HIPPO signaling provides a fail-safe for resolving embryonic cell fate conflicts during establishment of pluripotency in vivo

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Abstract

In mammals, the first few days of development entail segregation of pluripotent and extraembryonic trophectoderm cells. The challenge for the embryo at this time is to override cell plasticity to ensure that cells adopt distinct fates. Here, we identify novel mechanisms restricting expression of *Sox2* and *Cdx2* to mutually exclusive domains. We show that *Sox2* is repressed in the trophectoderm downstream of ROCK1/2 and HIPPO pathway LATS1/2 kinases. LATS1/2 kinases are thought to antagonize YAP1 and WWTR1, the transcriptional partners of TEAD4. However, the combined loss of *Yap1; Wwtr1* has not been reported. Using female germ line deletion and null alleles, we show that YAP1/WWTR1/TEAD4 simultaneously antagonize *Sox2* and promote *Cdx2* expression in trophectoderm. However, *Cdx2* is less sensitive to *Yap1* and *Wwtr1* dosage, which can lead cells to aberrantly coexpress CDX2 and SOX2, reminiscent of conflicted cell fate. We show that HIPPO resolves cell fate conflicts by elevating *Sox2* expression and driving cells to the inner cell mass, but *Sox2* is not required for HIPPO-mediated cell repositioning. Rather, HIPPO signaling represses cell polarity components PAR and aPKC, facilitating cell internalization upstream of *Sox2*. We propose that HIPPO engages in negative feedback and PAR-aPKC providing a fail-safe to ensure lineage segregation.

Introduction

During embryogenesis, cells gradually differentiate, adopting distinct gene expression profiles and fates. In mammals, the first cellular differentiation is the segregation of trophectoderm and inner cell mass. The trophectoderm, which comprises the outer surface of the blastocyst, will mainly produce cells of the placenta, while the inner cell mass will produce pluripotent cells, which are progenitors of both fetus and embryonic stem cells. Understanding how pluripotent inner cell mass cells are segregated from non-pluripotent cells will therefore reveal how pluripotency is first induced in the biologically significant setting of the embryo.

The first cell fate decision has been studied mainly from the perspective of trophectoderm specification, because the transcription factor CDX2, which is expressed in and essential for trophectoderm development (Strumpf et al., 2005), has provided a way to distinguish future trophectoderm cells from non-trophectoderm cells (Beck et al., 1995) and identify the mechanisms that break the symmetry in the mouse embryo. For example, knowledge of CDX2 as a marker of trophectoderm cell fate enabled the discovery of mechanisms by which the HIPPO signaling pathway interprets cellular differences in polarity and position to restrict *Cdx2* expression to trophectoderm. Briefly, polarization of outer embryonic cells around the 16-cell stage leads to inactivation of HIPPO pathway kinases LATS1/2. LATS1/2 inactivation enables *Cdx2* expression in outer cells since LATS1/2 can antagonize activity of the transcriptional complex upstream of *Cdx2*, which is thought to include YAP1, WWTR1 and TEAD4 (Anani et al., 2014; Cockburn et al., 2013; Hirate et al., 2013; Kono et al., 2014; Korotkevich et al.,

2017; Leung and Zernicka-Goetz, 2013; Lorthongpanich et al., 2013; Mihajlović and Bruce, 2016; Nishioka et al., 2009, 2008; Posfai et al., 2017; Rayon et al., 2014; Watanabe et al., 2017; Yagi et al., 2007; Zhu et al., 2017). However, the specific requirements for *Yap1* and *Wwtr1* in regulation of cell fate has been inferred from overexpression of wild type and dominant-negative variants, neither of which provide the standard of gene expression analysis that null alleles can provide. Moreover, studies of *Cdx2* regulation do not provide direct knowledge of how pluripotent cells are first formed, because the absence of *Cdx2* expression does not necessarily indicate acquisition of pluripotency. As such, our knowledge of how symmetry is broken in the early mouse embryo is incomplete.

Progenitors of inner cell mass are first morphologically apparent at the 16-cell stage as unpolarized cells residing inside the morula (reviewed in Frum and Ralston, 2018). However, at this stage, pluripotency genes, such as *Oct4* and *Nanog*, are expressed in both trophectoderm and inner cell mass (Dietrich and Hiiragi, 2007; Niwa et al., 2005; Palmieri et al., 1994; Strumpf et al., 2005). Moreover, *Oct4* and *Nanog* only become restricted to inner cell mass in response to CDX2, which represses their expression in the trophectoderm (Strumpf et al., 2005). Therefore, despite the widespread usage of OCT4 and NANOG as pluripotency markers, during the stages that pluripotent progenitors first emerge in the embryo, OCT4 and NANOG do not provide specific readouts of pluripotency.

In contrast to *Oct4* and *Nanog*, *Sox2* is expressed specifically in inside cells at the 16-cell stage and is therefore a specific marker of pluripotency (Guo et al., 2010; Wicklow et al., 2014). Importantly, expression of *Sox2* is not regulated by CDX2 (Wicklow et al., 2014), consistent with the suggestion that loss of *Cdx2* does not necessarily indicate acquisition of pluripotency. Thus, identifying how *Sox2* expression is regulated addresses the question of how symmetry is broken in the early mouse embryo from the unique perspective of when and how pluripotency is first established *in vivo*.

Results

Patterning of *Sox2* is ROCK-dependent

We sought to identify the mechanisms by which the patterned expression of SOX2 is achieved in the blastocyst. We therefore focused on how *Sox2* expression is normally repressed in the trophectoderm, to achieve the inside cell-specific SOX2 expression pattern. We previously showed that SOX2 patterning is normal in the absence of the trophectoderm factor CDX2 (Wicklow et al., 2014), suggesting that mechanisms that repress SOX2 in the trophectoderm act upstream of CDX2. Rho-associated, coiled-coil containing protein kinases (ROCK1 and 2) are thought to act upstream of *Cdx2* because embryos developing in the presence of a ROCK-inhibitor (Y-27632) exhibit greatly reduced *Cdx2* expression (Kono et al., 2014). Additionally, quantitative RT-PCR showed that *Sox2* mRNA levels are elevated in ROCKi-treated embryos (Kono et al., 2014), suggesting that ROCK1/2 repress expression of SOX2 in the trophectoderm. However, the role of ROCK1/2 in regulating the patterned expression of *Sox2* has not been investigated.

To evaluate the roles of ROCK1/2 in repressing *Sox2* expression in the trophectoderm, we collected 8-cell stage embryos prior to compaction (E2.5), and then cultured these either in control medium or in the presence of ROCK-inhibitor (Y-27632) for 24 hours (Fig. 1A). Embryos cultured in control medium exhibited normal cell polarity, evidenced by the apical localization of PARD6B in outside cells, and the basolateral localization of E-cadherin (CDH1) in both outside and inside cells (Fig. 1B, C) as expected (Vestweber et al., 1987; Vinot et al., 2005). Additionally, SOX2 was detected only in inside cells in control embryos (Fig. 1C, D), as expected (Wicklow et al., 2014). By contrast, embryos cultured in ROCK inhibitor exhibited defects in cell polarity (Fig. 1B', C'), consistent with prior studies (Kono et al., 2014). Moreover, ROCK-inhibited embryos exhibited ectopic *Sox2* expression in outside cells (Fig. 1C', D). These observations strongly suggest that ROCK1/2 regulate expression of *Cdx2* and *Sox2*, promoting expression of the former, and repressing the latter, in the trophectoderm.

YAP1 is sufficient to repress expression of SOX2 in the inner cell mass

We next investigated how ROCK1/2 repress expression of SOX2 in outside cells. ROCK1/2 kinases are required for nuclear localization of YAP1 (Kono et al., 2014), a transcriptional activator that partners with TEAD4 in the blastocyst (Nishioka et al., 2009). Thus, the gain of *Sox2* in outside cells of ROCKi-treated embryos could be due to loss of nuclear YAP1. Moreover, we previously showed that TEAD4 represses expression of *Sox2* in the trophectoderm (Wicklow et al., 2014), raising the possibility that YAP1 partners with TEAD4 to repress *Sox2* expression in the trophectoderm. To

test this hypothesis, we utilized a constitutively active variant of YAP1 (YAP1^{CA}). Normally, LATS-dependent phosphorylation of YAP1 on serine 112 leads YAP1 to interact with 14-3-3 protein and become tethered in the cytoplasm. Therefore substitution of alanine at serine 112 (YAP1^{S112A} or YAP1^{CA} hereafter) leads YAP1 to be constitutively nuclear and constitutively active (Dong et al., 2007; Nishioka et al., 2009; Zhao et al., 2007). We injected mRNAs encoding *YAP1^{CA}* and *GFP* into one of two blastomeres at the 2-cell stage, and then cultured these to morula or blastocyst stages (Fig. 1E). Mosaic overexpression permitted comparison of *YAP1^{CA}*-overexpressing and non-injected cells, which served as internal negative controls. We first examined localization of YAP1 in these embryos at the morula stage, with the expectation that YAP1 would be detected in nuclei of both inside and outside cells in *YAP1^{CA}*-overexpressing cells (Nishioka et al., 2009). As expected, YAP1 was observed in nuclei of all *YAP1^{CA}*-overexpressing cells (Fig. 1F, G). By contrast, YAP1 was detected in nuclei only in outside cells. Additionally, pYAP1, which is normally cytoplasmic in inside cells, was also observed in nuclei of *YAP1^{CA}*-overexpressing cells, suggesting that *YAP1^{CA}* can still be phosphorylated on intact residues. These observations confirmed that S112 is essential for cytoplasmic retention of YAP1.

Having confirmed that YAP1^{CA} exhibits constitutively nuclear localization, we next evaluated the consequences of ectopic nuclear YAP1 on expression of SOX2 in inside cells. We observed that the majority of *YAP1^{CA}*-overexpressing cells (92%) exhibited no detectable SOX2 (Fig. 1G, H). Therefore, nuclear localized YAP1 is sufficient to repress *Sox2* expression in the inner cell mass. These observations, together with our previous

work (Wicklow et al., 2014) suggest that, YAP1/TEAD4 normally repress *Sox2* in trophectoderm and that a key determinant in *Sox2* repression is the nuclear localization of YAP1.

YAP1 and WWTR1 restrict *Sox2* expression to the inner cell mass

Our observation that nuclear YAP1 is sufficient to repress expression of *Sox2* in the inner cell mass raised the possibility that *Yap1* is required to repress expression of *Sox2* in in outside cells. However, it was previously reported that *Yap1* null embryos survive until E9.0 (Morin-Kensicki et al., 2006) and exhibit normal *Cdx2* expression at the blastocyst stage (Nishioka et al., 2009), suggesting that other factors compensate for loss of *Yap1* during preimplantation development. Accordingly, the closely related gene *Wwtr1*, which is coexpressed with *Yap1* in the blastocyst (Varelas et al., 2010), has been proposed to compensate for the loss of *Yap1* during preimplantation development (Nishioka et al., 2009). However, the *Yap1; Wwtr1* double knockout phenotype has not been reported.

To generate *Yap1; Wwtr1* double knockout embryos, we mated mice carrying null alleles of *Yap1* and *Wwtr1*, generated by CRE-mediated deletion of each conditional allele (Xin et al., 2013, 2011) (see Methods), and then examined embryos at E3.25. Surprisingly, CDX2 expression appeared normal in *Yap1; Wwtr1* double knockout embryos (Fig. 2A-D). In contrast, SOX2 was detected in most cells of *Yap1; Wwtr1* double knockout embryos, indicating that *Yap1* and *Wwtr1* are required to repress expression of SOX2 in outside cells. *Yap1* null embryos carrying only a single functional

copy of *Wwtr1* (or vice versa, *Wwtr1 -/-; Yap1 +/-*) exhibited minor defects in SOX2 expression, with ectopic SOX2 detected in a few outside cells (Fig. 2B, C, arrowheads). These observations indicate that SOX2 expression is normally repressed in outside cells in a *Yap1/Wwtr1*-dependent manner and indicate that regulation of *Sox2* expression is more sensitive to *Yap1/Wwtr1* dose than is the regulation of *Cdx2* expression.

Oocyte-expressed *Yap1* has been shown to be required for *Cdx2* expression in preimplantation embryos (Yu et al., 2016). We therefore hypothesized that maternally provided *Yap1* and/or *Wwtr1* contribute to regulation of *Cdx2* and *Sox2*. To test this hypothesis, we deleted *Yap* and *Wwtr1* from the female germ line by generating mice carrying conditional alleles of *Yap* and *Wwtr1* in the presence of the female germ line-specific *Zp3Cre* (de Vries et al., 2000). We then crossed these females to males carrying null alleles of *Yap1* and *Wwtr1.* From these crosses, we obtained embryos lacking maternally provided *Yap1* and *Wwtr1* and carrying all expected combinations of maternal and zygotic alleles (Table 1). Interestingly, embryos lacking maternal *Yap1* and *Wwtr1* and carrying wild type alleles of both zygotic *Yap1* and *Wwtr1* exhibited normal CDX2 and SOX2 expression patterns (Fig. 2E), arguing that maternal *Yap1* and *Wwtr1* are not required in the presence of zygotically expressed *Yap1* and *Wwtr1*. This observation contrasts with the published observation that expression of CDX2 is lost in the absence of maternal *Yap1* (Yu et al., 2016). However, we observed that loss of maternally provided *Yap1 and Wwtr1* worsened the *Yap1* zygotic null SOX2 phenotype (Fig. 2F compare to B). Conversely, loss of maternal *Yap1* and *Wwtr1* worsened the

Wwtr1 zygotic null SOX2 phenotype (Fig. 2G compare to C). Meanwhile, CDX2 expression appeared normal in embryos of these genotypes. These results are consistent with our proposal that regulation of SOX2 is more sensitive to the dose of *Yap1/Wwtr1* than is CDX2. In fact, CDX2 expression was disrupted only by loss of both maternal and zygotic *Yap1* and *Wwtr1* (Fig. 2H). We therefore conclude that *Yap1* and *Wwtr1* are functionally equivalent and that maternal *Yap1* and *Wwtr1* are genetically redundant with zygotic *Yap1* and *Wwtr1*. Additionally, these observations provide the first loss of function evidence using null alleles to show that YAP and WWTR1 promote expression of *Cdx2* while repressing expression of *Sox2* in the trophectoderm.

YAP1 and WWWTR1 maintain outside cell positioning

To examine the roles of *Yap1* and *Wwtr1* in blastocyst development we examined embryos lacking *Yap1* or *Wwtr1* at E3.75. We again observed a range of phenotypes, correlating with dose of *Yap1* and *Wwtr1*. Embryos with intact maternal *Yap1* and *Wwtr1* and at least two zygotic copies of *Yap1* or *Wwtr1* appeared normal, with CDX2-positive trophectoderm encapsulating a cavity containing SOX2 positive ICM cells (Fig. 3A). By contrast, embryos with intact maternal *Yap1* and *Wwtr1*, but with only one functional copy of zygotically-expressed *Wwtr1* exhibited low levels of ectopic SOX2 in trophectoderm cells (Fig. 3B, white arrowheads). Ectopic SOX2 was not detected in this genotype at E3.25, suggestive of rescue by maternal *Yap1* and *Wwtr1* at the earlier time point. This genotype was phenocopied in embryos that were lacking maternal *Yap1* and *Wwtr1* and were heterozygous for both *Yap1* and *Wwtr1* (Fig. 3C, white arrowheads). A more severe phenotyped was observed in embryos lacking maternal

Yap1 and *Wwtr1* with only one functional zygotic allele of *Yap1* or *Wwtr1*, which did not form a blastocyst and, in addition to exhibiting ectopic SOX2, contained CDX2 negative cells in the outside position (Fig. 3D, E, yellow arrowheads). Strikingly, the number of outside cells appeared severely reduced in embryos lacking maternal *Yap1* and *Wwtr1* with only one functional zygotic allele of *Yap1* or *Wwtr1*, suggesting that *Yap1* and *Wwtr1* are essential for maintaining outside cell position.

To quantify these phenotypes, we grouped embryos according to the number of functional doses of *Yap1* and *Wwtr1*, regardless of maternal or zygotic source, counting the maternal contribution of *Yap1* and *Wwtr1* as a single functional dose, and then compared the numbers of outside and inside cells among embryos with one, two, or more than two functional doses of *Yap1* and *Wwtr1*. To identify inside versus outside cell position, we used the cell polarity marker CDH1 and defined inside cells as both appearing internal and as having uniform CDH1 staining around their entire cell membrane. In some cases, cells appearing internal, but lacking uniform CDH1 staining were present (Fig. 3D, yellow arrowheads) and were therefore counted as outside positioned. This criterion may have led us to overestimate the number of outside cells, but in spite of this, we observed a major decrease in the number of outside cells and an increase in the number of inside cells in embryos with as *Yap1* and *Wwtr1* dosage decreased (Fig. 3F, G). Finally, we quantified expression of SOX2 and CDX2 among embryos of these genotypic categories. Ectopic SOX2 was detected in the outside cells of embryos with two or fewer function copies of Yap1 and *Wwtr1.* However, CDX2 expression followed a slightly different trend. While CDX2 was present in all outside

cells in embryos with 2 or more functional alleles of *Yap1* and *Wwtr1*, some outside cells were also CDX2-negative in embryos with only 1 functional allele of *Yap1* and *Wwtr1* (Fig 3H). These observations are consistent with the differential sensitivities of *Cdx2* and *Sox2* to the dose of *Yap1* and *Wwtr1* observed at earlier stages and demonstrate that *Yap1* and *Wwtr1* are required to maintain cell position on the surface of the embryo.

LATS kinase is sufficient to induce inside cell positioning

The observation that *Yap1* and *Wwtr1* are essential for maintaining cell positioning on the surface of the embryo led us to hypothesize that HIPPO signaling, which represses YAP1/WWTR1, maintain inside positioning within the embryo. We therefore aimed to hyperactivate HIPPO signaling in the embryo, which is typically achieved by overexpression of *LATS2* kinase (Nishioka et al., 2009). We previously showed that by injection of *Lats2* mRNA into both blastomeres of the 2-cell stage embryos leads all cells to induce expression of *Sox2* (Wicklow et al., 2014), consistent with the notion that HIPPO signaling induces inner cell mass fate. However, since all cells are converted to inner cell mass following *Lats2* overexpression, these experiments do not enable us to know whether *Lats2*-overexpressing cells would adopt an inside position were they in the context of a wild type embryo. We therefore injected only one of two blastomeres with *Lats2* mRNA, and then evaluated their gene expression and positioning at later stages (Fig. 4A). We observed that almost all *Lats2*-overexpressing cells ended up contributing to the inner cell mass (Fig. 4B, C, G). Notably, SOX2 was detected in all *Lats2*-overexpressing cells observed within the inner cell mass (Fig. 4D), suggesting

that *Lats2*-overexpressing cells were not only localized to the inner cell mass but had also acquired an inner cell mass fate.

The increased prevalence of *Lats2*-overexpressing cells in the inner cell mass was also associated with a striking underrepresentation of *Lats2*-overexpressing cells within the trophectoderm and a decrease in the number of outside cells compared to embryos injected with *GFP* mRNA alone (Fig. 4B, C, E), suggesting that *Lats2* overexpression led cells to move to the inside or to die on the outside. Consistent with this latter possibility, a smaller proportion of *Lats2*-overexpressing cells were observed in the total embryo population than in embryos in which one blastomere was injected with *GFP* mRNA only (Fig. 4C). Moreover, *Lats2* overexpression led to formation of cellular fragments within the trophectoderm (Fig. 4B, yellow arrowheads), suggestive of cell death among *Lats2*-overexpressing outside cells. Interestingly, SOX2 was detected in rare outside *Lats2*-overexpressing cells (Fig. 4D). Therefore, LATS2 is sufficient to induce expression of SOX2, regardless of cell position within the embryo. Notably, the kinase-dead variant of LATS2, which functions as a dominant-negative (Nishioka et al., 2009), did not alter the positioning, survival, or *Sox2* expression status of injected cells (Fig. 4F,G), consistent with a previous report (Posfai et al., 2017). Therefore, *Lats2*-overexpression acts on cell position and cell fate through target proteins normally phosphorylated by LATS2 in response to HIPPO signaling.

To pinpoint when *Lats2*-overexpressing cells come to occupy the inside of the embryo, we performed a time course, examining the position of injected and non-injected cells

from the 16-cell to the blastocyst stage (up to 80 cells). Interestingly, between the 16 and 32-cell stages, the proportion of injected and non-injected cells in the total, outside, and inside cell populations were comparable whether embryos had been injected with *Lats2* and *GFP* or *GFP* mRNA alone (Fig. 4H-J). In embryos injected with *GFP* mRNA alone, the proportion of injected and non-injected cells in the total, outside and inside cell populations remained constant throughout the time course. In contrast, starting after the 32-cell stage, the average proportion of injected *Lats2*-overexpressing cells occupying the inside position increased dramatically. The *Lats2*-overexpressing cells increased the total number of inside cells in the ICM of embryos injected with *Lats2/GFP* mRNA compared to embryos injected with GFP mRNA only, without a significant decrease in the number of non-injected cells in the ICM, arguing that the non cell-autonomous effects of *Lats2*-overexpression on the positioning of non-injected cells are minimal (Fig 4K). The increase in the proportion of *Lats2*-overexpressing cells in the inside position was concurrent with a severe decrease in the proportion of *Lats2*-overexpressing cells occupying the outside position, consistent with internalization of *Lats2*-overexpressing cells after the 32-cell stage. Additionally, *Lats2*-injected cells became underrepresented as a proportion of the total embryo population after the 32-cell stage, lending further support to the idea that *Lats2*-overexpressing cells that fail to internalize undergo cell death. We therefore conclude that *Lats2* overexpression acts on cell position and survival around the time of blastocyst formation.

LATS2 acts through YAP1 to induce inner cell mass fate

To examine the mechanism by which LATS2 overexpression drives cells into the inner cell mass, we first asked whether LATS regulation of YAP1/WWTR1 mediates cell positioning. To evaluate this possibility, we co-overexpressed mRNAs encoding *Lats2* and *YAP1*^{CA}. We predicted that, if *Lats2*-overexpression drives cells to adopt inner cell mass fate by repressing YAP1/WWTR1, then co-overexpression of *Yap1*^{CA} would enable *Lats2*-overexpressing cells to contribute to trophectoderm.

We injected mRNAs encoding *Yap1*^{CA} and *Lats2* into one of two blastomeres at the two-cell stage, and then examined cell positions at the blastocyst stage (Fig 5A). Consistent with our hypothesis, *Yap1*^{CA}-overexpression led to a significant decrease in the proportion of *Lats2*-overexpressing cells contributing to the inside cell position (Fig 5B-D). Additionally, *Yap1*^{CA}-overexpression significantly increased the proportion of *Lats2*-overexpressing cells remaining in the outside position (Fig 5D-F). Therefore, because overexpression of *Yap1*^{CA} attenuated the effects of *Lats2*-overexpression on cell positioning and cell survival, we propose that LATS2 alters cell position and cell survival by regulating YAP1, and possibly WWTR1, activity.

LATS2 induces positional changes independent of *Sox2*

Our observation that *Lats2*-overexpression induces expression of SOX2 and leads cells to adopt inner cell mass fate prompted us to investigate whether SOX2 helps drive cell position, downstream of *Lats2*. In support of this hypothesis, SOX2 has been proposed to determine inner cell mass fate (Goolam et al., 2016; White et al., 2016). We therefore investigated whether *Sox2* is required for the inner cell mass-inducing activity of LATS2

by overexpressing *Lats2* in embryos lacking *Sox2* (Fig. 5G). Surprisingly, however, *Lats2*-overexpressing cells were equally likely to occupy inside position in the presence and absence of *Sox2* (Fig. 5H, I). Moreover, *Lats2*-overexpressing cells were equally unlikely to occupy outside position in the presence and absence of *Sox2* (Fig. 5H, I) (Fig. 5H, J). Therefore, although *Lats2* overexpression induces expression of SOX2, LATS2 acts on cell positioning/survival upstream of, or in parallel to, *Sox2.*

LATS2 antagonizes formation of the apical domain

Cell position in the preimplantation mouse embryo has been proposed to be determined by differential inheritance of apically localized of membrane components that pattern myosin activity to either constrict future ICM cells to the inside of the embryo, or to maintain the position of future trophectoderm cells on the embryo surface (Anani et al., 2014; Korotkevich et al., 2017; Maître et al., 2016, 2015; Samarage et al., 2015; Zenker et al., 2018). Indeed, the apical membrane component aPKC is required for maintaining outside cell positon (Dard et al., 2009; Hirate et al., 2015; Plusa et al., 2005) and fate (Alarcon, 2010). We hypothesized that, because *Lats2* overexpression led cells to adopt an inside position, that LATS2 antagonizes formation of the apical domain in outside cells before these cells are lost from the outer surface of the embryo.

To determine if the apical domain becomes disrupted by *Lats2* overexpression, we examined the localization of PARD6B and aPKCz in embryos at the 16 to 32-cell stages. While apical membrane components PARD6B and aPKCz were detected at the apical membrane of non-injected cells and cells injected with *GFP* only, most *Lats2*-

overexpressing cells lacked detectable PARD6B and aPKCz (Fig. 6A-D). Notably, PARD6B and aPKCz appeared downregulated, rather than mislocalized, suggesting that LATS2-overexpressing cells could be properly polarized. To evaluate cell polarization, we examined localization of CDH1, which was restricted to the basolateral membrane of both *Lats2*-overexpressing and non-injected cells (Fig. 6E). Moreover, other apically localized proteins were also properly localized in LATS2-overexpressing cells, including filamentous Actin (Fig. 6F), and phospho-ERM (pERM) (Fig. 6G). These observations demonstrate that *Lats2*-overexpressing outside cells are properly polarized, but that apical membrane components required for outside cell position and fate are repressed in *Lats2*-overexpressing cells, providing a mechanism by which HIPPO/LATS drives cell positioning and cell fate assignment.

Discussion

During preimplantation development, many lineage-specific transcription factors, including CDX2, OCT4, NANOG, and GATA6 are initially expressed throughout the embryo before their expression refines to their lineage-appropriate domains (Chazaud et al., 2006; Dietrich and Hiiragi, 2007; McDole and Zheng, 2012; Ralston and Rossant, 2008; Schrode et al., 2014; Strumpf et al., 2005). In striking contrast to these genes, SOX2 is never detected in outside cells (Wicklow et al., 2014), indicating that robust mechanisms must exist to prevent its aberrant expression in trophectoderm. In the present study, we show that the YAP1/WWTR1/TEAD4 complex exerts tight control over *Sox2* expression, downstream of cell polarization and HIPPO signaling, to ensure that *Sox2* is not expressed in the trophectoderm. This model contrasts with models

proposing that SOX2 acts in select embryonic cells as early as the 4-cell stage to dictate inner cell mass positioning and fate (Goolam et al., 2016; White et al., 2016). However, these studies did not evaluate the requirement for *Sox2* in regulating cell position, in contrast to the present study.

We have provided the first analysis of *Yap1* and *Wwtr1* loss of function using null alleles. Interestingly, we find that the complete absence of both *Yap1* and *Wwtr1* disrupts expression of *Cdx2* completely, in contrast to the *Tead4* null phenotype, in which low levels of Cdx2 are still detected (Nishioka et al., 2008). This observation suggests a role for maternal *Tead4* or *Tead* paralogues in promoting expression of *Cdx2*. We note that YAP1 and WWTR1, which repress *Sox2* expression, are not known to act as transcriptional repressors. Therefore, YAP1/WWTR1 may induce expression of an as-yet unidentified transcriptional repressor.

We have shown that *Sox2* expression is more sensitive to the dose of functional *Yap1* and *Wwtr1* alleles than is *Cdx2*. In other words, moderate decreases in *Yap/Wwtr1* cause derepression of *Sox2* expression without affecting *Cdx2* expression. The differing sensitivities of *Sox2* and *Cdx2* to *Yap1*/*Wwtr1* dose raise the possibility that intermediate doses of active YAP1/WWTR1 could yield cells that coexpress both SOX2 and CDX2. This proposal is consistent with the observation that CDX2 is detected in inside cells of the embryo during blastocyst formation (Dietrich and Hiiragi, 2007; McDole and Zheng, 2012; Ralston and Rossant, 2008). The coexpression of these lineage markers could be indicative of conflicted trophectoderm/inner cell mass fate

(Fig. 7A, B). Therefore, to ensure robust developmental transitions, embryos must have a mechanism for resolving such cell fate conflicts and nudging cells into their correct and final positions.

The timing of HIPPO-induced cell internalization coincides with loss of cell fate plasticity around the 32-cell stage (Posfai et al., 2017). This timing is likely due to formation of tight-junctions form between outside cells (Sheth et al., 1997), which reinforce differences in HIPPO signaling activity between inside and outside compartments of the embryo (Hirate and Sasaki, 2014; Leung and Zernicka-Goetz, 2013). However, cell divisions around the 32-cell stage temporarily disrupt inside and outside regions. Occasionally, mislocalized cells are caught in the act, as unpolarized cells with elevated HIPPO signaling located outside the embryo (Anani et al., 2014; Hirate et al., 2015). We propose two mechanisms by which such cells are eliminated from the trophectoderm (Fig. 7C).

First, a small proportion of conflicted cells undergo cell death. This is consistent with low baseline levels of apoptosis in cells around the 32-cell stage (Copp, 1978). Notably, cell lethality due to elevated HIPPO can be rescued by increasing levels of nuclear YAP1, suggesting that YAP1 activity normally provides a pro-survival signal to trophectoderm cells, consistent with the proposed role of YAP1 in promoting proliferation in non-eutherian mammals (Frankenberg, 2018). Moreover, deletion of *Sox2* did not rescue survival of outside cells in which HIPPO signaling was artificially elevated, arguing that HIPPO resolves cell fate conflicts upstream of lineage-specific gene expression.

Second, outside cells with elevated HIPPO signaling drive their own internalization. This is consistent with the observation that cells in which *Tead4* has been knocked down become internalized (Mihajlović et al., 2015). However, in contrast to *Tead4* knockdown, which preserves cell polarity, we show that LATS2 overexpression leads to repression of PAR/aPKC. Par/aPKC are required for outside cell positioning (Dard et al., 2009; Hirate et al., 2015; Plusa et al., 2005), but HIPPO signaling was not previously known to be capable of regulating the expression of Par/aPKC in the mouse early embryo. Intriguingly, in *Drosophila,* the *Lats* orthologue *Warts* has been shown to regulate the localization of Par/aPKC components during development (Keder et al., 2015; Lucas et al., 2013). Therefore, our observation of reduced Par/aPKC in *Lats2*-overexpressing cells is consistent with conserved regulation of the Par/aPKC complex by HIPPO signaling.

Our data show that, in addition to moving cells inside the embryo, *Lats2* overexpression antagonizes the epithelial character of outside cells by antagonizing PAR-aPKC complex formation. Therefore, the ability of LATS2 to antagonize the PAR-aPKC complex could point to the existence of an exit route for cells to delaminate from the outer trophectoderm epithelium, and then internalize. Alternatively, LATS2 could impact spindle orientation and increase the proportion of cells undergoing asymmetric division, via PAR-aPKC (Korotkevich et al., 2017; Plusa et al., 2005). Identifying the downstream mechanisms by which HIPPO drives cells to inner cell mass will be an exciting topic of future study.

Methods

Mouse strains and genotyping

All animal research was conducted in accordance with the guidelines of the Michigan State University Institutional Animal Care and Use Committee. Wild type embryos were derived from CD-1 mice (Charles River). The following alleles or transgenes were used in this study, and maintained in a CD-1 background: *Sox2*$^{tm1.1Lan}$ (Smith et al., 2009), *Yap*$^{tm1.1Eno}$ (Xin et al., 2011), *Wwtr1*$^{tm1.1Eno}$ (Xin et al., 2013), *Tg(Zp3-cre)93Knw* (de Vries et al., 2000). Null alleles were generated by breeding mice carrying floxed alleles and mice carrying ubiquitously expressed *Cre*, *129-Alpl*$^{tm(cre)Nagy}$ (Lomelí et al., 2000).

Embryo collection and culture

Mice were maintained on a 12-hour light/dark cycle. Embryos were collected by flushing the oviduct or uterus with M2 medium (Millipore). For embryo culture, KSOM medium (Millipore) was equilibrated overnight prior to embryo collection. Y-27632 (Millipore) was included in embryo culture medium at a concentration of 80 μM with 0.4% DMSO, or 0.4% DMSO as control, where indicated. Embryos were cultured at 37ºC in a 5% CO_2 incubator under light mineral oil.

Embryo microinjection

LATS2 and YAPS112A mRNA was synthesized from cDNAs cloned into the pcDNA3.1-poly(A)83 plasmid (Yamagata et al., 2005) using the mMESSAGE mMACHINE T7 transcription kit (Invitrogen). EGFP or nls-GFP mRNA were synthesized from EGFP cloned into the pCS2 plasmid or the nls-GFP plasmid (Ariotti et al., 2015) using the

mMESSAGE mMACHINE SP6 transcription kit (Invitrogen). mRNAs were cleaned and concentrated prior to injection using the MEGAclear Transcription Clean-Up Kit (Invitrogen). *Lats2*, *Lats2*^{KD} and *YAP*^{CA} mRNAs were injected into one blastomere of two-cell stage embryos at a concentration of 500 ng/µl, mixed with 350 ng/µl *EGFP* or *nls-GFP* mRNA diluted in 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA.

Immunofluorescence and Confocal Microscopy

Embryos were fixed with 4% formaldehyde (Polysciences), permeabilized with 0.5% Triton X-100 (Sigma Aldrich), and blocked with 10% FBS, 0.2% Triton X-100. Primary Antibodies used were: mouse anti-CDX2 (Biogenex, CDX2-88), goat anti-SOX2 (Neuromics, GT15098), rabbit anti-PARD6B (Santa Cruz, sc-67393), rabbit anti-PARD6B (Novus Biologicals, NBP1-87337), mouse anti-PKCζ (Santa Cruz Biotechnology, sc-17781), rat anti-CDH1 (Sigma Aldrich, U3254), mouse anti-YAP (Santa Cruz Biotechnology, sc101199), rabbit anti phospho-YAP (Cell Signaling Technologies, 4911), chicken anti-GFP (Aves, GFP-1020). Stains used were: Phallodin-633 (Invitrogen), DRAQ5 (Cell Signaling Technologies) and DAPI (Sigma Aldrich). Secondary antibodies conjugated to DyLight 488, Cy3 or Alexa Flour 647 fluorophores were obtained from Jackson ImmunoResearch. Embryos were imaged using an Olympus FluoView FV1000 Confocal Laser Scanning Microscope system with 20x UPlanFLN objective (0.5 NA) and 5x digital zoom. For each embryo, z-stacks were collected, with 5 µm intervals between optical sections. All embryos were imaged prior to knowledge of their genotypes.

Embryo Genotyping

To determine embryo genotypes, embryos were collected after imaging and genomic DNA extracted using the Extract-N-Amp kit (Sigma) in a final volume of 10 µl. Genomic extracts (1-2 µl) were then subjected to PCR using allele-specific primers (Table 2).

Acknowledgements

We are grateful to Dr. Hiroshi Sasaki for providing overexpression constructs, to Dr. Randy L. Johnson for providing Yap1 and Wwtr1 conditional alleles, and to Dr. Jason Knott for microinjection training. We also thank Dr. Ripla Arora and members of the Ralston Lab for comments. This work was supported by NIH R01 GM104009.

Competing Interests

The authors declare no competing interests.

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Figure Legends

Figure 1. ROCK1/2 and nuclear YAP1 repress expression of SOX2.

A) Experimental design schematic: embryos were collected at E2.5 or E3.5 and treated with ROCK inhibitor (ROCKi) or DMSO (control) for 24 hours.

B) Confocal images of apical (PARD6B) and basolateral (CDH1) membrane components in control and ROCKi-treated embryos. As expected, PARD6B and CDH1 are mislocalized to the entire cell membrane of all cells in ROCKi-treated embryos, demonstrating effective ROCK inhibition.

C) In control embryos, SOX2 is detected only in inside cells, while in ROCKi-treated embryos, SOX2 is detected in inside and outside cells.

D) Quantification of ectopic SOX2 detected in outside cells of control and ROCKi-treated embryos (p, student's t-test).

E) Experimental design: embryos were collected at E1.5 and one of two blastomeres injected with mRNAs encoding *YAP*^{CA} and *GFP*. Embryos were cultured 48 or 72 hours, fixed, and then analyzed by immunofluorescence and confocal microscopy.

F) Localization of GFP, YAP1, and pYAP1 in *YAP*^{CA}-injected embryos. In *YAP*^{CA}-overexpressing cells, pYAP1 and YAP1 were detected in nuclei of injected cells, regardless of position, demonstrating that *YAP*^{CA} evades HIPPO regulation as predicted.

G) In non-injected cells, SOX2 is detected in inside cells, but SOX2 is not detected in *YAP*^{CA}-overexpressing inside cells (arrowheads).

H) Across multiple embryos (n = 10), all non-injected inside cells express SOX2, whereas the vast majority of *YAP*^{CA}-injected inside cells fail to express SOX2.

Figure 2. *Yap1 and Wwtr1* are required to repress SOX2 expression in outside cells.

A-D) Loss of zygotic (Z) *Yap1* and *Wwtr1* affects SOX2, and not CDX2 expression. CDX2 appears normal in all E3.25 embryos examined, while SOX2 expression is derepressed in outside cells (arrowheads) as the number of functional copies of zygotic *Yap1* or *Wwtr1* alleles decreases (B, C), with ectopic SOX2 detected in most outside cells in the double knockout embryos (D).

E-H) Loss of maternal (M) and zygotic (Z) *Yap1* and *Wwtr1* reveals that maternal *Yap1* and/or *Wwtr1* promote *Cdx2* expression (H), and that at least one intact copy of zygotic *Yap1* and *Wwtr1* is sufficient for *Cdx2* expression at E3.25 (F and G). In contrast, repression of *Sox2* expression is more sensitive to dose of *Yap1* and *Wwtr1*, since ectopic SOX2 is detected in embryos with one intact copy of zygotic *Yap1* or *Wwtr1* (F,G).

Figure 3. *Yap1* and *Wwtr1* maintain outside cell positioning.

A) Non-mutant (embryos with maternal *Yap1* and *Wwtr1* intact and at least one functional allele of *Yap1* and *Wwtr1*) exhibit trophectoderm-specific expression of CDX2 and inner cell mass-specific expression of SOX2.

B) Embryos with maternal *Yap1* and *Wwtr1* intact and one zygotic functional allele of *Wwtr1* have normal CDX2 and low levels of ectopic SOX2 (white arrowheads).

C) Embryos lacking maternal *Yap1* and *Wwtr1* with a single functional copy of each of *Yap1* and *Wwtr1* exhibit normal CDX2 and low levels of ectopic SOX2 (white arrowheads).

D, E) Embryos lacking maternal *Yap1* and *Wwtr1* with only one zygotic copy of *Yap1* or *Wwtr1* fail to form a blastocyst and contain outside cells that lack CDX2 (yellow arrowheads), with ectopic SOX2 (yellow and white arrowheads).

F) The number of inside and outside positioned cells in embryos with indicated numbers of functional copy of *Yap1* or *Wwtr1*. The number of inside cells is significantly increased in embryos with 1 or 2 functional copies of *Yap1* or *Wwtr1*, compared to embryos with >2 functional alleles of *Yap1* or *Wwtr1*. The number of outside cells is significantly decreased in embryos with only 1 functional copy of *Yap1* or *Wwtr1*.

G) The ratio of inside to outside cells significantly increases as the number of functional *Yap1* or *Wwtr1* copies are reduced.

H) Proportion of cells with indicated cell fate marker expression in the inside and outside populations of embryos with indicated numbers of functional copies of *Yap1* or *Wwtr1*. Repression of *Sox2* expression is more sensitive to *Yap1* and *Wwtr1* than is *Cdx2* expression.

Figure 4. LATS2 kinase is sufficient to direct cells to inner cell mass fate.

A) Schematic of *Lats2* overexpression experiments: embryos were collected at E1.5 and one of two blastomeres was injected with mRNAs encoding *Lats2* and *GFP* or *GFP* alone. Embryos were cultured for 72 hours, fixed, and then analyzed by immunofluorescence and confocal microscopy.

B) Cells injected with *GFP* contributed to trophectoderm and inner cell mass, while cells injected with *Lats2* and *GFP* contributed almost exclusively to the inner cell mass, and only cellular fragments were observed in the trophectoderm (arrows), suggestive of cell death (n = number of embryos examined).

C) Proportion of inside, outside, and total cell populations across multiple embryos, which were comprised of non-injected cells, or cells injected with either *GFP* or *GFP/Lats2* mRNAs. Cells injected with *GFP/Lats2* were overrepresented within the inside cell population and underrepresented in the outside and total cell populations, relative to cells injected with *GFP* alone (P, chi-squared test).

D) Percentage of SOX2-positive cells within non-injected and *GFP*-injected or *Lats2/GFP*-injected populations observed inside and outside of the embryo. SOX2 was detected in all of the Lats2/GFP-injected inside cells, and in half of the rare, *Lats2/GFP*-injected outside cells (same number of embryos as in panel C) (p, student's t-test).

E) Average number of cells per embryo. The average number of outside cells is reduced in embryos injected with *Lats2/GFP*, relative to *GFP*-injected (p, student's t-test).

F) *Lats2*^{KD}*/GFP*-injected cells contribute to trophectoderm and inner cell mass, and do not alter expression of SOX2 (n = number of embryos examined).

G) Contribution of injected cells to inside and outside compartments of the embryos such as those shown in panels B and F. Unlike *Lats2*-injected cells, *Lats2KD*-injected cells do not tend to localize to the inside of the embryo (P, chi-squared test).

H) Proportion of *GFP* and *Lats2/GFP*-injected cells, relative to total cell number, over the course of embryo development (Solid lines = average of indicated data point and four previous data points).

I) Data as shown in panel H, shown relative to outside cell number.

J) Data as shown in panel H, shown relative to inside cell number.

K) Contribution of injected and non-injected cells to the inside cell population, following injection with *GFP* or *Lats2/GFP*. *Lats2/GFP*, contribute more cells to the inner cell mass, without reducing the average contribution of non-injected cells, resulting in larger overall inner cell mass size following *Lats2* overexpression (p, student's t-test).

Figure 5. LATS2 directs inner cell mass fate via YAP, but independently of *Sox2*

A) Experimental design: *Lats2/Yap1*^{CA} co-overexpression. Embryos were collected at E1.5 and one blastomere was injected with mRNAs encoding *Lats2, Yap1*^{CA}, and *GFP*, and embryos were examined 72 hours after injection (shown in panels B-F).

B) Cooverexpression of *Yap1*^{CA} and *Lats2* restores the ability to contribute to trophectoderm.

C) *Yap1*^{CA} reduces the tendency of *Lats2*-overexpressing cells to over-contribute to the inner cell mass.

D) Proportion of inside cells comprised of non-injected cells or cells injected with mRNAs indicated (same embryos quantified in panel C). *Yap1*^{CA} overexpression reduces the proportion of *Lats2*-overexpressing cells observed in the inner cell mass. (P, chi-squared test)

E) Proportion of outside cells comprised of non-injected cells or cells overexpressing the mRNAs indicated. *Yap1*^{CA}-overexpression increases the proportion of *Lats2*-overexpressing cells observed in the inner cell mass (P, chi-squared test).

F) Another example, showing that *Yap1*^{CA}-overexpression rescues the ability of *Lats2*-overexpressing cells to contribute to the trophectoderm.

G) Experimental design: *Lats2* overexpression in embryos lacking *Sox2*.

H) *Lats2*-overexpressing cells contribute to inner cell mass in spite of the absence of *Sox2*.

I) Proportion of non-injected cells and cells injected with the indicated mRNAs contributing to inner cell mass in the indicated genetic backgrounds. No significant differences were observed based on embryo genotype, indicating that *Sox2* is dispensable for inside positioning (P, chi-squared test).

J) Proportion of non-injected cells and cells injected with the indicated mRNAs contributing to trophectoderm in the indicated genetic backgrounds. No significant differences were observed based on embryo genotype (P, chi-squared test).

Figure 6. LATS2 antagonizes formation of the apical domain

A) PARD6B is detectable in *GFP*-overexpressing and in non-injected cells, but not in *Lats2*-overexpressing cells (n = number of embryos examined).

B) aPKCz is detectable in *GFP*-overexpressing and in non-injected cells, but not in *Lats2*-overexpressing cells (n = number of embryos examined).

C) Quantification of embryos shown in panel A.

D) Quantification of embryos shown in panel B.

E) CDH1 is localized to the basolateral membrane in both *Lats2*-overexpressing and non-injected cells (n = number of embryos examined).

F) Phalloidin staining showing that F-actin localization is indistinguishable in *Last2*-overexpressing and non-injected cells. (n = number of embryos examined)

G) pERM is localized to the apical membrane in both *Last2*-overexpressing and non-injected cells (n = number of embryos examined).

Figure 7. Resolution of cell fate conflicts in the preimplantation mouse embryo. A) At the 16-cell stage ROCK1/2 maintains the apical domain (purple), resulting in low LATS2 activity in outside cells, permitting levels of YAP1/WWTR1 activity that repress *Sox2* and promote *Cdx2* expression. In inside cells, LATS2 activity is higher, leading to lower activity of YAP1/WWTR1, which supports both *Cdx2* expression and *Sox2* expression, consistent with the observation that CDX2 and SOX2 are both detected in inside cells during this stage (Ralston and Rossant, 2008; Wicklow et al., 2014).

B) *Sox2* and *Cdx2* have different sensitivities to YAP1/WWTR1 activity, leading to co-expression of both lineage markers in cells with intermediate levels of HIPPO signaling.

C) During division from the 16- to the 32-cell stage, cells that inherit the apical membrane repress HIPPO signaling and maintain an outside position. However, cells that inherit a smaller portion of the apical membrane would initially elevate their HIPPO signaling. We propose that elevated HIPPO then feeds back onto polarity by further antagonizing PAR-aPKC complex formation, leading to a snowball effect on repression of *Sox2* expression, and thus ensuring that SOX2 is never detected in outside cells because these cells are rapidly internalized or apoptosed. The formation of tight

junctions coincides with the timing at which elevated HIPPO drives cells inside, consistent with the role of tight junctions in regulating HIPPO signaling (Hirate et al., 2013; Kono et al., 2014).

Table 1. Summary of embryos recovered from *Yap1*; *Wwtr1* germ line null females

Maternal Genotype: *Zp3Cre*/+ ; *Yap1^{fl}*/*Yap1^{fl}* ; *Wwtr1^{fl}*/*Wwtr1^{fl}* Paternal Genotype: *Yap1$^{\Delta}$*/+ ; *Wwtr1 $^{\Delta}$*/+	
Embryo Genotypes	Number observed (% of embryos)
Yap1$^{\Delta}$/*Yap1$^{\Delta}$* ; *Wwtr $^{\Delta}$*/*Wwtr1 $^{\Delta}$*	2 (22.2%)
Yap1$^{\Delta}$/+ ; *Wwtr $^{\Delta}$*/*Wwtr1 $^{\Delta}$*	2 (22.2%)
Yap1$^{\Delta}$/*Yap1$^{\Delta}$* ; *Wwtr $^{\Delta}$*/+	3 (33.3%)
Yap1$^{\Delta}$/+ ; *Wwtr $^{\Delta}$*/+	2 (22.2%)

Table 2. Allele-specific primers used for determining embryo and mouse genotypes

Allele Name	Primer Name	Primer Sequence	Reference
129-Alpl$^{tm(cre)Nagy}$	Cre-F	ATCCGAAAAGAAAACGTTGA	(Lomelí et al., 2000)
	Cre-R	ATCCAGGTTACGGATATAGT	
Sox2$^{tm1.1Lan}$	SOX2 FL/WT R	TGGAATCAGGCTGCCGAGAATCC	(Smith et al., 2009)
	SOX2 F	TCGTTCTGGCAACAAGTGCTAAAGC	
	SOX2 KO R	AGTACTTTGCTGCCTCTTTAA	
Tg(Zp3-cre)93Knw	oIMR1084	GCGGTCTGGCAGTAAAAACTATC	(de Vries et al., 2000)
	oIMR1085	GTGAAACAGCATTGCTGTCACTT	
Wwtr1$^{tm1.1Eno}$	TAZ FL/WT F	GGCTTGTGACAAAGAACCTGGGGCTATCTGAG	(Xin et al., 2013)
	TAZ FL/WT R	CCCACAGTTAAATGCTTCTCCCAAGACTGGG	
	TAZ KO FS	TGACAAAGAACCTGGGGCTA	
	TAZ KO RS	AACTGCTAACGTCTCCTGCC	
Yap$^{tm1.1Eno}$	YAP F	ACATGTAGGTCTGCATGCCAGAGGAGG	(Xin et al., 2011)
	YAP FL/WT R	AGGCTGAGACAGGAGGATCTCTGTGAG	
	YAP KO R	TGGTTGAGACAGCGTGCACTATGGAG	

Figure 1: Frum and Ralston

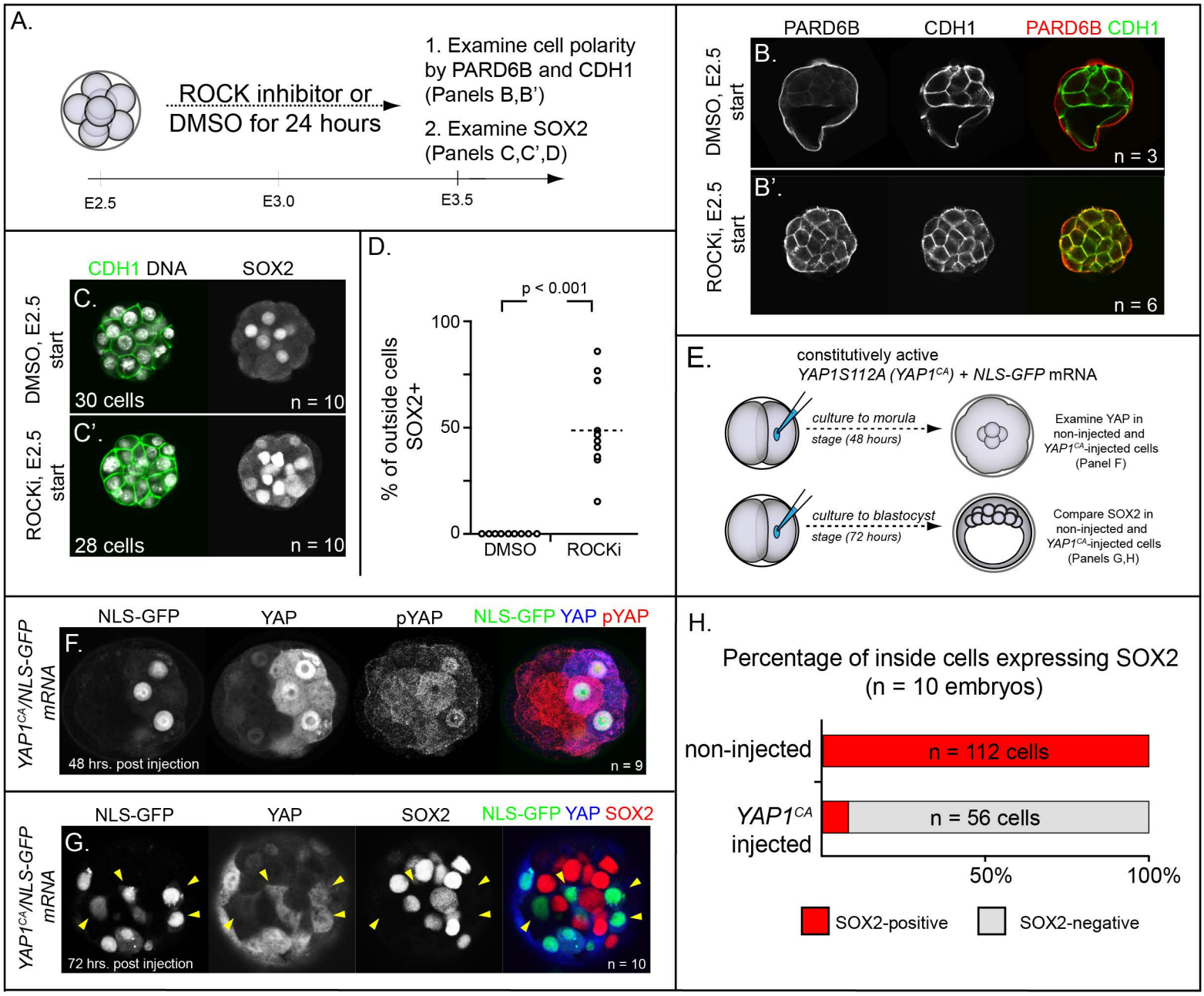

Figure 2. Frum and Ralston

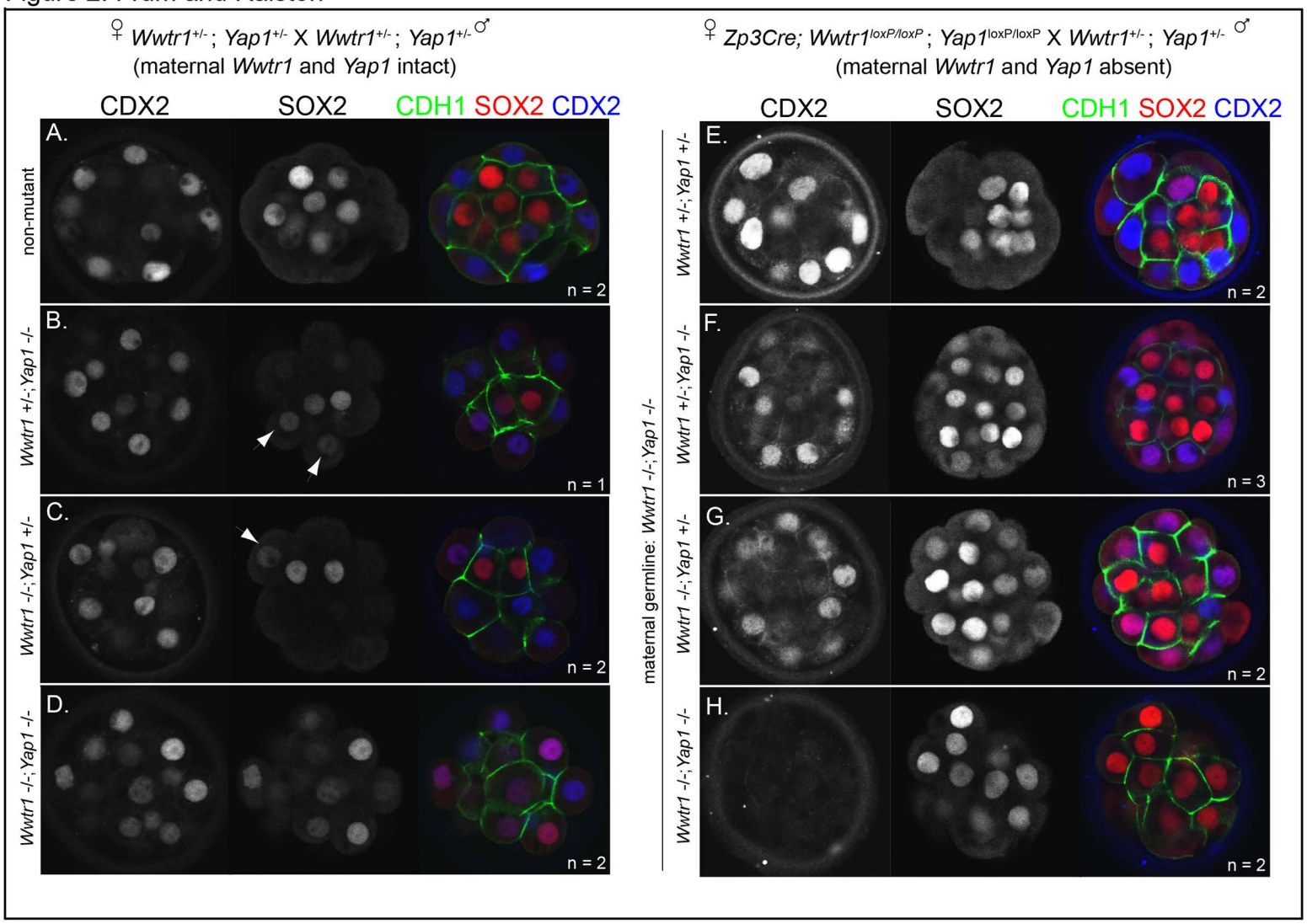

Figure 3. Frum and Ralston

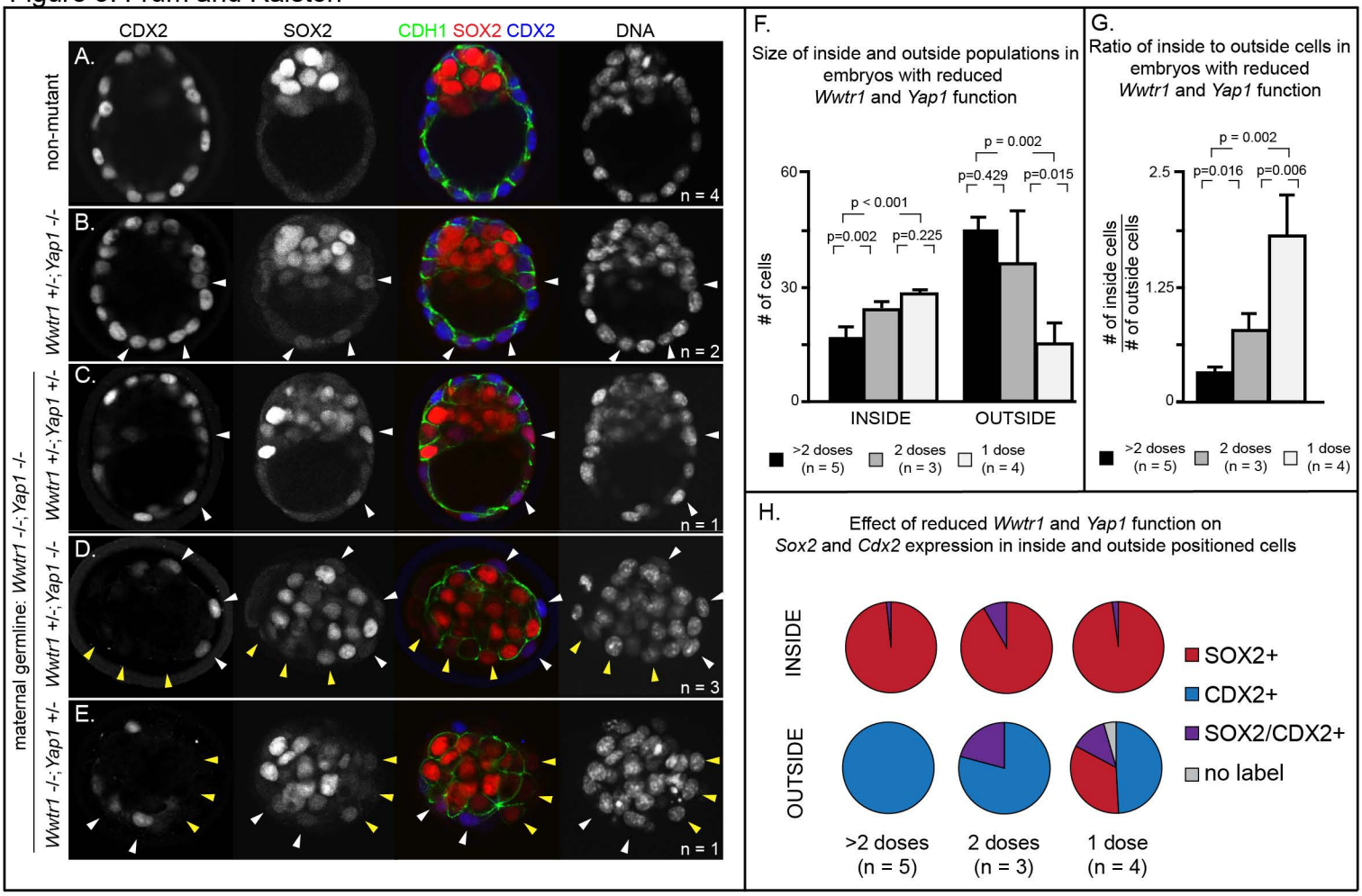

Figure 4. Frum and Ralston

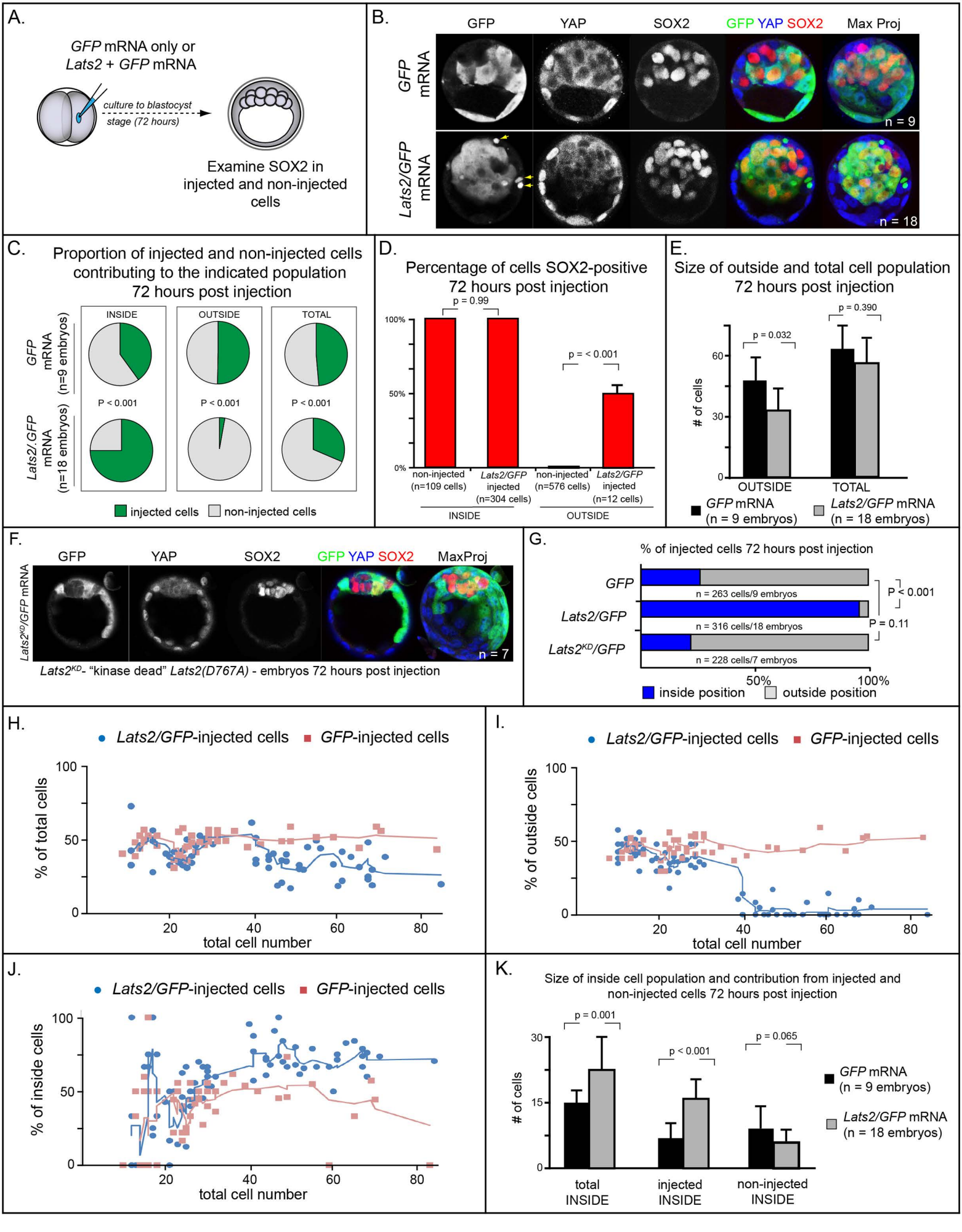

Figure 5. Frum and Ralston

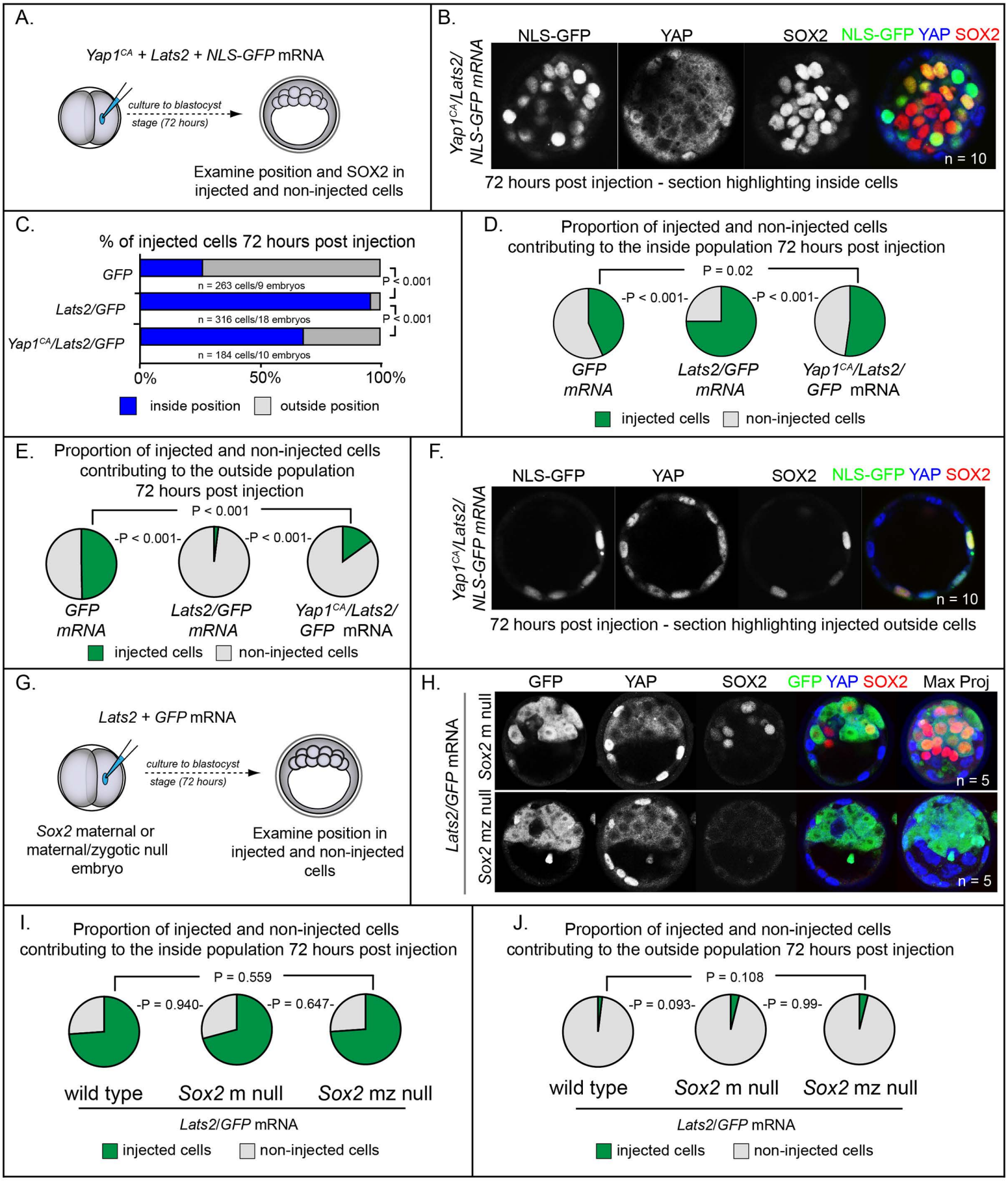

Figure 6. Frum and Ralston

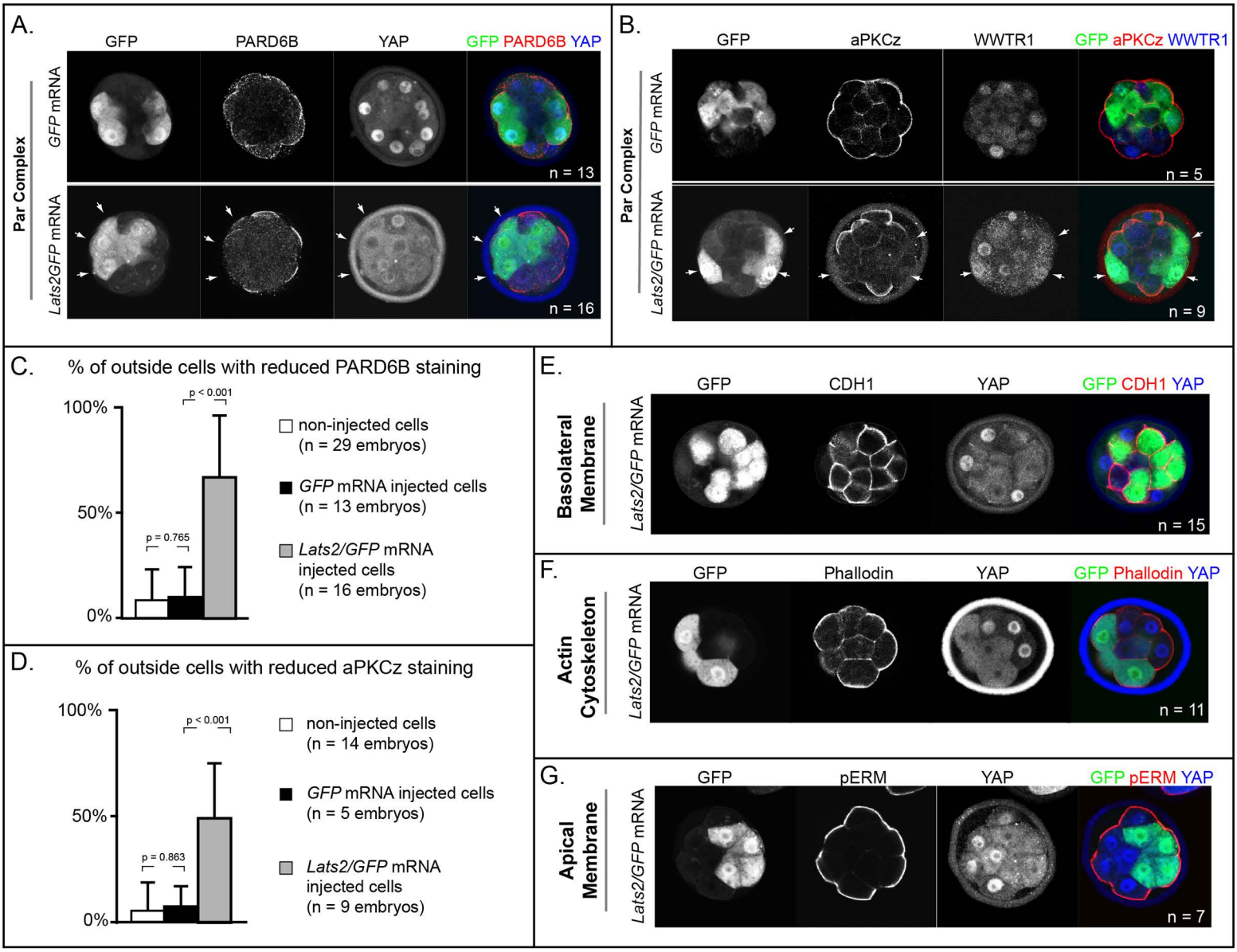

Figure 7. Frum and Ralston