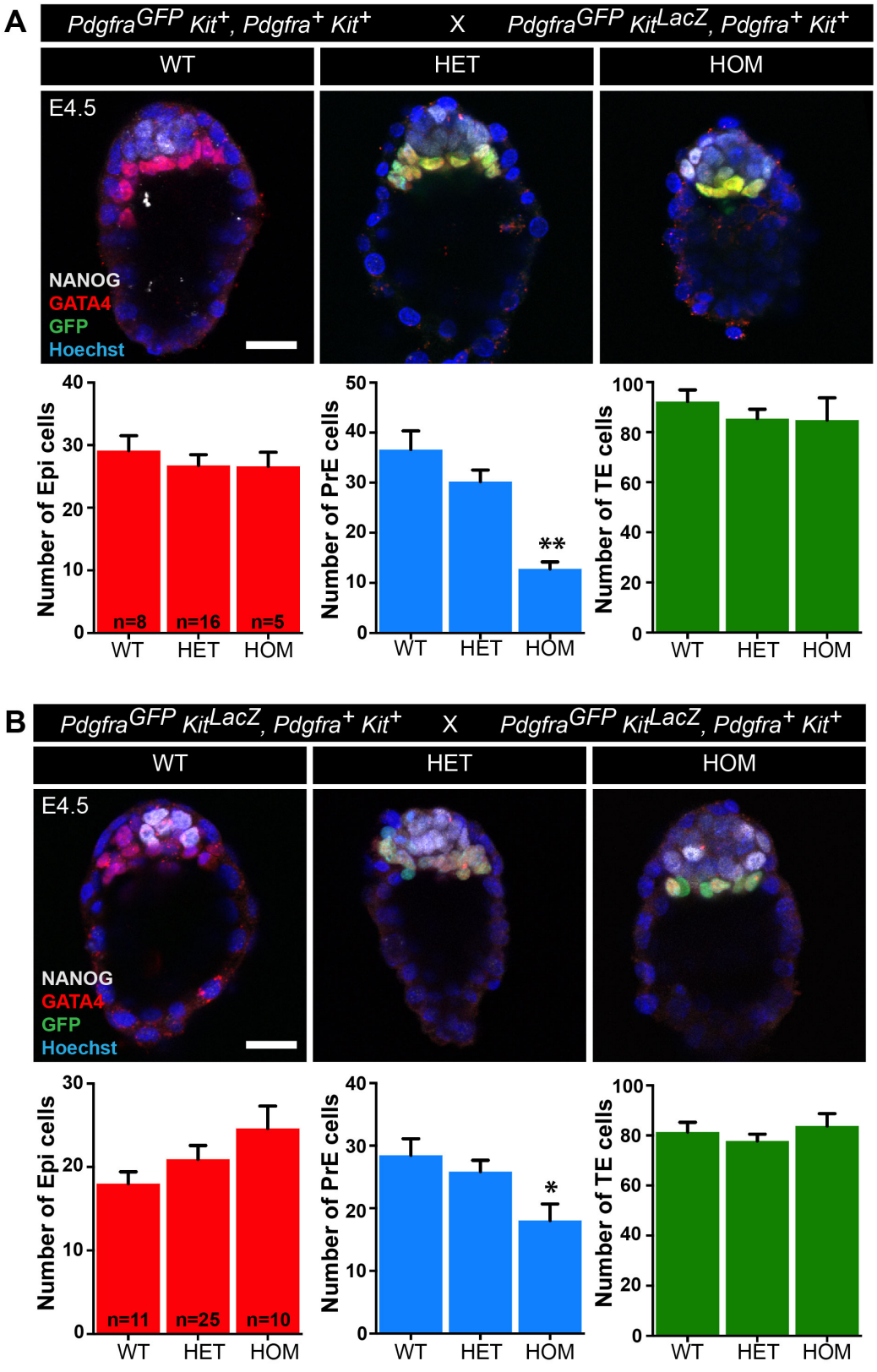
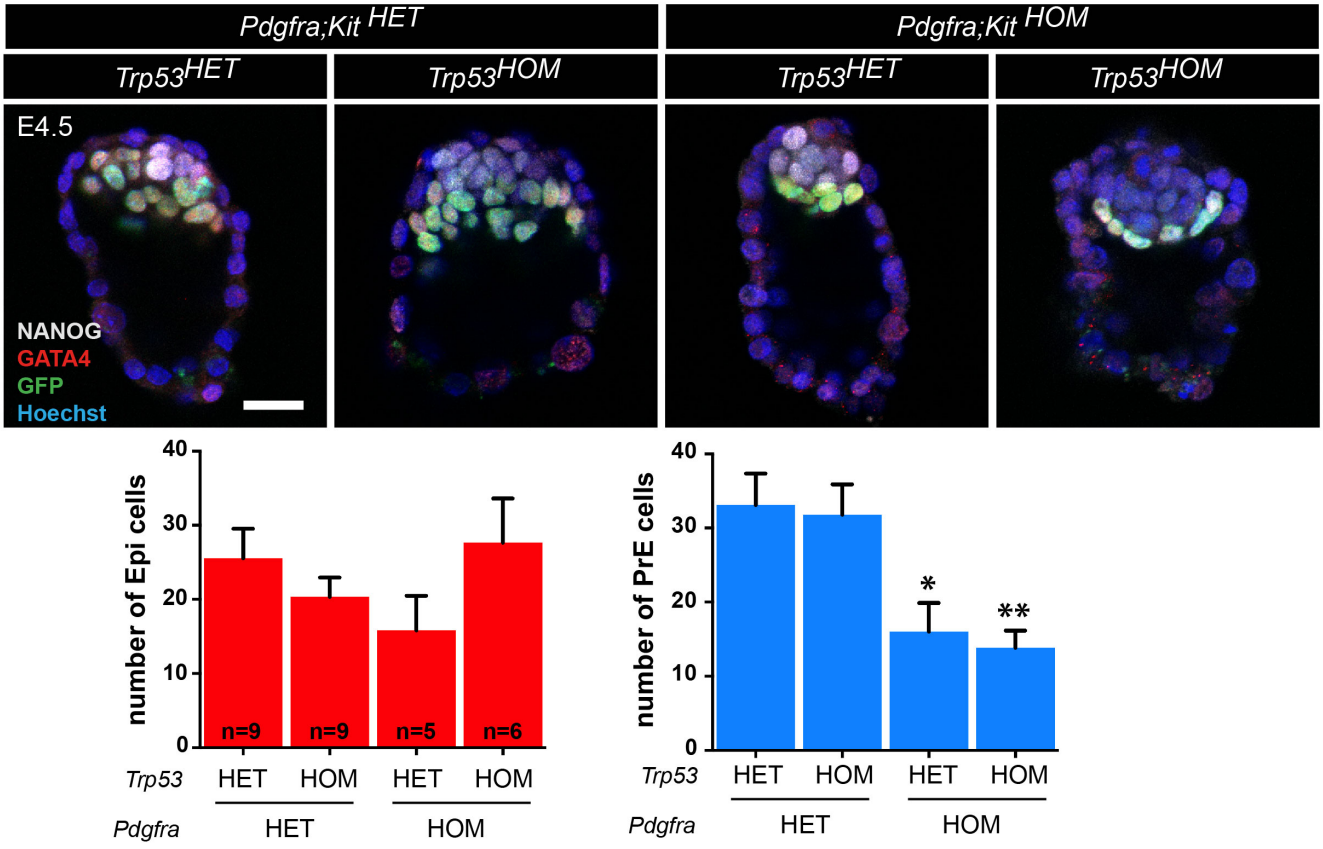


Figure 1. Bessonnard et al.



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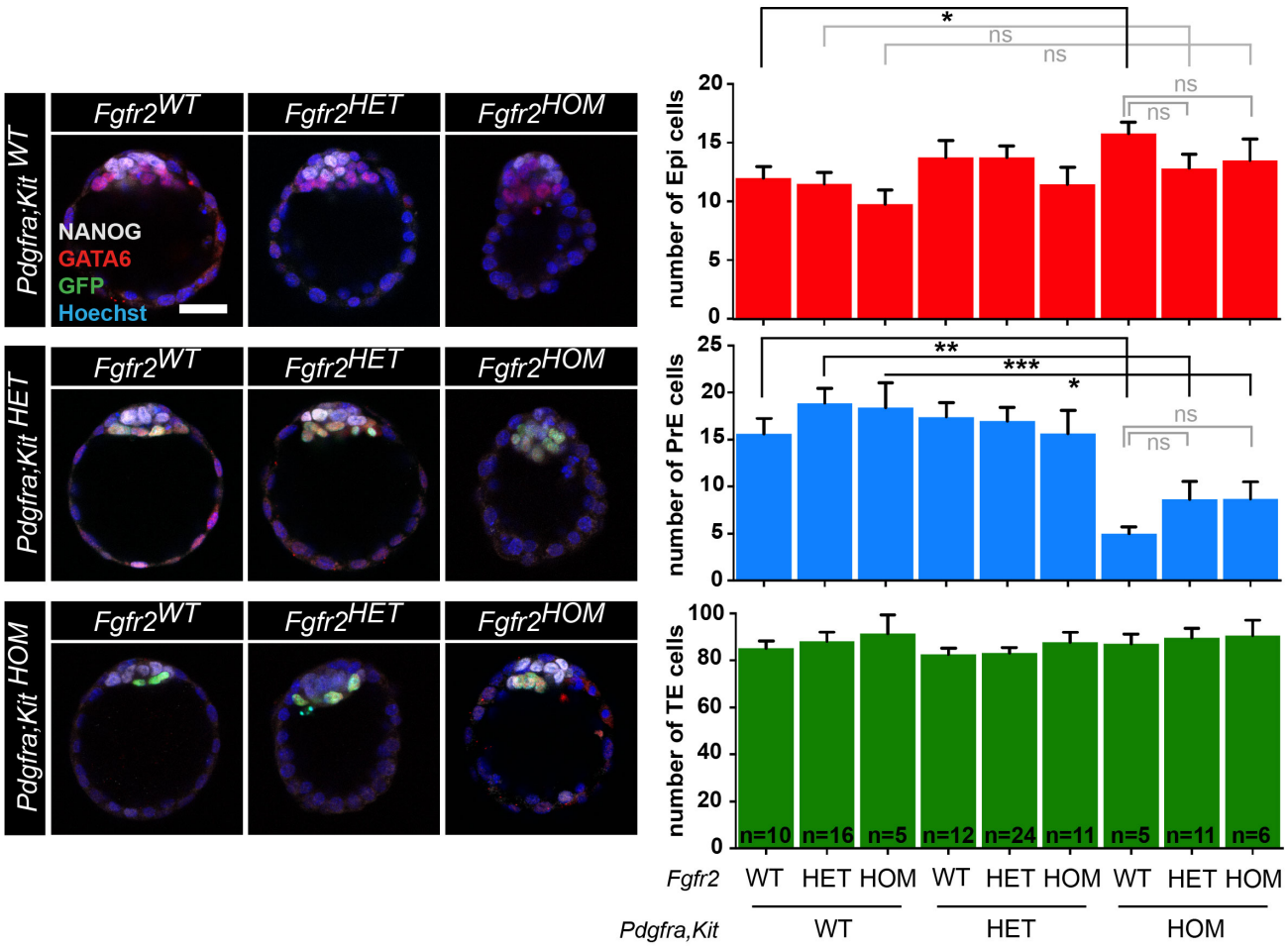

Figure 2. Bessonnard et al.

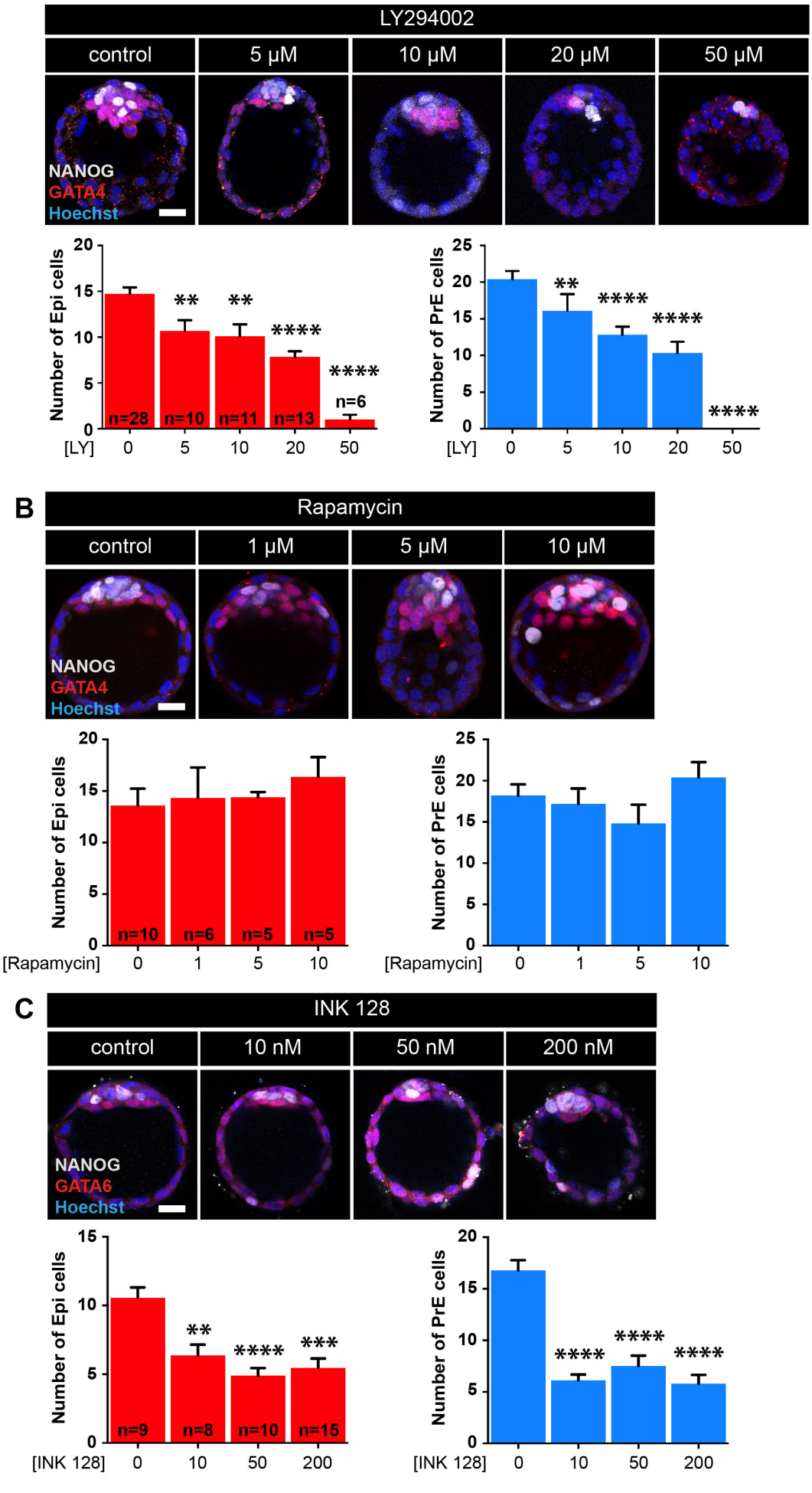

Figure 4. Bessonnard et al.

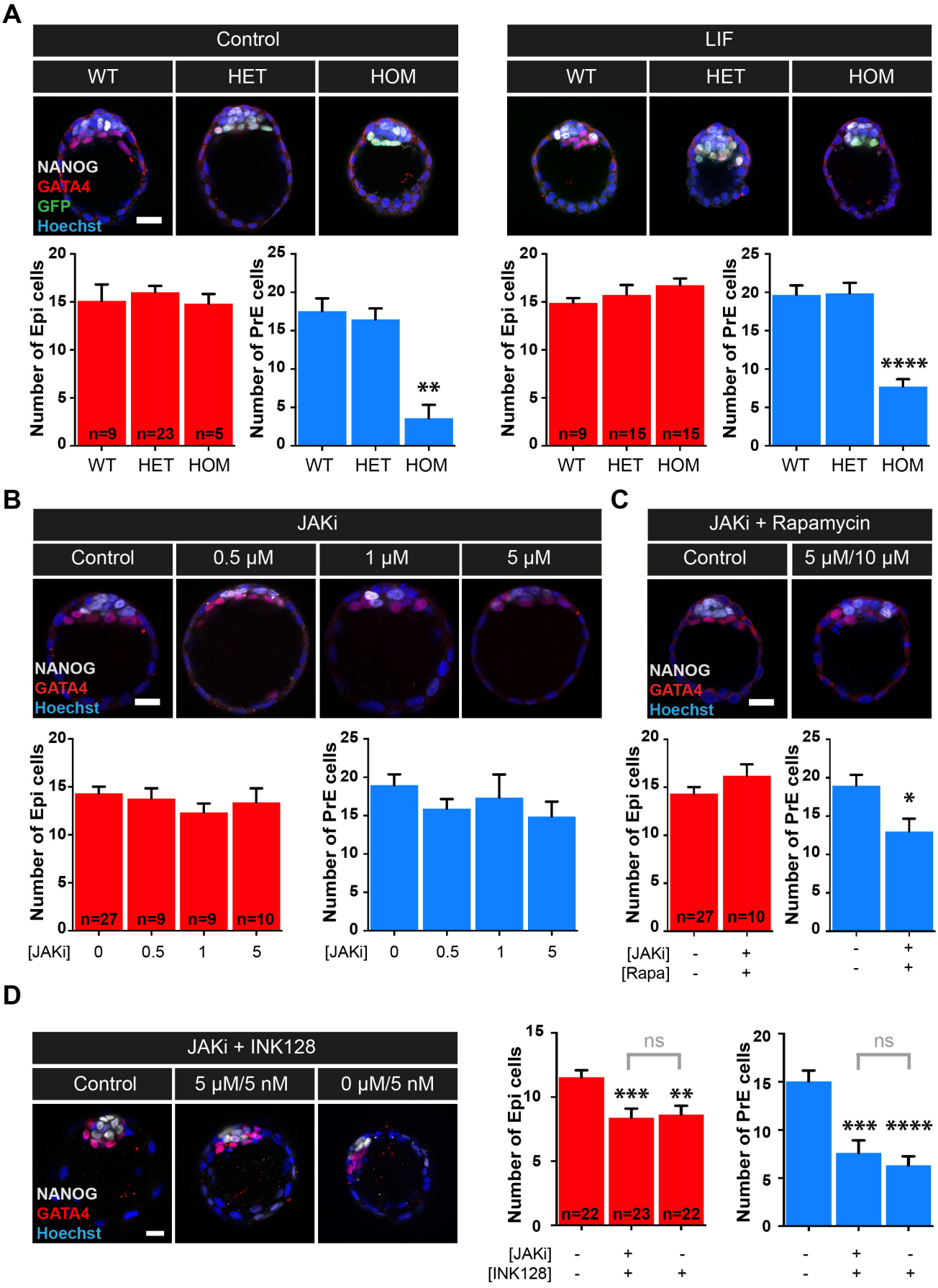

Figure 5. Bessonnard et al.

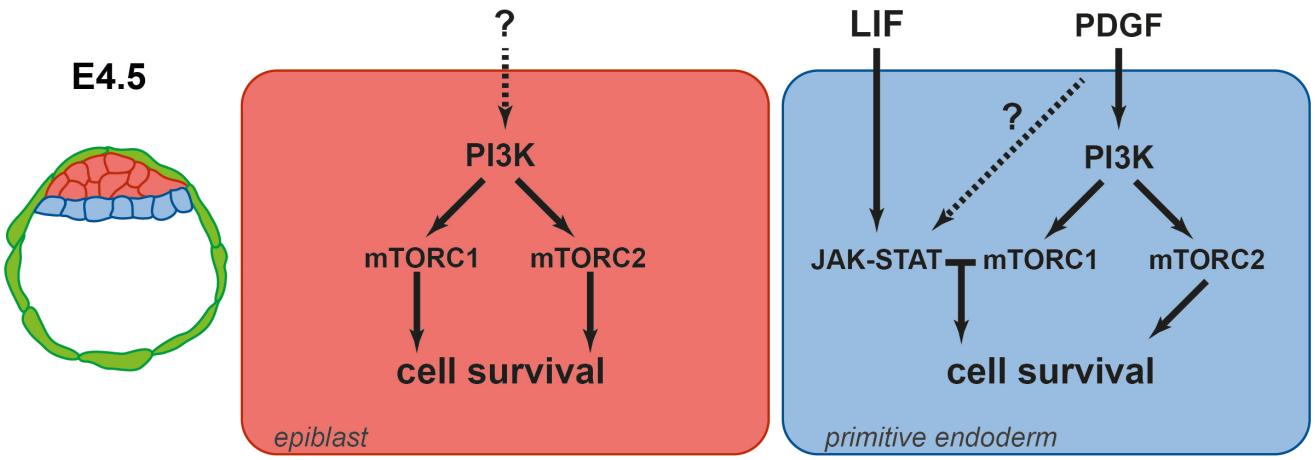

Figure 6. Bessonnard et al.

Supplementary Figures

PDGF signaling in primitive endoderm cell survival is mediated by PI3K-mTOR through p53-independent mechanism

This file contains 6 supplementary figures :

Figure S1: Validation of the pharmacological inhibitors.

Figure S2: Immunodetection of PrE and Epi markers in blastocyst carrying *Pdgfra* and *Kit*-combined deletion.

Figure S3: Differentiation of Kit$^{+/LacZ}$ and Kit$^{LacZ/LacZ}$ ES cells towards extraembryonic endoderm cell lineages.

Figure S4: Analysis of *Fgfr2*-KO embryos on late blastocyst composition.

Figure S5: Analysis of *Trp3* deletion on late blastocyst composition.

Figure S6: Effect of modulating LIF/STAT3 activity.

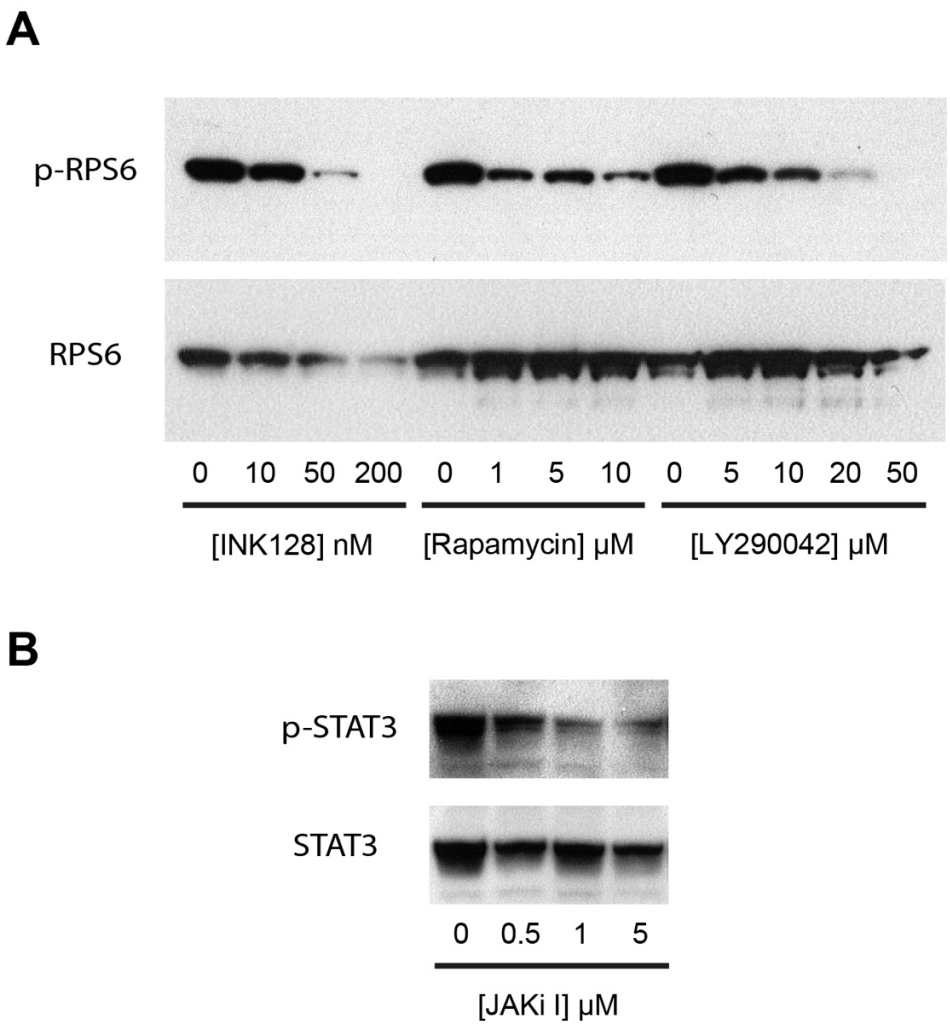

Figure S1: Validation of the pharmacological inhibitors. Immunoblotting for phospho-RPS6 (Ser235/Ser236), total RPS6, phospho-STAT3 (Tyr705) and total STAT3 was performed on protein extracts from ES cells treated for 16 hours with inhibitors at the concentrations indicated. **(A)** Decreased phospho-RPS6 signals upon INK128, Rapamycin and LY294002 treatments. **(B)** Decreased phospho-STAT3 signals upon JAK inhibitor I treatment.

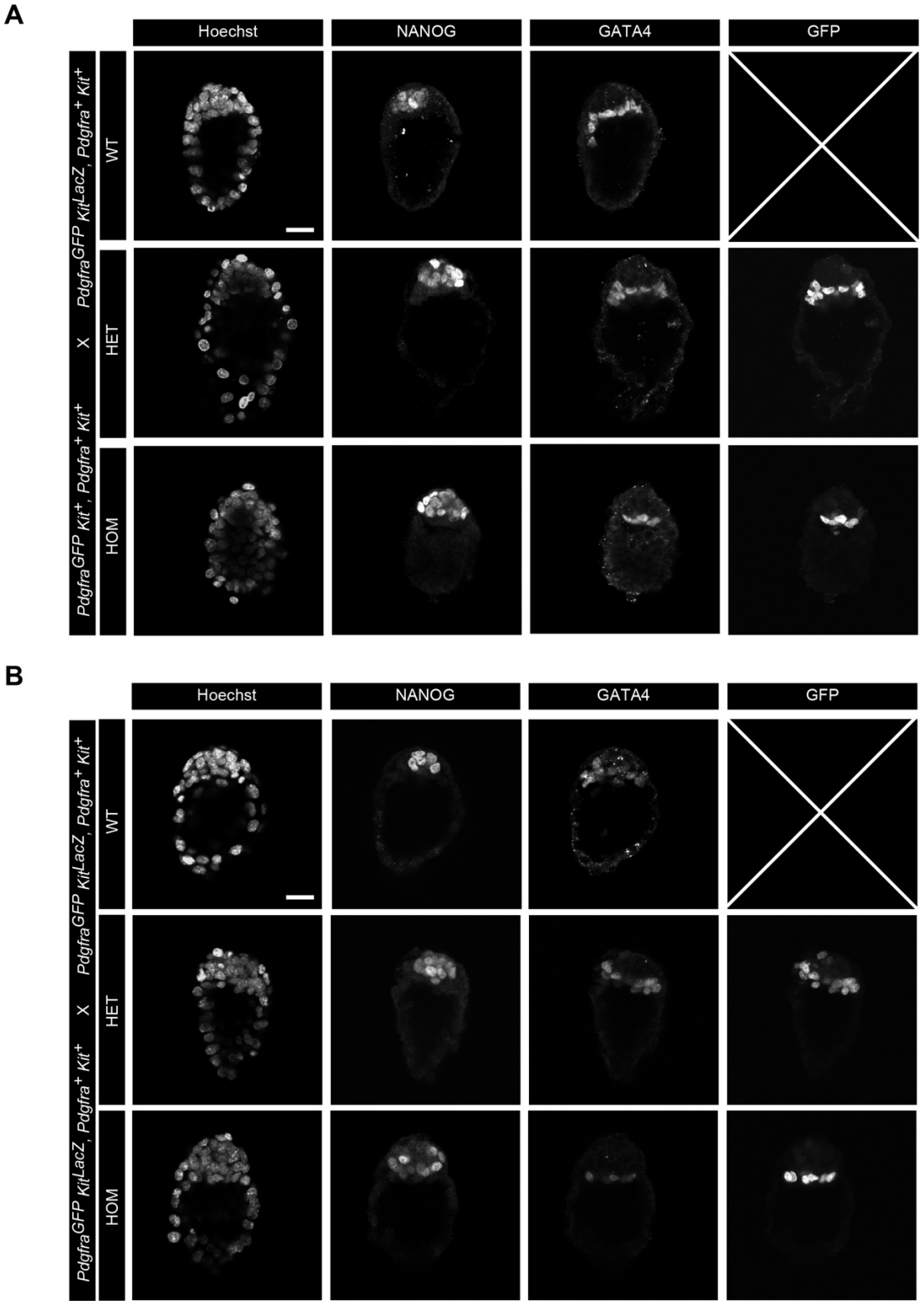

Figure S2: Immunodetection of PrE and Epi markers in blastocyst carrying *Pdgfra* and *Kit*-combined deletion. Hoechst, NANOG, GATA4 and GFP correspond to splitted channels represented in Figure 1. Scale bars: 20 µm. Genotypes are indicated as (A): WT (*Pdgfra*$^{+/+}$, *Kit*$^{+/+}$), HET (*Pdgfra*$^{+/H2B\text{-}GFP}$, *Kit*$^{+/+}$ or *Pdgfra*$^{+/H2B\text{-}GFP}$, *Kit*$^{+/wLacZ}$), HOM (*Pdgfra*$^{H2B\text{-}GFP/H2B\text{-}GFP}$, *Kit*$^{+/wLacZ}$) and (B): WT (*Pdgfra*$^{+/+}$, *Kit*$^{+/+}$), HET (*Pdgfra*$^{+/H2B\text{-}GFP}$, *Kit*$^{+/wLacZ}$), HOM (*Pdgfra*$^{H2B\text{-}GFP/H2B\text{-}GFP}$, *Kit*$^{wLacZ/wLacZ}$).

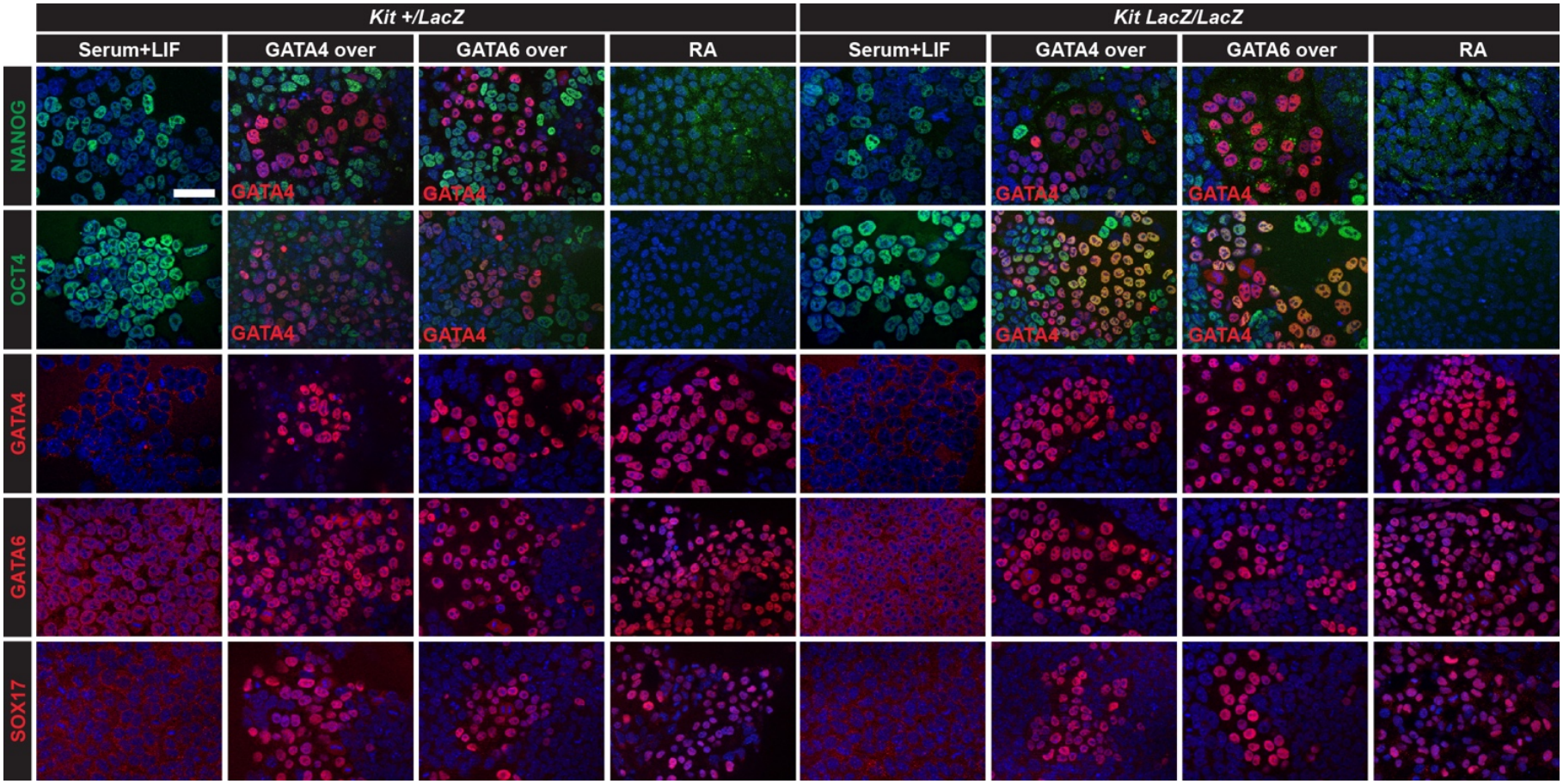

Figure S3: Differentiation of Kit$^{+/LacZ}$ and Kit$^{LacZ/LacZ}$ ES cells towards extraembryonic endoderm cell lineages. In standard Serum+LIF cell culture conditions, heterozygous and mutant ES cells express NANOG and OCT4 Epi markers (green) but no GATA4, GATA6 and SOX17 PrE markers (red). Extraembryonic endoderm cell differentiation was analyzed 48h after GATA4 or GATA6 overexpression or 4 days of 1µM retinoic acid treatment. Scale bar : 50µm.

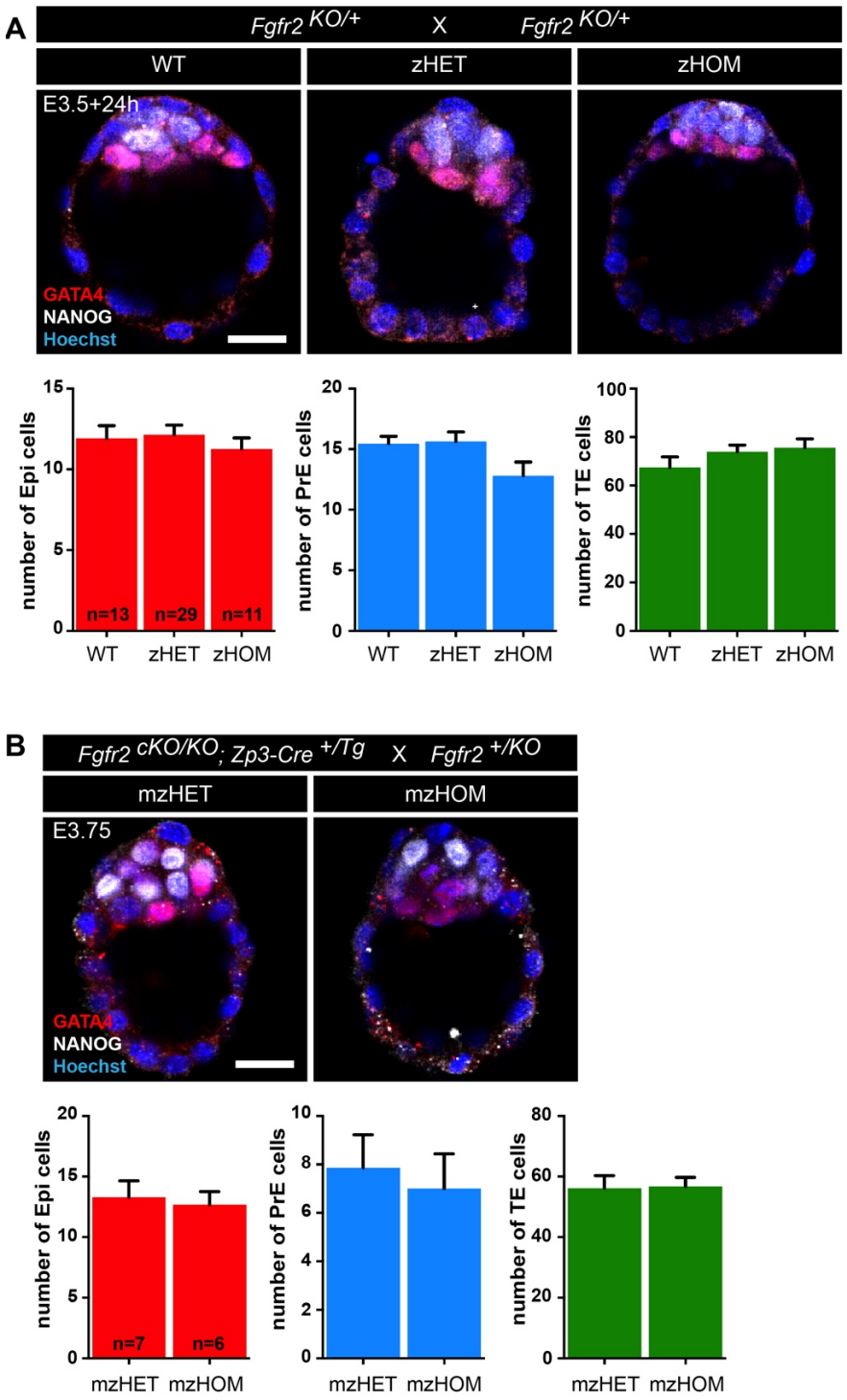

Figure S4: Analysis of *Fgfr2*-KO embryos on late blastocyst composition. E3.25 embryos from zygotic **(A)** and maternal-zygotic **(B)** deletion of *Fgfr2* were cultured 24 hours and immunostained with NANOG (grey) and GATA4 (red). Pictures correspond to a projection of 5 confocal optical slices. Scale bars: 20 µm. Number of NANOG-positive and GATA4-positive cells were quantified. Error bars indicate SEM. n, number of embryos analyzed; z, zygotic; mz, maternal and zygotic. *Fgfr2* genotypes are indicated as: WT ($Fgfr2^{+/+}$), HET ($Fgfr2^{+/KO}$), HOM ($Fgfr2^{KO/KO}$). Statistical Mann–Whitney tests are not significant.

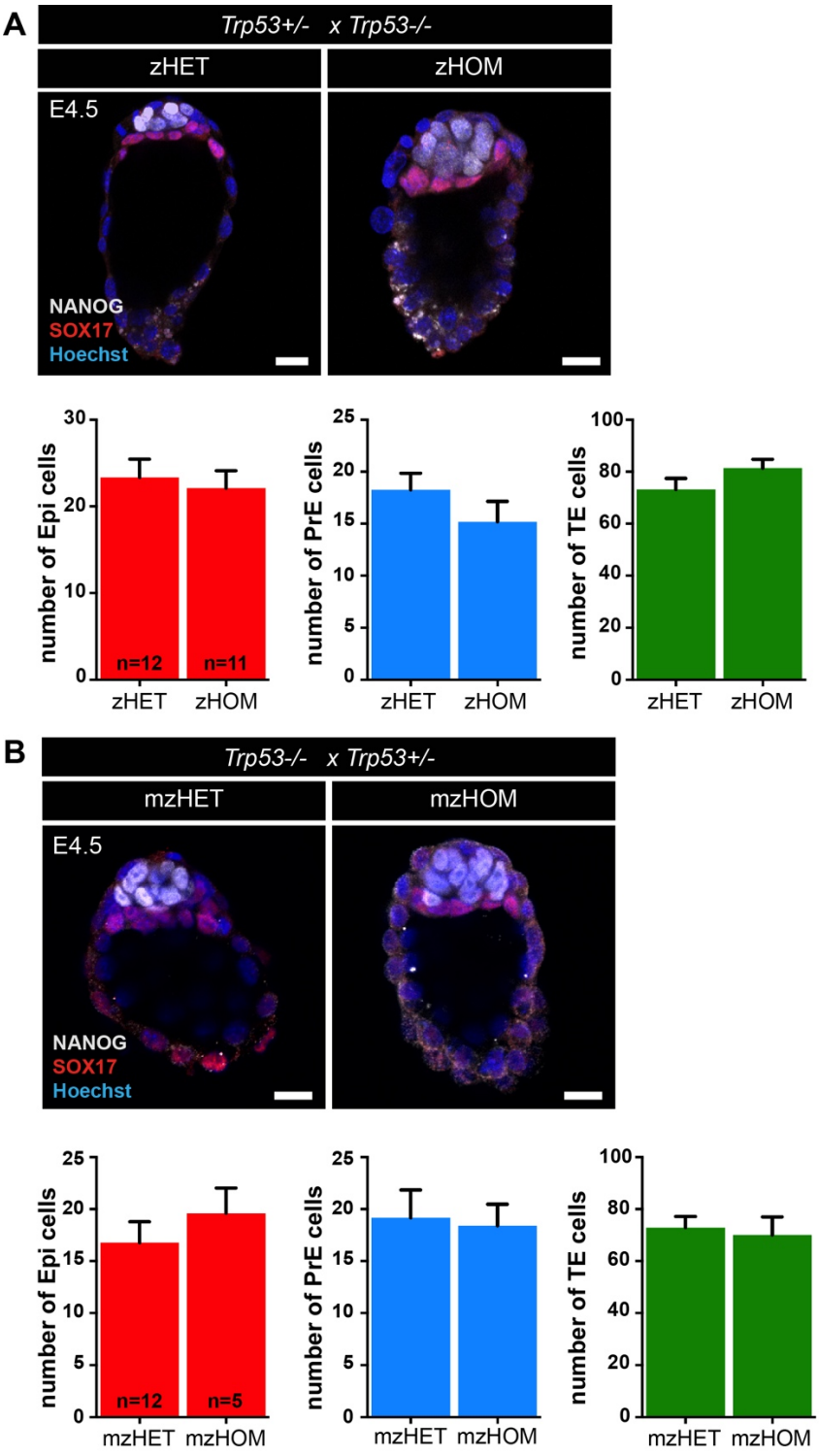

Figure S5: Analysis of *Trp3* deletion on late blastocyst composition. E4.5 embryos from zygotic **(A)** and maternal-zygotic **(B)** deletion were immunostained with NANOG (grey) and SOX17 (red) antibodies. Pictures correspond to a projection of 5 confocal optical slices. Scale bars: 20 µm. Number of NANOG-positive and GATA4-positive cells were quantified. Error bars indicate SEM. n, number of embryos analyzed; z, zygotic; mz, maternal and zygotic. *Trp53* genotypes as: HET (*Trp53*$^{+/-}$), HOM (*Trp53*$^{-/-}$). Statistical Mann–Whitney tests are not significant.

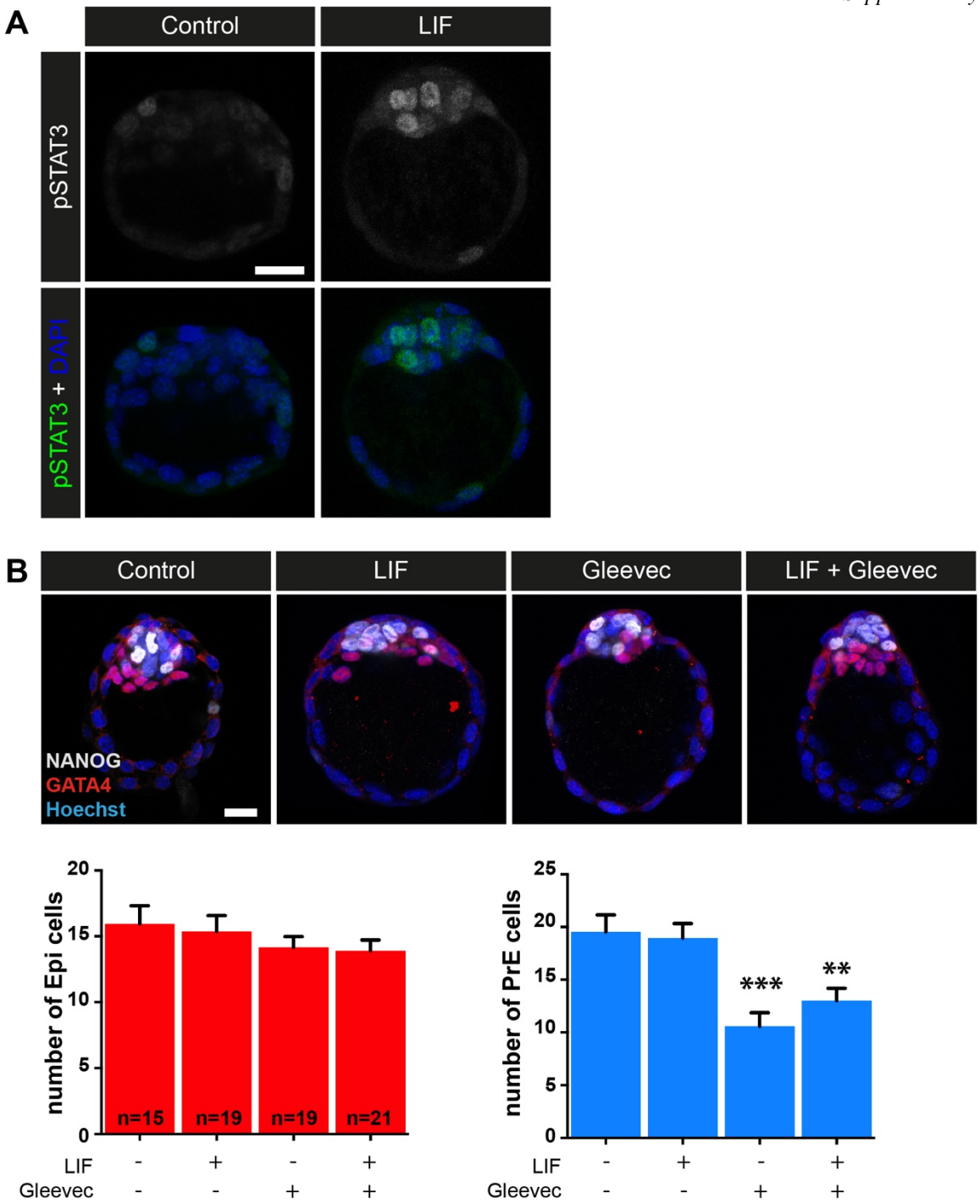

Figure S6: Effect of modulating LIF/STAT3 activity. **(A)** Effect of LIF on STAT3 signaling. Wild-type CD1 E3.25 embryos were cultured in presence or absence of LIF for 8 hours and pSTAT3 was immunostained and enriched in ICM cells of LIF-treated embryos. **(B)** Effect of LIF signaling activation on Gleevec-treated embryos. E3.75 Wild-type CD1 embryos were cultured in presence and/or absence of LIF and Gleevec. Number of NANOG-positive and GATA4-positive cells were quantified. Pictures correspond to a projection of 5 confocal optical slices. Scale bars: 20 µm. Error bars indicate SEM. n, number of embryos analyzed. Statistical Mann–Whitney tests are indicated when significant (**, $p < 0.01$; ***, $p < 0.001$).