Temporal reduction of LATS kinases in the early preimplantation embryo prevents ICM lineage differentiation

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Cellular localization of the Yes-associated protein [YAP] is dependent on large tumor suppressor [LATS] kinase activity and initiates lineage specification in the pre-implantation embryo. We temporally reduced LATS activity to disrupt this early event, allowing its reactivation at later stages. This interference resulted in an irreversible lineage misspecification and aberrant polarization of the inner cell mass (ICM). Complementation experiments revealed that neither epiblast nor primitive endoderm can be established from these ICMs. We therefore conclude that precisely timed YAP localization in early morulae is essential to prevent trophoderm marker expression in, and lineage specification of, the ICM.

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The first 4 d of mouse embryonic development set the stage for all embryonic and extraembryonic tissue formation. At the time of implantation, three distinct cell lineages—trophectoderm (TE), epiblast (EPI), and primitive endoderm (PrE)—are defined. Differential expression of lineage-specific transcription factors such as CDX2, OCT4/NANOG, and GATA6, respectively, drive and enforce these segregations [for review, see Rossant and Tam 2009], yet the upstream events driving the earlier segregation of TE and the inner cell mass (ICM), the common precursor of EPI and PrE, is the Hippo signaling pathway. The Hippo pathway has been associated with regulation of organ size, cell proliferation, and cancer in both Drosophila and mammals (Jia et al. 2003; Dong et al. 2007; Bando et al. 2009). In the mouse preimplantation embryo, the Hippo pathway is involved in cell fate determination, especially during inner/outer cell type formation at the morula stage (Nishioka et al. 2009). In its outer cells, the Hippo pathway is inactive, and Yes-associated protein [YAP] shuttles to the nucleus and binds TEAD4 to induce Cdx2 expression. Conversely, in inner cells, the extensive cell–cell contacts result in activation of the Hippo signaling pathway, causing cytoplasmic retention of YAP when it is phosphorylated by large tumor suppressor 1/2 (LATS1/2) kinases. Loss of LATS1/2 kinases apparently results in a developmental bias toward the TE-like lineage, as CDX2 is no longer restricted to the outer cells of the morula and TE of the blastocyst (Nishioka et al. 2009). However, the consequences of LATS1/2 kinase depletion and the ensuing CDX2 activation in ICM cells upon peri- and post-implantation development were never investigated in detail.

We showed previously that, when deprived of cell–cell interactions, blastomeres of the preimplantation embryo cannot adopt their respective future lineages. Many of these blastomeres grown in isolation randomly coexpress multiple lineage-specific markers, often including CDX2 (Lorthongpanich et al. 2012). Here we aimed to retain the intercellular architecture of the embryo but interrupt the “position-sensing” Hippo pathway to address lineage specification, differentiation, and pluripotency in early embryos. By injecting siRNA into mouse zygotes, we achieved temporary reduction of LATS1 and LATS2 kinases at early stages, which phenocopies the lineage misspecification of embryonic cells toward a TE-like lineage in the morula. As a consequence, the induced epithelialization of all embryonic cells and misexpression of lineage-specific genes result in failure of ICM lineage commitment and post-implantation development.

Results and Discussion

Early, transient inhibition of the Hippo signaling pathway causes post-implantation lethality

To address the function of the Hippo signaling pathway in early mouse embryos, we targeted LATS kinases by injecting siRNAs into zygotes and then culturing the embryos for 4 d. Efficient knockdown was observed for both Lats1 and Lats2 transcripts (Supplemental Fig. 1A,B). However, normal YAP phosphorylation (p-YAP) is detectable in the inner cells of treated morulae and blastocysts, comparable with non- or mock-injected (scrambled siRNA) controls (Supplemental Fig. 1C). Upon close examination, we found a dose-dependent up-regulation of Lats2 transcripts in Lats1 siRNA-injected embryos and vice versa throughout preimplantation development, suggesting functional redundancy and autoregulatory loops between Lats1 and Lats2 (Supplemental Fig. 1D,E). To overcome this redundancy, we established a double knockdown for Lats1 and Lats2 (Lats1/2 KD) in the embryo (Fig. 1A). Efficient knockdown was observed for both transcripts, however, mRNA levels slowly increased over time and...
were partially restored at the blastocyst stage [Fig. 1A]. The specificity and efficiency of both knockdowns was further confirmed on the protein level in NIH3T3 cells [Supplemental Fig. 2A,B]. Remarkably, however, even though efficient knockdown of both kinases was achieved, the embryos developed into morphologically normal-looking blastocysts in vitro [Fig. 1B].

To determine their in vivo developmental potential, we transferred Lats1/2-KD zygotes into one oviduct of a pseudopregnant female, while scrambled siRNA-treated embryos were transferred into the other. At the preimplantation stage [embryonic day 4.75 [E4.75]) control embryos had attached to the uterine wall and showed normal morphology [all of the 14 recovered of 20 transferred embryos] [Fig. 1C]. Lats1/2-KD embryos, however, formed atypical, collapsed clumps of cells, which nonetheless had initiated implantation [all of the 16 recovered of 20 transferred embryos] [Fig. 1C]. At E7.5, of 20 transferred control embryos, 17 had implanted and were morphologically normal. In contrast, of 20 transferred Lats1/2-KD embryos, 15 had implanted, yet only resorbed remnants were found in the respective deciduomas [Fig. 1D,E].

The lack of normal, developing embryos at E7.5 prompted us to investigate the capacity of Lats1/2-KD ICMs to give rise to embryonic stem cell (ESC) lines. When Lats1/2-KD blastocysts were cultured in ESC derivation medium, only trophoblast giant cells, but no ESC clumps, could be observed [0%, n = 0 of 15] [Fig. 1F]. In contrast, 67% [n = 8 of 12] of control blastocysts gave rise to ESC lines [Fig. 1F]. Taken together, these data suggest that depletion of Lats1/2 impairs ICM pluripotency and self-renewal.

Human and mouse LATS kinases are highly homologous and functionally conserved [Visser and Yang 2010] but differ sufficiently on the RNA level to render human LATS transcripts immune to the siRNA knockdown. Therefore, we decided to confirm the specificity of the double knockdown, exclude off-target effects, and rescue the phenotype by coinjecting expression plasmids encoding human LATS1 and/or LATS2 with mouse Lats1/2-siRNA into zygotes. These were transferred into pseudopregnant females and reisolated at E7.5. Noninjected controls were recovered with 88% efficiency, whereas only empty deciduae were found in Lats1/2-siRNA-injected embryos. In contrast, coexpression of LATS1, LATS2, or both rescued 25%, 44%, and 22% of the embryos, respectively [Fig. 1G; Supplemental Fig. 3].

Knockdown embryos fail to establish a normal ICM

The Lats1/2-KD phenotype is highly reminiscent of Oct4/Nanog knockout embryos, which form apparently normal blastocysts and implant but fail to maintain an embryo properly post-fertilization [Nichols et al. 1998; Mitsui et al. 2003]. We therefore addressed lineage specification on a molecular level at the preimplantation stages, focusing on the Hippo pathway, lineage marker gene expression, and cell polarization.

We first addressed Hippo pathway signaling by YAP/p-YAP staining and found, as expected, that knockdown of LATS1/2 resulted in a strong reduction of p-YAP in inner cells of the morulae and the ICM of the blastocyst [Fig. 2A]. This reduction coincides with ectopic activation of CDX2 in inner cells [Fig. 2A–C]. Unphosphorylated YAP localizes to the nucleus, where it induces CDX2 expression, enforcing TE differentiation [Nishioka et al. 2009]. In normal morulae, nuclear YAP colocalizes with CDX2 in outer cells only, whereas overlap of these proteins was found in all cells of Lats1/2-KD embryos [Fig. 2B]. In the E3.5 blastocyst, nuclear YAP staining is greatly reduced in the TE of both control and knockdown blastocysts, yet CDX2 expression is maintained. Similarly, although ICM cells in Lats1/2-KD embryos show only remnant nuclear YAP, CDX2 remains highly expressed in every cell. These results suggest that preventing YAP phosphorylation by Lats1/2-KD causes high nuclear accumulation of YAP in the inner cells of the morulae, which
CDX2 expressed in every cell of Lats1/2 in ICM and TE, respectively (Niwa et al. 2005). We found that antagonistic reciprocal repression then reinforces (Dietrich and Hiiragi 2007). It is generally accepted the late eight-cell stage, when lineage specification initiates sufficient to bias the embryonic cell fate toward a TE-like, CDX2-expressing cell lineage.

Normally, CDX2 is initially coexpressed with OCT4 at the late eight-cell stage, when lineage specification initiates (Dietrich and Hiiragi 2007). It is generally accepted that antagonistic reciprocal repression then reinforces a mutually exclusive OCT4 and CDX2 expression pattern in ICM and TE, respectively (Niwa et al. 2005). We found CDX2 expressed in every cell of Lats1/2-KD-derived, compacted morulae regardless of their inside/outside positioning [Fig. 2A-C]. Similarly, in blastocysts, all Lats1/2-KD embryonic cells, including ICM cells, express CDX2. Interestingly, however, OCT4 expression was also maintained in the ICM cells of Lats1/2-KD embryos [Fig. 2C]. Overall, CDX2 expression in Lats1/2-KD embryos suggests that even though the inside cells are physically present and expressing OCT4, the ICM cell fate—and thus pluripotency—is lost. To further address the phenotype of these OCT4/CDX2-positive ICMs, we studied Sox2 expression, another early inner cell-specific marker (Guo et al. 2010). Interestingly, Sox2 was absent in both Lats1/2-KD morulae and blastocysts, confirming the loss of “inner cell” characteristics (Supplemental Fig. 4). Coinjection of human LATS1/2, however, restored Sox2 expression, phosphorylation of YAP, localization of unphosphorylated YAP, and TE-restricted expression of CDX2 in most of the inside cells in the majority of rescued embryos [Supplemental Figs. 4, 5A–D].

Polarization of outer embryonic blastomeres at the morula stage is an early indication of TE commitment and epithelialization. At the blastocyst stage, TE cells remain polarized, whereas ICM cells are not. This polarization can be visualized by the asymmetric distribution of PRKCA (also known as atypical protein kinase C), which is tightly correlated with CDX2 expression (Plusa et al. 2005; Jedrusik et al. 2008). We frequently observed Lats1/2-KD ICMs with a flattened appearance [Fig. 2D, arrow], and the majority [80%; n = 16 of 20] showed a remarkable asymmetric distribution of PRKCA in ICM cells [Fig. 2D, arrow], with the remaining [20%; n = 4 of 20] being at least partially polarized. This is in clear contrast to unpolarized control ICMs and the restoration of an unpolarized ICM after rescue by LATS1/2 coinjection [Supplemental Fig. 5E]. We therefore suggest that ectopic activation of CDX2 and its downstream transcriptional network in the inner cells of morulae and blastocysts induces epithelial differentiation. To further confirm this observation, we studied the tight junction protein ZO1. Normally only expressed in the TE, we found additional, focal accumulation of ZO1 in ICMs of all analyzed Lats1/2-KD blastocysts [n = 8 of 8] but never in control ICMs [n = 0 of 10] [Fig. 2E].

Nonetheless, a morphologically distinct, OCT4-positive ICM is present in these embryos, even in expanded blastocysts. This observation puts a simple reciprocal repressive CDX2/OCT4 regulative network in doubt and further raises the question of the developmental potential of these cells.

Knockdown ICMs fail to differentiate into EPI and PrE

Normally, ICM cells of E3.5 blastocysts show mosaic, progressive complementary expression of the EPI marker NANOG versus the PrE marker GATA6, a segregation known as the “salt-and-pepper” model of EPI/PrE lineage determination (Chazaud et al. 2006; Plusa et al. 2008). A day later [at E4.5], these two distinct populations will sort out from each other and form the EPI or PrE marked by high NANOG or GATA6 levels, respectively [Plusa et al. 2008; Lanner and Rossant 2010; Kuikj et al. 2012].

In the absence of LATS1/2 kinase in the early morula, the ICM cells of the blastocyst express CDX2 ectopically but also retain OCT4 expression. To determine whether Lats1/2-KD ICMs retain the capacity to segregate into EPI or PrE, we examined NANOG and GATA6 expression. Remarkably, the typical, mutually exclusive expression of these two markers was lost in all Lats1/2-KD ICM cells; they all coexpressed the EPI-, PrE-, and TE-specific lineage markers NANOG, GATA6, and CDX2, respectively [Fig. 3A,B]. In control E4.5 embryos, GATA6-positive cells sorted to form a monolayer covering the EPI toward the blastocoel in all controls, yet such sorting was never observed in Lats1/2-KD embryos [Fig. 3B]. In contrast, the number of GATA6-positive cells in Lats1/2-KD ICMs was greatly reduced [1.6 ± 1.5 cells per embryo; n = 5 embryos] compared with the controls [9 ± 0.6 cells per embryo; n = 5 embryos].

We reasoned that Lats1/2-KD embryos could be developmentally delayed, and thus PrE segregation might still be ongoing. To address this hypothesis, we extended the period of preimplantation development by transferring two-cell stage [E1.5] Lats1/2-KD embryos into E0.5 pseudopregnant females to allow prolonged preimplantation development. Embryos were then collected at E4.75 [Fig. 3C]. We found the embryos had indeed developed further and contained more cells than in vitro-cultured embryos [Fig. 3D]. However, this extra time did not result in adequate EPI/PrE segregation. In contrast, we found a complete loss of GATA6 expression in these knockdown embryos compared with the strong expression and normal sorting in controls [Fig. 3C].
Proper PrE lineage induction and commitment requires fibroblast growth factor 4 (FGF4). It has recently been shown that this requirement is to promote PrE differentiation beyond the initial activation of GATA6 in the early blastocyst (Kang et al. 2013). Fgf4 knockout blastocysts are therefore highly reminiscent of the Lats1/2-KD embryos. FGF4 is provided by the EPI-committing cells within the ICM, while its receptor, FGFR2, is expressed by the PrE precursors and TE cells (Guo et al. 2010). We reasoned that Lats1/2-KD and misexpression of CDX2 could affect this network and indeed found a striking reduction of Fgf4 expression in these embryos, suggestive of a nonfunctional EPI lineage (Fig. 3H). A moderate reduction of Fgfr2 levels (60% of control) could be an indication of loss of its expression in PrE precursors but maintenance of its expression in TE cells [Fig. 3H].

Treatment of embryos with high levels of exogenous FGF4 blocks EPI differentiation but promotes a fate switch toward PrE [Yamanaka et al. 2010]. We therefore attempted to rescue PrE differentiation in Lats1/2-KD embryos by FGF4 supplementation during in vitro development from the eight-cell to the late expanded blastocyst stage [Fig. 3I]. In contrast to controls in which most cells lost NANOG but gained GATA6 and SOX17 expression in an FGF4 dose-dependent manner, treatment induced neither GATA6 nor SOX17 expression in Lats1/2-KD embryos [Fig. 3I]. One possible explanation could be that Lats1/2-KD embryos have lost their PrE formation potential, perhaps due to the loss or reduced expression of Fgfr2. Alternatively, the Lats1/2-KD inner cells are simply incapable of proper EPI/PrE segregation due to the multiple, aberrant lineage markers that they express. FGF4 expression in the ICM is also essential for continuous proliferation of polar TE and formation of the ectoplacental cone (Tanaka et al. 1998). Although Lats1/2-KD embryos are capable of inducing decidual reactions, we never observed ectoplacental cones in the implantation sites but only disorganized cellular remnants [Fig. 1D,E], further supporting an FGF4 loss in Lats1/2-KD embryos.

Failed rescue by embryo complementation

To resolve whether Lats1/2-KD embryo-derived ICMs have any developmental capacity, we performed embryo complementation experiments, supplying the knockdown cells with a wild-type niche, including endogenous signaling and cell contacts. GFP-positive, eight-cell stage morulae were aggregated with the untreated control or Lats1/2-KD, nonfluorescent eight-cell stage embryos [Fig. 4A]. We addressed how Lats1/2-KD cells would respond to exposure to extensive cell interactions with neighboring wild-type cells, in particular focusing on CDX2 expression across inner/outer cells of the aggregated embryo. Analysis 24 h after aggregation revealed reduction of CDX2 expression in the inner cells of controls and the wild type [Fig. 4B, dotted line], whereas CDX2 expression remains high in Lats1/2-KD embryo-derived cells [Fig. 4B, bottom row, solid line] despite the presence of wild-type cells, excluding non-cell-autonomous effects.

We next addressed the potential of Lats1/2-KD cells to contribute to cell lineages in chimeric blastocysts. In 56% of the embryos, Lats1/2-KD cells contributed to the TE and PrE [type I] [Fig. 4A], in 37.5% of the embryos, Lats1/2-KD cells contributed to the TE alone [type II], and in only 6.2% of the embryos was a contribution to the EPI found [type III]. We never observed exclusively Lats1/2-KD

We further examined the PrE capacity for lineage differentiation by analyzing the expression of the later endoderm marker SOX17 in E4.5 blastocysts [Fig. 3E]. Virtually all cells in Lats1/2-KD ICMs showed robust NANOG, but no SOX17, expression. In contrast, control embryos displayed two populations of either NANOG-positive or SOX17-positive cells [Fig. 3F-G]. Thus, ICMs of Lats1/2-KD embryos transiently express the early PrE marker GATA6, which largely overlaps with NANOG expression. However, GATA6 expression is rapidly lost, the second wave of GATA6 expression is never activated, and mature PrE markers are not expressed. The knockdown of Lats1/2 therefore causes long-lasting effects in the ICM lineage, preventing commitment to a mature PrE fate even though Lats1/2 expression is gradually recovering in the blastocyst by that time [Fig. 1A]. Importantly, GATA6 expression was restored in the expanded blastocyst upon coinjection of LATS1/2 and Lats1/2-siRNA into the zygote [n = 8 of 10] [Supplemental Fig. 5F].

Figure 3. EPI/PrE segregation failure in Lats1/2-KD ICMs. NANOG, GATA6, and CDX2 expression in in vitro-derived control and Lats1/2-KD E4.5 (A) and E4.5 (B) blastocysts, respectively. (C) GATA6 expression in delayed implantation E4.75 blastocysts. (D) Comparison of cell numbers of in vivo control but not Lats1/2-KD embryos (n = 5 for each treatment). (E) SOX17 expression was detected in E4.5 control but not Lats1/2-KD embryos (n = 15 per treatment). Number of NANOG-positive cells [8 ± 1 and 14 ± 1] (P) and SOX17-positive cells [6 ± 2 and 0] (G), found in ICM cells of control and knockdown E4.5 blastocysts, respectively. (F) Relative expression of Fgf4 and Fgfr2 in Lats1/2-KD or control E4.5 blastocysts. NANOG, GATA6, and CDX2 (J) and NANOG, SOX17, and CDX2 (J) immunodetection in control or Lats1/2-KD embryos after culturing in medium supplemented with increasing concentrations of FGF4. The insert shows an optical, transversal section of the ICM. Bar, 50 μm. (*) P < 0.05, Student’s t-test.
embryo before and after immunostaining. Bar, 50 μm. (control and a knockdown aggregation blastocyst. Arrows indicate Lats1/2 demarcate the PrE layer; arrowheads and solid lines indicate the demarcate inner cells derived from control/wild-type and CDX2 expression 1 d after aggregation. Dotted and solid lines Lats1/2 aggregating ESCs with we performed additional complementation experiments, knockdown-derived cells contributing to the PrE by loca-
tionally allocated to extraembryonic lineages. However, a competitive environment, knockdown cells were prefer-
entially directed to extraembryonic cell fate, as expected. ESCs are known to contribute only to the EPI lineage,
weakened ICM expressing OCT4 and NANOG at the blastocyst stage, but instead form a nonfunctional, morphologically nor-
mal ICM expressing OCT4 and NANOG at the blastocyst stage. Thus, the Hippo signaling pathway normally func-
tions to induce TE marker expression in outer cells, but additional position-defining mechanisms act to define the
ICM lineage. As a consequence, Hippo pathway inhibition by Lats1/2-KD results in long-lasting coexpression of
CDX2 and OCT4 in ICMs. This observation in turn indicates that the often hypothesized, mutually exclu-
sive negative feedback loop between Lats1/2-KD-derived cells localizing to the probable PrE position. (F) Lineage contribution of ESCs in a control E7.5 embryo before and after immunostaining. Bar, 50 μm.

**Conclusion**

Inhibition of the Hippo signaling pathway affects ICM lineage commitment, pluripotency, and gastrulation by interfering with the reading mechanism necessary to identify cell position and intercellular communication. As a consequence, despite correct spatial organization, the inner cells are unable to acquire proper ICM lineage fate and express the TE marker CDX2.

Remarkably, the inner cells of Lats1/2-KD morulae do not adopt a bona fide TE fate despite CDX2 expression but instead form a nonfunctional, morphologically normal ICM expressing OCT4 and NANOG at the blastocyst stage. Thus, the Hippo signaling pathway normally functions to induce TE marker expression in outer cells, but additional position-defining mechanisms act to define the ICM lineage. As a consequence, Hippo pathway inhibition by Lats1/2-KD results in long-lasting coexpression of CDX2 and OCT4 in ICMs. This observation in turn indicates that the often hypothesized, mutually exclusive negative feedback loop between Cdx2 and Oct4 is likely an oversimplification.

Lats1/2-KD cells are incapable of forming or even contributing to EPI or PrE lineages. The atypical polarization of PRRCA in ICM cells suggests that Hippo pathway inhibition results in activation of an epithelial program. This likely causes the sorting of Lats1/2-KD-derived cells to the PrE location in chimera complementation experiments despite their failure to achieve proper endoderm differentiation. Interestingly, the ICM cells of knockdown embryos display a first, cell-autonomous wave of GATA6 expression. However, it seems their undefined state precludes progression of a subset of ICM cells to mature EPI, resulting in the failure of the second, non-cell-autonomous progression to PrE in neighboring cells. Likewise, potential PrE precursors lose the ability to respond to FGF4 signaling presumably because FGF2R expression is never induced. Just as blastomeres growing in isolation express an undefined mixture of early lineage markers with a propensity to CDX2 expression [Lorthongpanich et al. 2012], inhibition of the Hippo pathway results in aberrant coexpression of TE and ICM lineage-specific transcription factors in the ICM. Both manipulations trap cells in contradictory, incompatible developmental programs, resulting in developmental failure. This transient early prevention of YAP phosphory-
lation demonstrates that a timely, brief disturbance in lineage commitment causes irreversible, long-lasting developmental defects.

**Materials and methods**

*Embryo collection, culture, and transfer*

Embryos were collected from superovulated B6D2F1 females as previously described [Lorthongpanich et al. 2008]. For exogenous FGF4 treatment, different concentrations of FGF4 [R&D Systems] were used [Yamanaka et al. 2010]. All mouse work was approved by the BRC IACUC [A*STAR, Biopolis].
RNAi and microinjection
SMARTpool siRNA oligonucleotides to mouse Lats1 (L-063467-00-0005) and Lats2 (L-044602-00-0005) were purchased from Dharmacon. The siRNAs were microinjected at ~10 pL of siRNA into zygotes and analyzed at pre- and post-implantation stages.

RNA preparation and real-time PCR
RNA was extracted from pools of three embryos of different stages. Total RNA extraction and real-time PCR were done as previously described (Lorthongpanich et al. 2012).

Immunocytochemistry
Embryo immunofluorescence staining was performed as previously described (Dietrich and Hiiragi 2007). Primary antibodies include those to OCT4 (1:250, Santa Cruz Biotechnology), NANOG (Bechtel Laboratories), CDX2 (1:250, BioGenex), PRKCA (1:200, Santa Cruz Biotechnology), ZO1 (1:200, Invitrogen), p-YAP (1:200, Cell Signaling Technology), YAP (1:200, 4912S, Cell Signaling Technology), Sox17 (1:200, R&D Systems), and GATA6 (1:250, Santa Cruz Biotechnology).

Rescue experiment using hLATS2
Various concentrations of hLATS1 and hLATS2 plasmids (SW Chan and W Hong, unpubl.) were coinjected with SMARTpool Lats1/2 siRNAs into zygotes. Injected embryos were separated into two groups for in vitro culture or transferred to pseudopregnant females for post-implantation development.

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