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Signaling pathways in mammalian preimplantation development: linking cellular phenotypes to lineage decisions

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Running title: Signaling pathways in the preimplantation embryo

Abstract

The first stages of mammalian development, before implantation of the embryo in the maternal uterus, result in the establishment of three cell populations in the blastocyst: trophectoderm, epiblast, and primitive endoderm. These events involve only a small number of cells, and are initiated by morphological differences among them related to cell adhesion and polarity. Much attention has been paid to the master transcription factors that are critical for establishing and maintaining early lineage choices. Nevertheless, a large body of work also reveals that additional molecular mechanisms are involved. Here, we provide an updated view of the role of different signaling pathways in the first stages of mouse development, and how their cross-talk and interplay determine the initial lineage decisions occurring in the blastocyst. We will also discuss how these pathways are critical for translating cellular phenotypes -the product of the morphogenetic events occurring at these stages- into transcriptional responses and expression of lineage-specifying transcription factors.

From totipotency to the blastocyst.

The onset of cell differentiation in the mammalian embryo entails the loss of totipotency to favor the appearance of the first lineage-committed cell populations. The unicellular zygote undergoes a series of roughly equal and synchronic divisions to generate cells that will then make fate decisions to become specialized. The first three rounds of cell division generate an 8-cell embryo in which each of the cells, termed blastomeres, is morphologically identical and retains full capacity to develop into any tissue or population. At this point, the embryo undergoes a process known as compaction (Fig. 1). An increase in intercellular adhesion is manifested in a smoother outer embryo surface, and cells start to get polarized along their apico-basal axis. During the following two division rounds, cells lose synchronicity and can divide symmetrically or asymmetrically. As a result, two populations are distinguished in the embryo: an outer layer of polarized cells enclosing an inner group of apolar cells (Tarkowski and Wroblewska, 1967; Ziomek and Johnson, 1980).

The relationship between division angles, cell position and the fate of individual blastomeres has been widely studied. Recent reports show that there are substantial relocations of cells within the embryo and that division-angle and the initial blastomere positions cannot consistently predict their fate in a consistent manner (McDole et al., 2011; Anani et al., 2014; Watanabe et al., 2014; Toyooka et al., 2016). Apical constriction driven by cortical tension also seems to contribute to blastomere internalization (Samarage et al., 2015). A recent study shows that asymmetric divisions generate inter-blastomere heterogeneities in contractility. Apolar cells have a higher amplitude of contraction than polar cells, and this drives apolar cells to internalize (Maitre et al., 2016).

At the ~32-cell stage, a fluid-filled cavity known as the blastocoel begins to form within the embryo. The blastocoel is maintained by a seal formed by tight junctions established between outer cells. This process is called cavitation and implies a physical constraint on the embryo. As the blastocoel expands, blastomeres deform and inner cells are driven

to one end of the embryo, forming the blastocyst (Fig. 1). At this stage, which occurs 3.5 days post coitum (dpc) in the mouse, the two first distinct cellular populations can be clearly distinguished. The outer epithelial layer forms the trophectoderm (TE), which will give rise to extraembryonic structures, mainly the placenta. The inner cell mass (ICM) sits beneath the TE at one point of the cavity and undergoes the second lineage choice a day later. The cells forming the ICM are sorted into two populations: an extraembryonic monolayer lying in contact with the blastocoel, known as primitive endoderm (PrE) or hypoblast; and the epiblast (EPI), which remains enclosed between the TE and the PrE, and gives rise to the embryo proper (Fig. 1). The TE differentiates into polar (cells lying on top of the ICM) and mural (cells that are not in contact with the ICM). The polar TE maintains its proliferative capacity while cells of the mural TE exit the cell cycle and differentiate into trophoblast giant cells that help to invade the endometrium (Underhill and Robins, 2016). At this point, the embryo is ready to implant in the uterus.

Underlying the emergence of these populations is a transcriptional circuitry orchestrating the fate of every cell. Transcription factors (TFs) are the key elements that trigger specific programs to promote one lineage or another. In the last decade, the core set of transcription factors involved in the first lineage choices in the mammalian embryo has been identified. *Cdx2* is the main component in the regulatory network determining the TE lineage (Strumpf et al., 2005). Other transcription factors linked to the TE are *Gata3* (Home et al., 2009; Ralston et al., 2010) and *Eomes* (Russ et al., 2000). The key elements of ICM establishment are *Oct4* (official gene symbol *Pou5f1)* (Nichols et al., 1998), *Sox2* (Avilion et al., 2003), and *Nanog* (Chambers et al., 2003; Mitsui et al., 2003). The expression of most of these TFs is initially ubiquitous or stochastic, and it is only at the blastocyst stage that they become restricted to their definitive domains and the two first lineages are properly established (Dietrich and Hiiragi, 2007). CDX2 is first detected upon compaction in a salt-and-pepper fashion and is later restricted to the TE. CDX2 is the first core element that delimits its spatial expression domain. OCT4 is ubiquitously

expressed in the early morula, and restriction to the ICM does not occur until the late blastocyst stage. SOX2 is the first marker of ICM fate, showing enriched expression in inner cells from the 16-cell stage (Guo et al., 2010; Wicklow et al., 2014). NANOG also exhibits variable levels of expression at the morula stage. Despite being an ICM marker, NANOG expression is maintained in the TE until the late blastocyst stage, similar to the situation with OCT4 (Guo et al., 2010; Xenopoulos et al., 2015). Remarkably, there is no initial correlation between the mosaic and variable expression of CDX2 and that of OCT4 or NANOG (Dietrich and Hiiragi, 2007), although reciprocal inhibition between CDX2 and OCT4 has been proposed to segregate the TE and ICM lineages (Niwa et al., 2005).

NANOG is not only a marker of the ICM. Its heterogeneous expression within the ICM and its complementary expression with that of another transcription factor, GATA6, is a key to determine the segregation of the EPI and PrE. NANOG and GATA6 are the earliest markers that define the EPI and PrE lineages respectively (Koutsourakis et al., 1999; Chambers et al., 2003; Mitsui et al., 2003; Chazaud et al., 2006). However, both genes are expressed in the same cells from the early morula stage, and they become mutually exclusive only later, at the mid-blastocyst stage (Chazaud et al., 2006; Plusa et al., 2008). Cells expressing either factor are initially dispersed in the ICM in a salt-and-pepper manner, and blastomeres are subsequently sorted to define the two populations.

The heterogeneous and variable transcription factor expression levels in the preimplantation mouse embryo reveal that no single factor *per se* is predictive of lineage segregation. This insight has forced researchers to probe deeper to identify the first differences arising among blastomeres related to lineage TFs. A recent study suggests that OCT4 protein kinetics, rather than transcription or protein levels, differs among blastomeres before compaction, and this behavior correlates with the adoption of an inner or outer position at later stages (Plachta et al., 2011). In addition, inter-blastomere heterogeneities have been found as early the 4-cell stage. SOX2-DNA interaction dynamics varies between blastomeres and creates a bias in cell fate (White et al., 2016).

SOX21, a target of *Oct4* and *Sox2*, is heterogeneously expressed in the 4-cell embryo and its levels could initiate cell-fate decisions (Goolam et al., 2016). However, evidence gathered so far suggests that restricted expression patterns of core transcription factors are not the initial drivers of lineage decisions, but rather that TFs are in charge of maintaining and executing lineage-specific programs.

Signaling pathways driving the first lineage choices.

The mouse embryo undergoes major morphological changes during the preimplantation phase, including compaction, polarization, and cavitation. The molecular links between these processes and cell fate is crucial to understanding the behavior and commitment of the first populations. Although the main TFs of the regulatory networks involved in lineage establishment have been identified, it is not fully understood how these elements become spatially restricted. The study of the signaling pathways potentially involved in cell-to-cell communication and that might act upstream of these markers is essential to getting a clearer picture of the molecular mechanisms operating at these early stages.

In recent years, many studies have analyzed the role of different pathways in preimplantation development. Here, we review the main pathways that have been shown to be involved in determining the first lineage decisions (Fig. 2).

The Hippo pathway

The Hippo pathway is the best characterized signaling cascade involved in the first lineage choice. It was first identified in *Drosophila* as a pathway implicated in tissue growth control, and its core consists of a kinase cascade targeting the expression of genes involved in cell proliferation and survival (Pan, 2010; Meng et al., 2016; Sun and Irvine, 2016). Mammals have an analogous and more complex network that includes the main components discovered in *Drosophila*.

During preimplantation development, a novel role has been assigned for this pathway based on the observation that perturbations in some of its components do not result in changes in cell number. The Hippo pathway has been shown to play a key role in determining the establishment of the TE and the ICM in the mouse (Fig. 2A, B). The first evidence for the importance of this pathway was the phenotype of embryos lacking the transcription factor TEAD4. *Tead4*$^{-/-}$ embryos fail to cavitate properly, do not form a mature TE, and are unable to implant (Yagi et al., 2007; Nishioka et al., 2008). More importantly, TEAD4 has been shown by genetic means to regulate TE lineage factors such as *Cdx2* (Yagi et al., 2007; Nishioka et al., 2008) and *Gata3* (Ralston et al., 2010). *Tead4*$^{-/-}$ morulae present a reduced expression of CDX2, and this effect is exacerbated at the blastocyst stage, when CDX2 is absent in most of the embryos (Nishioka et al., 2008; Rayon et al., 2014).

However, TEAD4 alone cannot be responsible for triggering *Cdx2* expression because, in contrast to *Cdx2*, *Tead4* is expressed in the nucleus of every cell in preimplantation embryos (Nishioka et al., 2008). *Cdx2* expression is instead determined by the differential intracellular location of the transcriptional coactivator YAP (Yes Associated Protein 1, and almost certainly the Yap-related protein TAZ) in inner and outer cells (Nishioka et al., 2009). This differential location depends on the activation of several Hippo pathway components that end up in phosphorylation of YAP, what maintains it in the cell cytoplasm (Fig. 2B). When the pathway is off, YAP is not phosphorylated and thus translocates to the nucleus. The kinase LATS1/2 (Large Tumor Suppressor kinase 1/2) is responsible for YAP phosphorylation and its consequent retention in the cytoplasm of inner cells (Nishioka et al., 2009). The junction-associated protein AMOT (Angiomotin) localizes at adherens junctions of apolar inner cells in the embryo (Hirate et al., 2013; Leung and Zernicka-Goetz, 2013). There, AMOT interacts with another two Hippo components: LATS1/2 and NF2 (Neurofibromin 2). LATS1/2 phosphorylates AMOT and stabilizes this complex, which is essential for the activation of the Hippo

pathway (Hirate et al., 2013). Disruption of *Lats1/2* (Nishioka et al., 2009), *Amot* (and *Amotl2*) (Hirate et al., 2013; Leung and Zernicka-Goetz, 2013), or *Nf2* (Cockburn et al., 2013) leads to ectopic nuclear YAP accumulation and CDX2 expression in inner cells. In polar outer cells, AMOT is sequestered at the apical domain, which impairs its activation and the activation of the Hippo pathway. YAP is thus translocated into the nucleus where it binds to the transcription factor TEAD4, leading to the expression of their target genes, such as *Cdx2* (Hirate et al., 2013; Leung and Zernicka-Goetz, 2013).

The role of Hippo in the first lineage decision seems not to be restricted to TE-related genes. It has been shown that Hippo is also involved in restricting *Sox2* to the inner cells, although it is not known if this effect is direct or indirect (Wicklow et al., 2014). Ectopic SOX2 is detected in *Tead4*$^{-/-}$ embryos and in 2-cell embryos injected with *Lats2* mRNA, and hence YAP is retained in the cytoplasm of every cell.

Despite the consistent evidence for the role of the Hippo pathway in establishing cell fate, a recent study suggests that it is dispensable under low oxygen conditions. The study shows that TEAD4 controls energy homeostasis by preventing accumulation of reactive oxygen species (Kaneko and Depamphilis, 2013). The phenotype of *Tead4*$^{-/-}$ embryos can be bypassed if those embryos are cultured in low oxygen (5%) conditions. Therefore, under these culture conditions, the Hippo pathway would not be required for the first lineage choice between the TE and ICM.

Although Hippo has not been directly linked to the segregation of the EPI and PrE, a recent report proposes that clonal downregulation of *Tead4* biases cells towards EPI rather than PrE (Mihajlovic et al., 2015). However, no nuclear YAP has been detected within the ICM, precluding a direct role of *Tead4* in promoting PrE fate.

Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) are a family of cell surface receptors that bind several extracellular factors and activate various signaling cascades, including MAPK/ERK and PI3K. RTKs often function as entry points, integrating external inputs and driving intracellular responses. The RTKs include several important subfamilies of receptors such as FGFRs, EGFRs, VEGFRs, and PDGFRs, and are implicated in a broad range of cellular responses including embryonic development, angiogenesis, oncogenesis, and metabolism (McKay and Morrison, 2007; Li et al., 2016; Sewduth and Santoro, 2016).

RTK signaling has been linked to the two first differentiation events that take place in the mammalian embryo: the TE/ICM distinction and the later segregation of the ICM to EPI and PrE (Fig. 2A).

ERK2, the effector of Ras-MAPK signaling, is detected at the apical surface of blastomeres at the 8-cell stage, whereas at later stages it is homogeneously distributed throughout all cells. Inhibition of MAPK at the 8-cell stage significantly decreases *Cdx2* expression (Lu et al., 2008). Induction of *Ras* in mouse embryonic stem (ES) cells cultured under conditions for trophoblast stem (TS) cells (with Fgf4 and heparin) produces TS-like cells. When injected into morulae to form chimeras, these TS-like cells are excluded from the embryo proper and only contribute to the TE and at later stages to the placenta (Lu et al., 2008). Thus, Ras-MAPK signaling may act in the early embryo to promote TE fate.

Temporal control of this pathway is also required for the segregation of EPI and PrE within the ICM (Fig. 2A, C). Embryos lacking GRB2, an RTK-associated adaptor protein, fail to differentiate endoderm lineages (Cheng et al., 1998). *Grb2*$^{-/-}$ blastocysts do not express *Gata6*, whereas *Nanog* can be found throughout the ICM rather than the normal salt-and-pepper distribution (Chazaud et al., 2006). Interestingly, the requirement of RTK signaling for *Gata6* expression is not constant during preimplantation stages, shown by the different effects of RTK inhibitors depending on the timing of administration (Nichols

et al., 2009; Frankenberg et al., 2011). If RTK signaling is inhibited from the morula stage, either in the presence or absence of *Nanog*, *Gata6* is not expressed, reproducing the *Grb2*$^{-/-}$ phenotype. However, if treatment begins after cavitation, *Gata6* is only expressed in the absence of *Nanog*. RTK is thus crucial for *Gata6* initiation, whereas its maintenance is RTK-independent and requires *Nanog* downregulation. Complementing these observations, inhibition of RTK signaling leads to NANOG upregulation both in wildtype and in *Gata6* mutant embryos, suggesting a dual role for this pathway, not only promoting PrE fate, but also maintaining *Nanog* levels within a physiological range (Schrode et al., 2014).

Similarly, opposing temporal controls on the EPI and PrE lineages are exerted by members of the Fgf family (Fig. 2A, C). FGF4 is required for PrE lineage restriction, it is not required for the initial triggering of the PrE program (Yamanaka et al., 2010; Kang et al., 2013). Lack of *Fgf4* does not affect *Gata6* expression until the ~64-cell stage, when NANOG is detected in all cells forming the ICM, at the expense of GATA6, PDGFRα, SOX17, and GATA4. Moreover, treating *Gata6*$^{-/-}$ embryos with FGF4 does not rescue their inability to specify PrE (Bessonnard et al., 2014; Schrode et al., 2014). These data suggest that there may be another mechanism responsible for the initial induction of *Gata6* expression and PrE specification. Single-cell transcriptome profiling has provided more details about the emergence of differential gene expression in EPI and PrE cells (Guo et al., 2010; Ohnishi et al., 2014). *Fgf4* and *Fgfr2* are the first genes whose expression correlates inversely among the cells of the ICM. FGF4 is produced by pre-EPI cells, and activates the ERK pathway in pre-PrE cells, presumably through FGFR2. FGF4 leads to the downregulation of *Nanog* and activation of the PrE program. Other Fgf ligands (*Fgf3*, *Fgf13*) and receptors (*Fgfr1, Fgfr4*) are differentially expressed within the ICM (Ohnishi et al., 2014), but their putative role in lineage segregation has not been explored. *Fgf2* and *Fgfr2* are also expressed in the TE (Haffner-Krausz et al., 1999; Kunath et al., 2014; Yang et al., 2015), and they may be implicated in the proper

cavitation of the blastocyst (Yang et al., 2015). Furthermore, the differential expression of *Fgf4* and *Fgfr2* is implicated in the later specification of TE derivatives, as well as in the establishment and maintenance of TS cells (Tanaka et al., 1998; Haffner-Krausz et al., 1999).

PDGFRα has been identified as one of the markers of the PrE lineage in the blastocyst (Plusa et al., 2008). It is first detected in some cells in the morula, and is subsequently restricted to the PrE layer (Fig. 2A, C). Lack of *Pdgfra* does not cause a severe phenotype at preimplantation stages (Soriano, 1997); however, *Pdgfra* mutant blastocysts have significantly fewer PrE cells than wildtype counterparts (Artus et al., 2010), as a result of increased cell death in *Pdgfra* mutant embryos, an effect also seen upon inhibition of PDGF signaling (Artus et al., 2013). However, cell specification and sorting are not affected (Artus et al., 2013). *Pdgfra* expression is regulated by GATA6 (Artus et al., 2010), in line with the idea that PDGF signaling is required not for PrE fate determination but for its maintenance. Four PDGF ligands have been identified in mammals, but no clear role has been assigned to any of them at these stages, although the presence of PDGF-A has been reported (Palmieri et al., 1992).

The Notch pathway

Notch signaling has been widely studied and is known to be involved in multiple cell fate decisions during development (Koch et al., 2013). Nevertheless, its role in preimplantation development was dismissed since loss of function mutants of the Notch pathway develop normally until postimplantation stages (Conlon et al., 1995; Souilhol et al., 2006).

Notch is a cell-to-cell signaling pathway that takes its name from the receptor on the receiving cell. Interaction of the receptor with a ligand of the Delta or Jagged family expressed on the signaling cell leads to a cascade of proteolytic events that result in the release of the Notch intracellular domain (NICD). The NICD translocates into the

nucleus, where it binds the transcription factor RBPJ (Recombination signal Binding Protein for immunoglobulin Kappa J region) and leads to activation of target genes. In the absence of the NICD, RBPJ associates with various corepressors to inhibit gene expression (Wang et al., 2015).

Through the characterization of a regulatory element upstream of *Cdx2*, we recently showed that both Notch and Hippo converge on this element to regulate *Cdx2* expression (Fig. 2A, B) (Rayon et al., 2014). Double mutants for *Tead4* and *Rbpj*, respectively the effectors of the Hippo and Notch pathways, die before 3.5 dpc, thus showing a phenotype more severe than that of *Tead4* mutant embryos. At the blastocyst stage, Notch activity is restricted to the TE, and blockade of the pathway with a γ-secretase inhibitor (which blocks production of NICD) leads to a decrease in *Cdx2* levels. Furthermore, overexpression of the Notch1 ICD (N1ICD) unbalances the proportion of TE/ICM cells (Rayon et al., 2014). Although these results show that Notch induces *Cdx2* expression, how and when the pathway is activated remains unsolved.

Aside from *Notch1*, mammals express three additional receptors (*Notch2-4*), as well as five ligands classified in two families (*Jagged1-2* and *Delta-like1, 3* and *4*). RT-PCR surveys and transcriptomic studies have revealed the expression of multiple Notch pathway components in preimplantation mouse development (Cormier et al., 2004; Wang et al., 2004; Tang et al., 2011; Boroviak et al., 2015; Goolam et al., 2016). Further studies of these factors will help to clarify the role of Notch during the first lineage choice.

LIF – JAK/STAT

Leukemia inhibitory factor (LIF), a member of the interleukin-6 cytokine family, supports mouse ES cell self-renewal (Smith et al., 1988; Williams et al., 1988). It binds to a heterodimeric receptor formed by a low-affinity cell surface LIF-receptor (LIFR) and Glycoprotein 130 (gp130). These receptors constitutively bind members of the janus kinase (JAK) family of tyrosine kinases in their cytoplasmic domain. JAK kinases are

inactive in the absence of the ligands. Upon ligand-receptor interaction, the receptors induce JAK kinase auto-phosphorylation and subsequent phosphorylation of STAT3, which can then translocate into the nucleus and activate downstream target genes.

LIF, LIFR, and gp130 become detectable at the blastocyst stage (Fig. 2A, B). LIF is expressed in the TE, whereas both LIFR and gp130 receptors are restricted to the ICM (Nichols et al., 1996). STAT3 is localized in the cytoplasm in oocytes, zygotes and two-cell stage embryos, but is phosphorylated and present in the nucleus from the four-cell stage (Do et al., 2013). Until the early blastocyst stage STAT3 is present in the nucleus of all cells, but by the late blastocyst stage (E4.5) it is present at higher levels in the nucleus of PrE and TE cells (Morgani and Brickman, 2015). In addition, mRNA expression of *Jak1*, *Jak2*, *Jak3*, and *Tyk2* tyrosine kinases has been detected from the oocyte up to the blastocyst stage (Onishi and Zandstra, 2015).

Knockout studies for individual pathway components do not reveal preimplantation phenotypes (Stewart et al., 1992; Li et al., 1995; Ware et al., 1995; Yoshida et al., 1996; Neubauer et al., 1998; Rodig et al., 1998). The lack of phenotype associated with knockout of LIF signaling components during early embryo development suggests that the pathway is not required for pluripotency *in vivo*, contrasting its action *in vitro*. However, an early effect of the pathway could be masked in knockout studies due to redundancy in signaling molecules or maternal contribution. For instance, IL-6 is also expressed from the 8-cell stage (Gerwin et al., 1995) and in the EPI of E4.5 embryos (Ohnishi et al., 2014). IL-6 sustains ESC self-renewal in the absence of LIF (Nichols et al., 1994) and could compensate the lack of LIF *in vivo* in *Lif*-null embryos. Similarly, other JAK family members could override JAK1 defects in *Jak1* knockout embryos. Also, a role for the LIF pathway in maintaining pluripotency during preimplantation development is suggested by the observations that $Lif^{-/-}$ females do not support implantation (Stewart et al., 1992) and that $Gp130^{-/-}$ embryos develop normally but fail to undergo diapause (Nichols et al., 2001).

Recent evidence identifies a role for JAK/STAT signaling in early development and reveals the maternal contribution of JAK/STAT signaling components. *Stat3* zygotic null embryos survive until E6.0 (Takeda et al., 1997), but materno-zygotic deletion of *Stat3* arrests development at E4.5 due to complete loss of the EPI lineage. In addition, treatment with JAK1 inhibitors decreases STAT3 and OCT4 in the blastocyst (Do et al., 2013), showing that the effect observed in *Stat3* materno-zygotic mutants is downstream of the JAK signaling pathway.

The mechanism by which the JAK/STAT pathway regulates ICM gene expression remains unsolved. Careful analysis of pSTAT3 in the early embryo shows higher expression in PrE cells than EPI cells at E3.5 and E4.5 (Morgani and Brickman, 2015). Moreover, JAK/STAT pathway blockade (with low concentrations of inhibitors in a time-controlled manner) leads to ICM defects, including increased apoptosis in the PrE. Conversely, exposure of cultured embryos to LIF from morula to late blastocyst stages increases the number of PrE cells (Morgani and Brickman, 2015). These findings suggest a role for the JAK/STAT pathway in PrE formation, rather than supporting pluripotency through EPI maintenance. Nonetheless, these studies do not tackle the contribution of LIF signaling from the TE or the possible contribution of IL-6. A possibility to be explored is that LIF signaling has different functions in the ICM and EPI/PrE lineages. The generation of mouse reporter lines downstream of LIF and/or IL-6 could help to address these questions. Furthermore, studies in ES cells have shown that the PI3K-Akt and the MAPK intracellular pathways are downstream of LIF (Schiemann et al., 1997). It would be of interest to analyze this relationship in the preimplantation embryo.

The TGFβ superfamily

The transforming growth factor beta (TGFβ) superfamily is a large family of structurally related proteins involved in many cellular processes such as cell growth, cell differentiation, and apoptosis. These proteins interact with a family of serine/threonine

protein kinase receptors, and transduce the signals to the nucleus via SMAD proteins. The TGFβ superfamily comprises TGFβ isoforms, Activins, NODAL, and bone morphogenetic proteins (BMPs). The downstream effectors SMAD2/3 are common to all TGFβ pathways, and these pathways are thus considered to have similar functions.

Nodal signaling is important for body axis establishment and differentiation (Shen, 2007). NODAL is secreted as an inactive dimerized precursor that is cleaved by the convertases SPC1 and SPC4 to generate the active protein. Activins are homodimers or heterodimers of beta subunits shared with Inhibins. Activins and NODAL bind to type II Activin receptors (ACTRII/IIB), triggering recruitment, phosphorylation and activation of type I Activin receptors (Activin receptor-like kinases, or ALKs, including ALK1-7). ALK activation induces phosphorylation of cytoplasmic SMAD2/3. ALK activation by NODAL requires the co-receptor CRIPTO, and this distinction between Nodal and Activin signaling generates different specificities between these pathways. Upon phosphorylation, SMAD2/3 dimers form a ternary complex with SMAD4, which can then translocate to the nucleus. SMADs in the nucleus recruit chromatin modifiers and cofactors to activate target gene expression (Ross and Hill, 2008). Some of the targets of this pathway are *Lefty1* and *Lefty2* (which encode divergent TGFβ superfamily members) and *Nodal* itself (Besser, 2004; Guzman-Ayala et al., 2009). TGFβ is expressed from fertilization throughout preimplantation development (Rappolee et al., 1988) and may have functions redundant with the Activin/Nodal signaling pathway.

Activin is expressed maternally in the oocyte and disappears during the first divisions. Zygotic *Activin* expression begins upon morula compaction and by E3.5 is restricted to the ICM. One day later, at E4.5, *Activin* is lost from the epiblast and is detected only in the TE of the hatched blastocyst (Albano et al., 1993; Lu et al., 1993). *Nodal* expression starts in the ICM of the E3.5 embryo and persists in the EPI and PrE as the tissues segregate (Takaoka et al., 2006; Granier et al., 2011; Park and Dufort, 2011). SMAD2/3 is detected from the 4-cell stage onward (James et al., 2005).

Activin/Nodal signaling pathway mutants do not have preimplantation phenotypes (Papanayotou and Collignon, 2014). However, no mutant affecting *Activin* and *Nodal* in combination has been reported, and potential maternal contributions have not been analyzed yet. Our understanding of Activin/Nodal regulation in preimplantation development comes from the analysis of *Nodal* cis-regulatory elements. The *Nodal* ASymmetric Enhancer (ASE) contains two sites for FoxH1-Smad2/3 and acts as an auto-regulatory element activating its own expression (Granier et al., 2011). The ASE directs reporter expression in EPI cells and remains active after *Nanog* expression disappears upon implantation. The recently identified highly bound enhancer (HBE) recapitulates the onset of *Nodal* expression in the ICM of preimplantation embryos. The HBE is regulated by OCT4, KLF4, and by Activin/Nodal signaling itself (Papanayotou et al., 2014).

More interestingly, the Activin/Nodal signaling pathway seems to regulate antero-posterior axis formation from the blastocyst stage (E4.5), much earlier than previously expected. Although SMADs do not show any restricted distribution in the blastocyst, the downstream target *Lefty1* is expressed asymmetrically in a subset of PrE cells (Takaoka et al., 2006). These LEFTY1-expressing cells have been shown by lineage tracing analysis to later form the distal visceral endoderm, a tissue essential for the establishment of the mouse embryo antero-posterior axis (Takaoka et al., 2011). Remarkably, these observations suggest that initial AP polarity is specified autonomously within cells of the ICM.

BMPs are involved in all major developmental processes (Whitman, 1998; Massague et al., 2000). BMPs are synthesized as dimeric precursors that are cleaved by convertases to generate N-terminal and C-terminal fragments. BMP receptors are heterotetrameric complexes composed of type I receptors (BMPR-1A, BMPR-1B, ActR-1A) and II receptors (BMPR-2, ActR-2A and ActR-2B). These receptors are serine/threonine kinases, and several complexes can assemble depending on particular BMPs. The downstream effectors for BMP are SMAD1/5/8, which form a complex with SMAD4

(Moustakas and Heldin, 2002). SMADs then translocate to the nucleus where they function as transcription factors with coactivators and corepressors to regulate gene expression (Massague, 1998; Whitman, 1998; Walsh et al., 2010).

Several BMP pathway components are expressed at early stages of development (Fig. 2A, B). In the blastocyst, *Bmp4* and *Bmp7* are specifically located in the ICM, whereas *Bmpr2* is expressed in the TE (Coucouvanis and Martin, 1999; Guo et al., 2010). The effector SMAD1 is detected in all blastocyst cells, but pSMAD1 is expressed at higher levels in TE cells (Reyes de Mochel et al., 2015). Mouse mutants for BMPs and its receptors show a range of developmental defects, but none of them has demonstrated that the pathway is crucial for preimplantation development (Reyes de Mochel et al., 2015). This may be explained by redundancy among BMP components. Supporting this, double heterozygotes for *Smad1*/5 die at ~E9.0 and display pleiotropic abnormalities; homozygous mutants have not been identified, most likely due to earlier death (Arnold et al., 2006). Pharmacological inhibition of BMP signaling has helped to underscore its effect in TE and PrE development. BMP inhibition causes a reduction in the number of TE and PrE cells (Graham et al., 2014). In addition, culture of embryos with BMP antagonists has shown an effect on cell division (Reyes de Mochel et al., 2015).

Wnt/β-catenin

β-CATENIN is a multifunctional protein that plays two roles in cells: one in cell adhesion through interactions with cadherins and another as the main effector of the canonical Wnt signaling pathway. WNT proteins bind to the receptor FRIZZLED and the co-receptor LRP5/6 (low-density lipoprotein receptor-related protein 5/6), resulting in activation of DISHEVELLED (DVL), which inhibits GSK3 activity and leads to the stabilization and accumulation of β-CATENIN. Accumulated β-CATENIN translocates into the nucleus where it interacts with members of the TCF/LEF family of transcription factors and activates downstream target genes (Barker and Clevers, 2000). In the

absence of WNT proteins, β-CATENIN associates with a cytoplasmic protein complex composed of AXIN, APC (adenomatous polyposis coli), GSK3β (glycogen synthase kinase 3 β) and CSNK1A1 (casein kinase 1 α), in which it is phosphorylated and rapidly degraded by the ubiquitin-proteasome pathway.

Several reports have demonstrated that multiple Wnt signaling pathway members are expressed in early embryos (Lloyd et al., 2003; Hamatani et al., 2004; Mohamed et al., 2004; Wang et al., 2004; Kemp et al., 2005), suggesting a possible role during preimplantation development. *β-catenin* is expressed at all stages from the fertilized zygote to the blastocyst. Active β-CATENIN is mostly localized in the nuclei of all embryonic cells before the morula stage and mainly in the TE of blastocysts. However, *β-catenin*-null mutation studies revealed that lack of zygotic *β-catenin* does not significantly impair the blastocyst formation (Haegel et al., 1995; Huelsken and Birchmeier, 2001). Even embryos with maternal truncation of β-CATENIN protein, which affects blastomere adhesion, develop up to the blastocyst stage (De Vries et al., 2004). Silencing the Wnt/β-catenin pathway blocks the competency of blastocysts to implant, without affecting uterine receptivity, showing that nuclear β-CATENIN is essential for blastocyst activation prior to implantation (Xie et al., 2008). Moreover, recent studies using a null-allele show that embryos lacking both maternal and zygotic *β-catenin* develop until blastocyst without affecting lineage specification. After hatching, the embryonic portion is defective, and embryos undergo fission forming trophoblastic vesicles that decidualize but no longer develop (Messerschmidt et al., 2016). WNT proteins can also signal through β-catenin-independent (non-canonical) pathways via FRIZZLED receptors, regulating Ca^{2+}/planar cell polarity (PCP) and Rho signaling (Veeman et al., 2003; Barrow, 2006). Coordination of canonical Wnt/β-catenin signaling activation with attenuation of the non-canonical Wnt/RhoA signaling pathway in TE cells ensures blastocyst competency for implantation (Veeman et al., 2003; Xie et al., 2008). Moreover, a possible role in regulating cell–cell adhesion during preimplantation embryo

development has been reported for DVL family proteins, which have been reported as intermediate transducers of divergent Wnt pathways (Capelluto et al., 2002; Itoh et al., 2005; Na et al., 2007). A role for Wnt pathway has been assigned in the segregation of the ICM in marmoset embryos since inhibition of WNT leads to an increase in the number of NANOG cells at the expense of a reduced number of GATA6 cells (Boroviak et al., 2015). Therefore, whereas canonical Wnt activities seem to be dispensable for the preimplantation development of zygotes to blastocysts, definitive roles of non-canonical Wnt pathways during early embryo development remain elusive.

Rho/ROCK

The small GTP-binding proteins of the Rho family, consisting of the Rho, Rac, and Cdc42 subfamilies, reorganize the actin cytoskeleton and are involved in various cell functions such as endocytosis/exocytosis, cell shape change, cell motility, cell polarization, and cytokinesis. These proteins are inactive when associated with GDP and become active upon exchange of GDP for GTP, which induces conformational changes that promote association with downstream effector proteins (Jaffe and Hall, 2005). The ROCK1 and ROCK2 serine/threonine kinases are activated by association with active GTP-bound RHO protein. Activated ROCK triggers the phosphorylation of myosin light chain (MLC) and its phosphatase, inducing cytoskeletal reorganization by increasing actin-myosin interaction. Simultaneously, ROCK also phosphorylates the LIM kinases, which modulate filamentous actin stability. The Rho/ROCK signaling pathway can be activated by various intracellular receptors and membrane receptors, such as G protein-coupled receptors (GPCRs) and RTKs.

During preimplantation development, the presence of RHO and ROCK has been reported from the oocyte to the blastocyst (Clayton et al., 1999; Kawagishi et al., 2004). *In vivo* studies have shown that neither *Rock1* nor *Rock2* can compensate the loss of the other isoform during mouse embryonic development. *Rock1* deletion is embryonically

lethal due to cardiac fibrosis, whereas *Rock2*$^{-/-}$ mice die due to placental dysfunction (Shimizu et al., 2005; Zhu et al., 2011). Moreover, *Rock1* and *Rock2* double mutant embryos die before E9.5 (Kamijo et al., 2011). Nevertheless, treatment of preimplantation embryos with inhibitors results in apparent conflict with the post-implantation lethality phenotype. Disrupting Rho activity with the specific inhibitor *Clostridium botulinum* C3-transferase from the 2-cell stage impairs cell polarization and compaction at the 8-cell stage (Clayton et al., 1999). In contrast, inhibition of ROCK from 2-cell stage only has a significant effect after the 8-cell stage, inhibiting the formation of the blastocoel cavity (Kawagishi et al., 2004). These results suggest that ROCK contributes to blastocyst formation and that impairment of compaction at the 8-cell stage by inhibition of Rho is ROCK-independent. However, higher concentrations of the ROCK inhibitor cause defects in the first cleavage and failure in compaction at the 8-cell stage (Duan et al., 2014). Furthermore, inhibition of ROCK activity during blastocyst cavity expansion affects cohesion of ICM cells and the segregation of epiblast and PrE tissues, without compromising lineage specification. Treated blastocysts are able to implant but postimplantation development is impaired (Laeno et al., 2013), similar to the phenotype observed in zygotic *Rock1/2* double mutant embryos (Kamijo et al., 2011). Therefore, both the maternal and the zygotic contributions of Rho/ROCK signaling should be further investigated to clarify its role in early developmental stages.

G protein–coupled receptors (GPCRs)

GPCRs are members of a large superfamily of membrane proteins that bind a wide variety of extracellular ligands and transduce signals into cells via heterotrimeric G proteins (Gα, Gβ/Gγ subunits). Upon activation, GPCRs undergo a conformational change that promotes the exchange of GDP/GTP associated with the Gα subunit. This leads to dissociation of the Gβ/Gγ dimer from Gα, which further transduces intracellular signaling responses. After the signal propagation, the GTP is hydrolyzed to GDP and Gα

becomes inactive, leading to its re-association with the Gβ/Gγ dimer to form the inactive heterotrimeric complex. GPCRs can signal to several downstream effectors, triggering multiple signaling pathways that directly depend on the Gα subunit type (Gαs, Gαi/o, Gαq/11, Gα12/13). The Gαs and Gαi/o families activate or inhibit the activity of adenylate cyclase (AC), affecting cyclic AMP (cAMP) production. Members of the Gαq/11 family activate phospholipase C (PLC), ultimately leading to intracellular calcium mobilization. The Gα12/13 family is involved in activation of Rho family GTPases. GPCRs can also signal through non-G-protein mediated events that involve scaffolding proteins and transactivation of RTKs or G-protein receptor kinases that regulate the GPCR signal.

Expression of some GPCRs such as opioid, serotonin, histamine, and adrenergic receptors has been reported in preimplantation embryos (Cikos et al., 2011). However, although activation upon agonist treatment has been described in some cases, the specific function and cellular targets of GPCRs during early development remain largely unknown. Below we briefly discuss two selected examples.

Histamine receptor type 2 (H2R) is detected in blastocysts, and ligand-binding assays reveal a predominant localization in TE cells, suggesting a role in implantation (Zhao et al., 2000). Moreover, incubation of blastocysts with an H2R agonist stimulates cAMP accumulation and blastocyst hatching, whereas H2R antagonists lead to implantation failure (Zhao et al., 2000).

Similarly, transcripts for β2- and β3-adrenergic receptors are detected in oocytes and all stages of preimplantation development. Protein expression of β2-adrenoceptor has been confirmed, with expression stronger in the ICM (Cikos et al., 2005). Treatment with a β-adrenergic agonist inhibits cell proliferation and impairs early development. Interestingly, β-adrenergic receptors are coupled to Gαs subtype proteins, which upon activation in cell assays regulate Hippo signaling by inducing YAP/TAZ phosphorylation (Yu et al., 2012). Stimulation of cells with epinephrine (the natural adrenergic receptor ligand) or overexpression of β2-adrenergic receptors activates LATS1/2 and inhibits YAP function.

Moreover, in a screen for YAP-inhibitors, another β-adrenergic agonist, dobutamine, was found to block YAP nuclear translocation (Bao et al., 2011). Taken together, this evidence suggests a possible role for β-adrenergic receptors in Hippo signaling modulation during preimplantation development.

Finally, expression of *Rgs2*, a negative regulator of Gα, has been reported in early embryos from the zygote to the blastocyst (Zhu et al., 2014). Reduced levels of *Rgs2* expression led to abnormal embryonic development *in vitro*, with embryos arrested at the 2- or 4-cell stage, and a significant decrease in the expression of zygotic activation-related genes.

Estrogen receptors

Estrogens elicit diverse biological responses on growth, differentiation, inflammation, pregnancy, and homeostasis. Traditionally, estrogen receptors (ER) have been considered cytosolic receptors that upon hormone binding undergo a conformational change and translocate to the nucleus, where they act as transcription factors binding to DNA motifs known as estrogen response elements (EREs). Two nuclear estrogen receptor isoforms have been described: ERα and ERβ. More recently, several putative membrane estrogen receptors mediating non-genomic effects have been reported, including mERα and mERβ, ER-X, GPR30, and Gq-mER (Micevych and Kelly, 2012). These receptors bind estrogenic compounds and activate intracellular signaling pathways such as the phospholipase C, protein kinase C (PKC), protein kinase A (PKA), and MAP kinase signaling cascades.

Neither *Erα* nor *Erβ* knockout is embryonically lethal (Lubahn et al., 1993; Krege et al., 1998; Lee et al., 2012), although ERα mRNA and protein expression is dynamic during early development (Hou and Gorski, 1993; Hou et al., 1996; Cheng et al., 2016). *Erα* transcripts appear in the zygote and then decline, reappearing in the blastocyst stage. ERα protein is detected in all the cells at the 8-cell and morula stages and in the TE cells

of blastocysts. In contrast with results from *Erα* mutant mice, cavitation and TE specification are inhibited in 8-cell embryos treated with an ERα-selective antagonist (Cheng et al., 2016). Conversely, treating embryos with an ERα-selective agonist promotes cavitation and blastocoel expansion without affecting ICM and TE lineage differentiation, suggesting that maternal contribution might overcome the effects of a lack of ERα in earlier studies.

Analyzing signaling pathways in blastocyst-derived stem cells

An important property of the mouse blastocyst is that TE, PrE, and EPI populations can be isolated and cultured *in vitro* under conditions that preserve stemness. Moreover, these populations can also be directed to differentiate, thereby providing additional tools for studying the gene regulatory and signaling networks operating in these lineages.

Mouse embryonic stem (ES) cells are derived from the epiblast (Evans and Kaufman, 1981; Martin, 1981), trophoblast stem (TS) cells from the trophectoderm (Tanaka et al., 1998), and extraembryonic endoderm (XEN) cells from the primitive endoderm (Kunath et al., 2005). Chimera assays have demonstrated that each stem cell type contributes to the lineage to which it belongs (Beddington and Robertson, 1989; Tanaka et al., 1998; Kunath et al., 2005).

The core elements of the gene regulatory networks of the stem cell populations and their corresponding lineage in the embryo are also preserved. Accordingly, ES cells cannot be derived from *Oct4* mutant embryos (Nichols et al., 1998) and neither can TS cells be derived from *Cdx2* mutant embryos (Strumpf et al., 2005). However, some important elements in these circuitries do not behave in the same way, and this needs to be considered if stem cells are to be used as a proxy in the analysis of mechanisms taking place in the embryo.

How signaling pathways define and maintain pluripotency in ES cells has been extensively studied over the past decades. LIF was demonstrated to preserve stemness

(Smith et al., 1988; Williams et al., 1988). Later, the inhibition of ERK and GSK3 signaling (known as 2i conditions) was shown to be sufficient to achieve a ground state of pluripotency (Ying et al., 2008). The combination of LIF and ERK inhibitors or LIF and GSK3 inhibitors (or exogenous Wnt3a) is sufficient to maintain self-renewal capacity of cells (Ying et al., 2008; ten Berge et al., 2011). These findings suggest that pluripotency *in vitro* is not achieved through a single pathway but rather that overlapping or redundant mechanisms may be at play. Equally, these findings highlight differences between our understanding of the role of different pathways *in vivo* and *in vitro*. Despite the importance of Wnt/β-catenin in ES cell pluripotency, so far there is no conclusive evidence showing a similar requirement in the blastocyst (Munoz-Descalzo et al., 2015). Furthermore, Wnt signaling can have different effects in different pluripotent cells (Tesar et al., 2007). In ES cells, it has a dual role, promoting mesoderm differentiation (Gadue et al., 2006; Bakre et al., 2007) or maintaining pluripotency (ten Berge et al., 2011), whereas in epiblast-derived stem cells (EpiSCs) it promotes differentiation (Kurek et al., 2015). Contrary to what happens in the blastocyst epiblast, EpiSC self-renewal capacity relies on Activin/Nodal Signaling (Tesar et al., 2007). Likewise, different effects have been described for Fgf/ERK signaling in the embryo and in stem cells. ERK inhibition improves the efficiency of ES cell derivation (Nichols et al., 2009), and while *Fgf4* mutant ES cells can be maintained in self-renewal conditions they fail to differentiate (Kunath et al., 2007), a finding compatible with their role in lineage selection within the ICM. However, the role of FGF signaling in the other stem cell populations is puzzling. FGF4 is required for PrE formation, but is dispensable for XEN cell propagation; XEN cells can be derived (with FGF supplied in the culture medium) and maintained from *Fgf4* mutants (Kang et al., 2013). Interestingly, TS cell maintenance requires FGF4 (Tanaka et al., 1998); however, there is no evidence attributing a role to FGF4 in establishing the TE lineage in the embryo.

Similar considerations apply to other signaling pathways. YAP is reported to maintain ES cell pluripotency and to positively regulate the expression of *Oct4* and *Sox2* (Lian et al., 2010), whereas it is excluded from cell nuclei in the ICM (Nishioka et al., 2009). However, a recent study claims that YAP is dispensable for self-renewal but necessary for ES cell differentiation (Chung et al., 2016). ES cells express various receptors and ligands of the Notch pathway (Lowell et al., 2006), and Notch activity has been detected heterogeneously in ES cells using a CBF:H2B-Venus transgene (Nowotschin et al., 2013); however, the pathway is inactive in the ICM (Rayon et al., 2014).

Other pathways show similar behavior in stem cells and embryos. For example, the role of the Rho/Rock pathway in cell adhesion seems to be similar in embryos and in ES cells. Inhibition of ROCK causes defects in ICM cell polarization, compaction, and cohesion (Clayton et al., 1999; Kawagishi et al., 2004; Laeno et al., 2013; Duan et al., 2014). In ES cells, ROCK inhibition results in colony disruption (Chang et al., 2010; Laeno et al., 2013), causes a reduction in stem like properties, including alkaline phosphatase activity and *Oct4* expression, and induces neural progenitor markers (Chang et al., 2010).

Embryos and stem cells also show differences in gene regulation. Two enhancers have been identified for *Oct4* in pluripotent cells, but only one of them (the Distal Enhancer, DE) is able to direct reporter expression in the blastocyst ICM (Yeom et al., 1996). Similarly, we recently identified elements that regulate *Cdx2* differently in TE and TS cells. The TEE (an enhancer upstream of *Cdx2*) drives reporter expression specifically in the TE of blastocysts but not in TS cells. Other regulatory elements, one in the first *Cdx2* intron and another downstream of *Cdx2*, are active in TS cells and in the blastocyst but not in a TE-restricted manner (Rayon et al., 2016). A possible explanation for these seemingly contradictory results is that blastocyst-derived stem cells do not exactly match the lineage from which they are derived. This may be due to culture conditions being

able to only partially reproduce conditions in the embryo. It is thus important not to assume that the same molecular mechanisms and circuitry will act *in vivo* and *in vitro*.

Manipulating signaling pathways in the embryo

An obvious advantage of studying signaling pathways is the possibility to modulate their activity through the use of pharmacological activators or inhibitors. This is a standard approach for cell culture assays, but is more challenging *in vivo*. Nevertheless, there is a strong tradition of using drugs and other compounds in embryos to alter the effects of specific pathways, exemplified by the use of broad FGFR inhibitors in the analysis of limb development (Rosello-Diez et al., 2011). The difficulties inherent to these approaches, such as problems with diffusion, localized delivery, or achieving effective concentrations, have limited a broader use of this approach in developmental studies.

The mammalian preimplantation embryo in fact provides an excellent system for the use of pharmacological modulators of signaling activity because they can be easily cultured *in vitro* up to the blastocyst stage, allowing efficient drug delivery in a time and dose controlled fashion. This property of mammalian embryos underlies the increasing use of pharmacological treatments, which have helped to provide insight into the role of different pathways during preimplantation development (Fig.3, Table 1). The specific temporal activities of certain pathways might be masked in full loss or gain of function models. A clear example is the different temporal requirements for FGF/ERK signaling (Nichols et al., 2009; Frankenberg et al., 2011; Kang et al., 2013; Schrode et al., 2014). Also, effects due to putative redundancy of specific components can be avoided with inhibitors that affect all the ligands or receptors of a pathway. Similarly, the maternal contribution of some components can be blocked with inhibitors, enabling analysis of zygotic contribution. These considerations predict some discrepancies between observations with inhibitors or knockout mice, as is the case for the Rho/Rock pathway (Clayton et al., 1999; Kawagishi et al., 2004; Duan et al., 2014). Another advantage of using

pharmacological modulators is that more than one pathway can be interrogated simultaneously, as we showed for the Hippo and Notch pathways (Rayon et al., 2014), or in successive or partially overlapping time windows. Nevertheless, it should always be kept in mind that a drug might not fully activate or inhibit the target pathway, and that genetic disruption should ideally be used to confirm findings obtained with pharmacological agents.

Signaling as a transducer of early asymmetries in the preimplantation embryo

A mouse embryo can be cultured in a dish from the one-cell stage up to the blastocyst in a chemically defined medium free of serum and growth factors. Furthermore, these cultured embryos are able to producing viable offspring if transferred to the uterus of a surrogate mother. This fact indicates that the embryo is a self-organizing system, equipped with all the information and signals needed to initiate development (Wennekamp et al., 2013). After a small number of cell divisions, morphological constraints and differential gene activity result in lineage decisions without external inputs. These differing cellular phenotypes and the trigger of regulatory networks must result from initial asymmetries followed by cell-to-cell communication in the embryo. We suggest that signaling pathways link these events and translate morphogenetic changes into transcriptional responses.

The first morphological distinction to appear in early mouse embryos is the establishment of apical-basal polarity in cells located on the embryo surface. Cells start to polarize around the 8-cell stage, after compaction (Ziomek and Johnson, 1980). The apical domain, located on the outer side of cells, is characterized by an enrichment of microvilli on the surface and rearrangements in the cortex, including F-actin, microtubules and clathrin (Ducibella et al., 1977; Maro et al., 1985). Furthermore, establishment of the apical domain has been shown to require EZRIN (Dard et al., 2004) and a protein

complex containing PARD3, PARD6b, and atypical protein kinase C (aPKC) (Pauken and Capco, 2000; Plusa et al., 2005; Vinot et al., 2005; Alarcon, 2010).

The Hippo pathway has been shown to be a readout of cellular polarity in the early embryo. In general, Hippo signaling is active in inner cells and inactive in outer cells. However, because polarization events are progressive, outer apolar cells can be observed in 16-cell embryos that still maintain pYAP in the cytoplasm (Hippo ON), therefore linking this pathway to polarity rather than to cell position (Anani et al., 2014; Hirate et al., 2015). As discussed above, the differential activity of this pathway in inner and outer cells is not due to transcriptional modifications but rather to the subcellular distribution of its components. Their location is dependent on cell polarity and cell-cell adhesion molecules. The junction-associated protein AMOT is sequestered by the apical complex in outer polarized cells, whereas it is located at the adherens junctions of apolar cells, where it can activate Hippo signaling. LATS1/2 phosphorylates AMOT in adherens junctions and YAP. As a consequence, pYAP is retained in the cytoplasm (Hirate et al., 2013).

Interestingly, although polarity and Hippo are closely related, the mechanisms through which they interplay and the response generated differ throughout early development. Disruption of the Par-aPKC system in 32-cell stage embryos excludes YAP from the nuclei of every cell, and CDX2 is not detected. In contrast, in 16-cell stage embryos, YAP is not completely excluded from the nuclei, even though its phosphorylated form is also detected in the cytoplasm, and *Cdx2* expression is slightly affected (Hirate et al., 2015). The transcription factor AP-2γ (TFAP2C) has been reported to be important for the apical domain because it regulates *Pard6b*. Even though TFAP2C does not directly regulate any Hippo signaling component, nuclear YAP accumulation is impaired in TFAP2C KD embryos (Cao et al., 2015). Building on the acquired knowledge about the Hippo pathway and its functions in the early mouse embryo, it will be interesting to assess the subcellular

distribution of the components of other signaling pathways that do not show an obvious differential distribution between cells of different positions or lineages.

The other main process that takes place when symmetry is broken is an increase in intercellular interactions. E-cadherin (*Cdh1*), a cell adhesion molecule, changes from being uniformly distributed along the surfaces of all the cells to being enriched in cell-cell contacts areas by the time of compaction (Vestweber et al., 1987). These regions are also enriched in PAR1 (Vinot et al., 2005) and JAM-1 (Thomas et al., 2004). Maternal/zygotic *E-cadherin* mutants fail to compact and form an epithelium, and the balance and positioning of TE/ICM cells is disturbed (Stephenson et al., 2010). It may be the broad distribution of apical domain markers that keeps the Hippo pathway inactive, so that CDX2 is detected in a higher percentage of cells. Epithelial integrity is essential for proper preimplantation development and may be important in cell-to-cell communication; however, no pathway has yet been shown to translate intercellular adhesions into a transcriptional response. p120-catenin, which stabilizes E-cadherin, is necessary for ES cell differentiation towards primitive endoderm, but its absence does not impede early development in the mouse (Pieters and Goossens, 2016).

How compaction begins is incompletely understood, but recent studies provide new information. Live imaging using an E-cadherin-GFP mouse reporter line has revealed the presence of filopodia that by the time of compaction extend from apical borders to the apical membrane of neighboring cells. These E-cadherin-dependent filopodia are important for cell shape changes during compaction, and their early formation causes premature compaction (Fierro-Gonzalez et al., 2013). Studies of tension dynamics demonstrate that pulsed actomyosin contractility beginning at 8-cell stage increases surface tension and, through cooperation with E-cadherin, decreases intercellular tension. These periodic cortical waves, whose propagation is constrained by cell-cell contacts, drive compaction in the embryo (Maitre et al., 2015). The source of tension heterogeneities in the embryo is generated after the first asymmetric divisions and is

sufficient to direct cell internalization (Maitre et al., 2016). Compaction in embryos lacking the maternal *Myh9* allele is delayed due to their inability to generate sufficient tensions. When wildtype blastomeres are confronted with others lacking the maternal *Myh9* allele, the wildtype cells are internalized (Maitre et al., 2016). It would be interesting to analyze if any pathway integrates mechanical forces with the lineage programs triggered after compaction.

Crosstalk and interplay of signaling pathways

The regulative capacity of the mammalian embryo suggests that complementary and compensatory mechanisms may serve to circumvent any disturbances and ensure normal embryonic development. This early plasticity will complicate the study of these mechanisms, because eliminating an individual signaling component will not be deleterious for the embryo. It seems likely that the activation of lineage programs will rely on overlapping inputs to ensure their proper functioning. It is also possible that the initial heterogeneous and progressively restricted TF expression in blastomeres is regulated by different signaling pathways acting in a complementary, sequential, or synergistic manner. Thus, analysis of combined inputs will be needed to unmask putative compensatory effects.

A clear example of this is the cooperation between the Hippo and Notch pathways in the regulation of *Cdx2*. The effectors of the two pathways, TEAD4 and RBPJ, converge on a *Cdx2* enhancer (TEE) to guarantee its expression. Notably, *Tead4*;*Rbpj* double mutants cause a more serious phenotype than single knockouts, leading to embryonic death before the blastocyst stage (Rayon et al., 2014). The Hippo and Notch pathways appear to act in parallel, but cross-regulation between them has not been ruled out.

A recent report shows that Rho/Rock signaling is an essential regulator of Hippo signaling and cell polarization, enabling ICM and TE lineage specification (Kono et al., 2014). Inhibition of Rho/Rock signaling affects LATS1/2 activity, YAP nuclear

accumulation, and the localization of apico-basal regulators such as PARD6B, PRKCZ, SCRIB, and LLGL1 (Kono et al., 2014; Mihajlovic and Bruce, 2016). The link between Rho and Hippo seems to involve AMOT, which is mislocalized in the basolateral domains of outer cells when Rho signaling is blocked (Mihajlovic and Bruce, 2016), thus affecting LATS activity. Other studies using cultured cells show that Rho GTPases modulate YAP activity in response to activation of GPCRs or to mechanical cues such as extracellular matrix stiffness (Dupont et al., 2011; Mo et al., 2012; Yu et al., 2012).

A link has also been established between Nodal and β-catenin signaling, through the *Nodal* proximal epiblast enhancer (PEE). The PEE responds to Wnt pathway signals before implantation (Granier et al., 2011). Mutant blastocysts lacking β-*catenin* show no expression of a PEE-GFP reporter. Thus, the regulation of *Nodal* through the PEE enhancer is the earliest effect to be detected in β-*catenin* mutants.

It is notable that the role of some pathways was initially dismissed because of the lack of a phenotype in loss-of-function mouse models. More detailed analysis has revealed roles, for instance, for the Notch and Rock pathways. These findings raise the question of whether other pathways with to date no implication in the first lineage choices, such as Sonic hedgehog, may in fact have cryptic roles that have yet to be deciphered. Research into the interplay among signaling pathways in the first phases of mouse development has barely begun. The combination of genetic and pharmacological tools, together with lineage-specific fluorescent reporter strains, will allow exploration of these relationships and the building of a more complete understanding of the molecular events underpinning the first differences and lineage choices during mammalian development.

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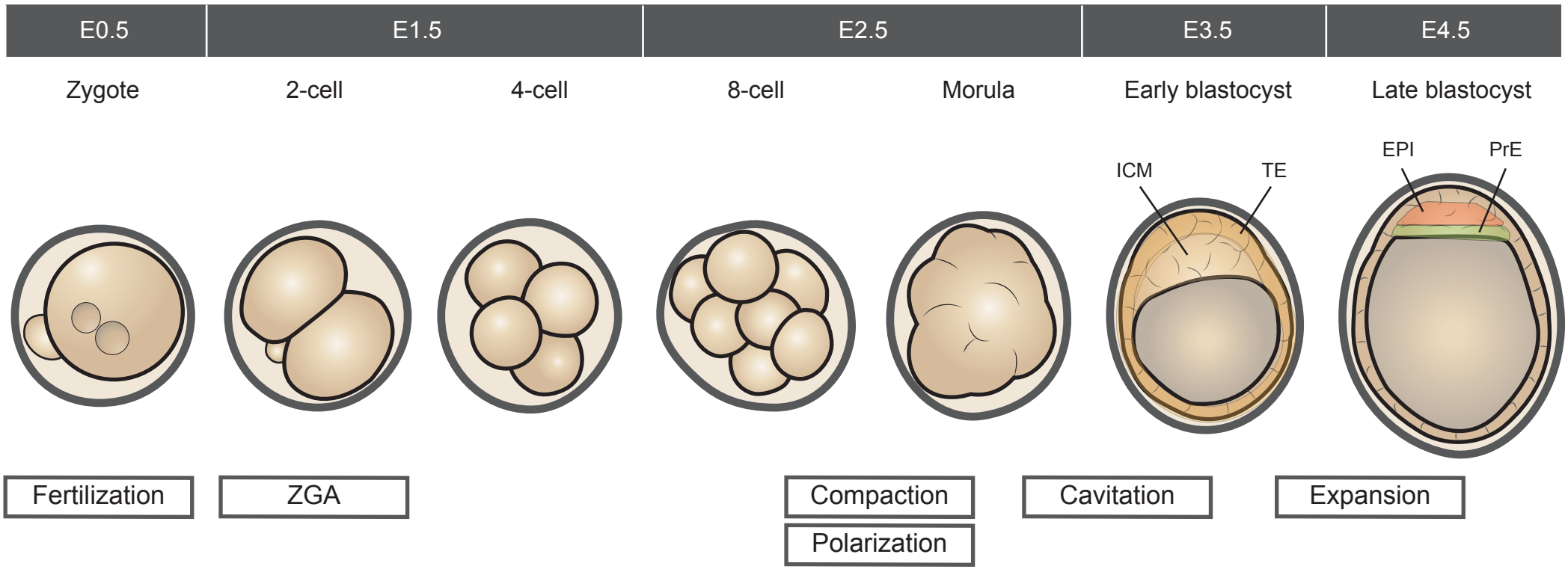
Figure legends

Figure 1. Mouse preimplantation development. Morphological changes, activation of the zygotic genome and the appearance of the first cell lineage populations in the mouse embryo, from fertilization at embryonic day (E) 0.5 to the expanded blastocyst (E4.5). Zygotic gene activation (ZGA) takes place at the 2-cell stage. The 8-cell stage embryo undergoes compaction, and outer cells become polarized. The blastocoel cavity begins to form at the 32-cell stage, giving rise to the blastocyst, in which the trophectoderm (TE) and the inner cell mass (ICM) can be distinguished. While the blastocyst expands, the ICM segregates into the primitive endoderm (PrE) and the epiblast (EPI).

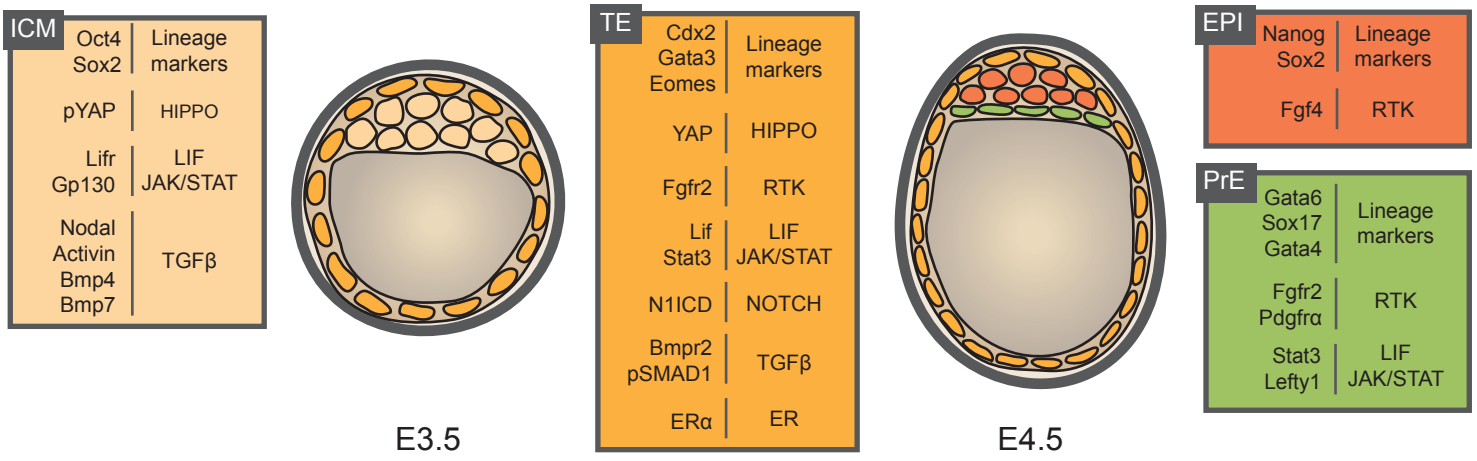
Figure 2. Signaling pathways in the mouse blastocyst. Lineage establishment and maintenance in the embryo are driven by heterogeneities in transcription factor expression and differential activity of signaling pathways. **A**: Differential expression of signaling pathway components in the ICM vs the TE at E3.5 and in the EPI vs the PrE at E4.5. **B-C**: Close-up of cells showing lineage markers and signaling pathway components distribution in TE/ICM at E3.5 (**B**) and in EPI/PrE at E4.5 (**C**).

Figure 3. Effects and lineages targeted after inhibition of signaling pathways. The role of several pathways has been clarified thanks to the use of pharmacological compounds. The lineage targeted, the molecular mechanisms affected, and the observed phenotype in preimplantation embryos for each of the pathways on the left are indicated.

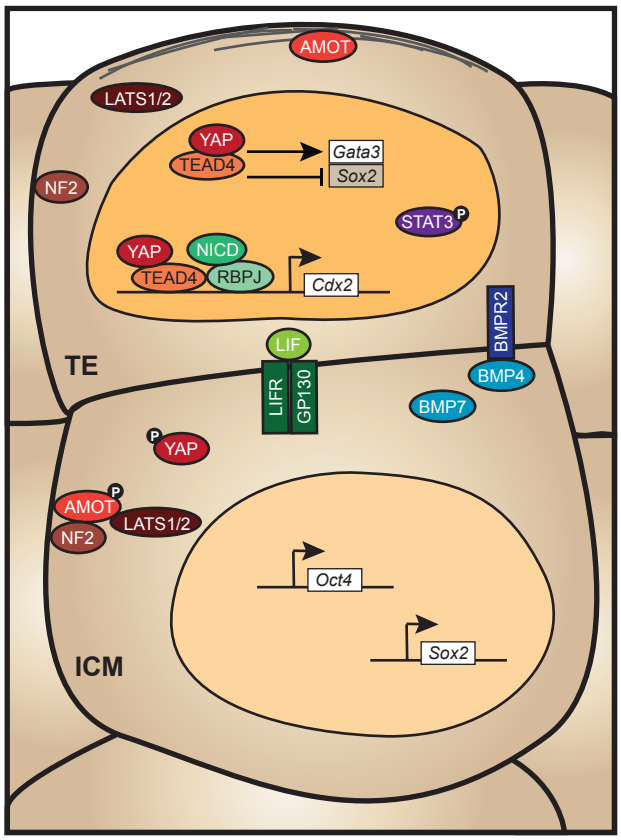
Table 1. Pharmacological components used to modulate signaling activity in the preimplantation embryo


E0.5
E1.5
E2.5
E3.5
E4.5
Zygote
2-cell
4-cell
8-cell
Morula
Early blastocyst
Late blastocyst
ICM
TE
EPI
PrE
Fertilization
ZGA
Compaction
Polarization
Cavitation
Expansion

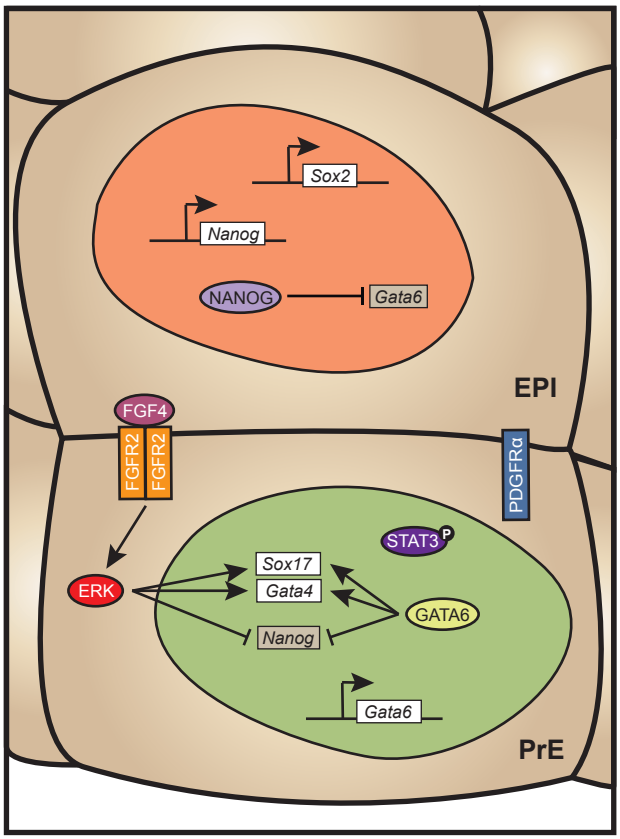
A.

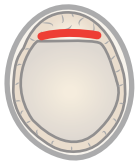
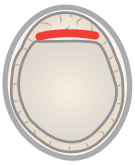

ICM
Oct4
Sox2
Lineage markers
pYAP
HIPPO
Lifr
Gp130
LIF JAK/STAT
Nodal
Activin
Bmp4
Bmp7
TGFβ
E3.5
TE
Cdx2
Gata3
Eomes
Lineage markers
YAP
HIPPO
Fgfr2
RTK
Lif
Stat3
LIF JAK/STAT
N1ICD
NOTCH
Bmpr2
pSMAD1
TGFβ
ERα
ER
E4.5
EPI
Nanog
Sox2
Lineage markers
Fgf4
RTK
PrE
Gata6
Sox17
Gata4
Lineage markers
Fgfr2
Pdgfrα
RTK
Stat3
Lefty1
LIF JAK/STAT

B.


AMOT
LATS1/2
YAP
TEAD4
Gata3
Sox2
NF2
STAT3
P
YAP
NICD
TEAD4
RBPJ
Cdx2
TE
LIF
LIFR
GP130
BMPR2
BMP4
BMP7
P
YAP
AMOT
P
NF2
LATS1/2
Oct4
Sox2
ICM

C.


Sox2
Nanog
NANOG
Gata6
EPI
FGF4
FGFR2
FGFR2
PDGFRα
STAT3
P
ERK
Sox17
Gata4
Nanog
GATA6
Gata6
PrE

Pathway	Lineage	Drug Effect	Phenotype
Hippo		TEAD-YAP inhibition	No cavitation ↓ *Cdx2*
RTK		MEK inhibition FGFR inhibition PDGFR inhibition	↓ *Gata6* ↑ *Nanog* ↓ PrE cells
Notch		Notch inhibition	↓ *Cdx2*
LIF JAK/STAT		JAK inhibition	↑ Apoptosis in PrE
TGFß		BMP inhibition	↓ TE cells ↓ PrE cells
Rho/ROCK		ROCK inhibition	No compaction

Signaling Pathway	Drug	Description	References
Hippo	Verteporfin	Inhibitor of TEAD-YAP association	(Rayon et al., 2014)
Receptor Tyrosine Kinases	PD98059	MEK inhibitor	(Lu et al., 2008)
	PD184352	MEK inhibitor	(Nichols et al., 2009)
	PD0325901	MEK inhibitor	(Nichols et al., 2009; Yamanaka et al., 2010; Frankenberg et al., 2011; Schrode et al., 2014; Morgani and Brickman, 2015)
	SU5402	FGFR inhibitor	(Nichols et al., 2009)
	PD173074	FGFR inhibitor	(Nichols et al., 2009; Yamanaka et al., 2010)
	Gleevec	PDGFR inhibitor	(Artus et al., 2013)
Notch	RO4929097	γ-secretrase inhibitor	(Rayon et al., 2014)
LIF JAK/STAT	JAKi	JAK inhibitor	(Do et al., 2013; Morgani and Brickman, 2015)
	LY294002	PI3K inhibitor	(Morgani and Brickman, 2015)
	CHIR99021	GSK3 inhibitor	(Ying et al., 2008; Nichols et al., 2009)
TGFβ superfamily	Noggin	BMP antagonist	(Graham et al., 2014)
	LDN193189	BMP antagonist	(Reyes de Mochel et al., 2015)
	Dorsomorphin	Bmpr1 inhibitor	(Graham et al., 2014)
WNT β-catenin	PKF115-584	Inhibitor of the β-catenin-TCF complex	(Xie et al., 2008)
	CGP049090	Inhibitor of the β-catenin-TCF complex	(Xie et al., 2008)
Rho/ROCK	Y-27632	ROCK inhibitor	(Kawagishi et al., 2004; Laeno et al., 2013; Duan et al., 2014; Kono et al., 2014; Cao et al., 2015; Mihajlovic and Bruce, 2016)
	Fasudil	ROCK inhibitor	(Laeno et al., 2013)
	Rho inhibitor I	*Clostridium botulinum* C3-transferase	(Clayton et al., 1999; Kono et al., 2014)
GPCR	Famotidine	H2 antagonist	(Zhao et al., 2000)
	Ranitidine	H2 antagonist	(Zhao et al., 2000)
	Pyrilamine	H1 antagonist	(Zhao et al., 2000)
	UK 14304	α2-adrenergic receptor agonist	(Cikos et al., 2007)
	Isoprotenerol	β-adrenergic receptor agonist	(Cikos et al., 2005)
	Dobutamine	β-adrenergic receptor agonist	(Bao et al., 2011)
Estrogen Receptors	G15	GPR30 antagonist	(Yu et al., 2015)
	G1	GPR30 agonist	(Yu et al., 2015)
	ICI 182,780	ER antagonist	(Saito et al., 2014)
	MPP	ERα antagonist	(Cheng et al., 2016)
	PPT	Erα agonist	(Cheng et al., 2016)