A conserved molecular cascade initiates a trophectoderm program in human, cow and mouse embryos prior to blastocyst formation

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Abstract

Current understanding of cell specification in early mammalian preimplantation development is mainly based on mouse studies. The first lineage differentiation event occurs at the morula stage with outer cells initiating a trophectoderm (TE) program to become the earliest placental progenitors. At subsequent developmental stages, the inner cell mass (ICM) arises from inner cells and is comprised of precursor cells of the embryo proper and yolk sac[1]. Notably, recent gene expression analyses suggest that the mechanisms regulating early lineage specification in the mouse may differ in other mammals, including human[2-5] and cow[6,7]. Here, we examined evolutionary conservation of cell dynamics and a molecular cascade initiating TE segregation in mouse, cow and human embryos using a comparative embryology approach. We discovered that the expression pattern of key TE lineage-associated factors shows a high degree of conservation among all three species. Specifically, at the morula stage outer cells acquire an apico-basal cell polarity, with expression of aPKC and PARD6B at the surface-free domain, nuclear expression of the Hippo signaling pathway effectors, YAP1 and WWTR1, and restricted expression of the transcription factor GATA3, suggesting initiation of a TE program. Furthermore, we demonstrate that inhibition of aPKC, by small-molecule pharmacological modulation and TRIM-Away protein depletion, impairs TE initiation at the morula stage. Altogether, our comparative embryology analysis provides novel insights into early lineage specification in human preimplantation embryos and suggests a similar mechanism initiating a TE program in mouse, cow and human embryos.

Main text

Our current understanding of cell specification during mammalian preimplantation development mainly relies on mouse studies. At the 8-cell stage, the mouse embryo undergoes a drastic morphological change, where blastomeres flatten and adhere to each other in a process known as compaction[8]. After subsequent rounds of cell division two distinct cell populations are discernible at the morula stage: inner and outer cells. Following this, a blastocyst is formed, whereby the inner cells give rise to the inner cell mass (ICM), and the outer cells become the trophectoderm (TE), a polarized epithelium that will form fetal components of the placenta. Subsequently, the ICM will further segregate into the epiblast (EPI), which gives rise to the fetus, and the primitive endoderm (PrE), which primarily contributes to the yolk sac[1].

Concomitant with compaction, cell polarity is established in the 8-cell mouse embryo. Inner and outer cells display different polarization states, which influence their cell fate acquisition. The contact-free surface of the outer cells acquires an apical domain, enriched with the Atypical protein kinase C (aPKC) that together with the proteins Partitioning defective homolog 6B (PARD6B) and homolog 3 (PARD3), forms the anterior PAR polarity complex, while PAR1 (EMK1 or MARK2), E-CADHERIN and other cell adhesion molecules localize to the basolateral

domain[9-12]. In the polar outer cells, the apical PAR proteins sequester Angiomotin (AMOT), a modulator of the Hippo pathway, from the junctional complexes. This interaction prevents activation of downstream Hippo pathway kinases, Large tumor suppressor kinases 1/2 (LATS1/2)[13]. Consequently, in outer cells, Yes-associated protein 1 (YAP1) and WW domain-containing transcription regulator protein 1 (WWTR1, also known as TAZ) accumulate in the nucleus where, together with TEA-domain family member 4 (TEAD4), they promote the expression of TE lineage-associated factors, such as Caudal type homeobox 2 (*Cdx2*) and Gata binding protein 3 (*Gata3*)[14-16]. By contrast, in the apolar inner cells, AMOT is free to interact with a large protein complex at the cell junction and is activated through phosphorylation by LATS1/2. In these cells, activation of the Hippo pathway results in YAP1 and WWTR1 phosphorylation and cytoplasmic retention, thus maintaining the inner cells in an unspecified state[13,17,18].

In the mouse, CDX2 is expressed in outer cells from the morula stage and *Cdx2* mutant embryos exhibit loss of epithelial integrity at the blastocyst stage, thus failing to maintain the blastocoel cavity or to implant[14]. Notably, in human, cow and pig embryos, CDX2 is detectable later in cavitating blastocysts[2,6,7,19]. A recent study in human embryos, using single-cell RNA-sequencing (scRNA-seq) analysis, suggests concurrent establishment of EPI, PrE and TE at the blastocyst stage[5]. These differences hint at a divergent molecular cascade controlling cell specification in mouse and other mammals. However, detailed protein expression and functional analyses of cow and human embryos are still missing. We hypothesize that, similar to the mouse[20,21], the outer cells of cow and human embryos initiate a TE program at the morula stage to form a functional epithelium that drives and supports cavitation to form a blastocyst.
To test this hypothesis, we combined morphokinetic analysis, molecular characterization and functional inhibition. We particularly focused our analysis at the morula stage, where the embryos are still a compacted group of cells without a single dominant cavity. Our results suggest a high degree of conservation of the molecular cascade initiating TE specification at the morula stage in mouse, cow and human embryos.

First, we performed morphokinetic analysis of mouse, cow and human embryos (**Movies 1-3**). As the length of preimplantation embryogenesis varies between these three species, we calculated the duration of each developmental stage as a percentage of time from the 8-cell stage to the end of cavitation (**Extended Data Fig. 1a**). We observed that mouse embryos remain at the 8-cell stage for a comparatively short period of time and rapidly undergo compaction. By contrast, we observed a prolonged 8-cell to compaction transition with multiple cell divisions in cow and human embryos (**Extended Data Fig. 1b-f**). We termed this stage “pre-compaction”. Mouse embryos exhibit a relatively long morula stage, while cow and in particular human embryos show a comparatively rapid transition between compaction and cavitation (**Extended Data Fig. 1b-d** and **Extended Data Table 1**). In addition, we quantified the number of inner and outer cells at

the morula stage and found that cow and mouse embryos have a similar percentage of inner cells, while human embryos show a strikingly reduced proportion of inner cells (**Extended Data Fig. 1g**). Similarly, we observed a difference in the number of ICM cells in mouse and cow compared to human blastocyst stage embryos (**Extended Data Fig. 1h**). These data suggest that the proportion of inner cells at the morula stage is indicative of the number of ICM cells found later in development and, moreover, that human blastocysts contain fewer cells fated to contribute to EPI and PrE. Taken together, this comparative morphokinetic analysis provides relevant information to consider when comparing similar developmental stages in different species.

Since CDX2 is detectable in TE cells only at later stages in cow and human embryos[2,6,14], we sought to determine whether alternative TE-associated genes may be expressed prior to blastocyst formation. We mined published human preimplantation scRNA-seq datasets[4,5,22] and were intrigued to observe heterogeneous expression of *GATA3* in human morula cells (**Fig. 1a, b** and **Extended Data Fig. 2a**). Moreover, we reanalyzed a human preimplantation chromatin accessibility dataset[23] and performed differential analysis of putative transcription factor binding sites enriched in accessible regions between the morula and 8-cell stage. Interestingly, we identified enrichment of GATA and TEAD motifs at the morula stage, whereas at the 8-cell stage we observed motif enrichment of genes involved in embryonic genome activation, such as DUXA/DUX4[24] and ZSCAN4[25] (**Fig. 1c**). In the mouse, GATA3 expression overlaps with CDX2 in outer cells at the morula stage, and an elegant genetic analysis demonstrated that GATA3 acts downstream of TEAD4/YAP1 and in parallel to CDX2[15]. The protein localization of TEAD4 and YAP1 is different between inner and outer cells, with TEAD4 detected in all nuclei, while YAP1 is localized only in the nuclei of outer cells[26,27]. By immunofluorescence analysis, we confirmed that TEAD4 shows a similar expression pattern in the human, with nuclear expression detected in all cells of morula stage embryos (**Extended Data Fig. 3**). Next, we analyzed the expression of YAP1 and GATA3 in mouse embryos and observed co-localized nuclear expression of these factors in outer cells at the morula stage, while in inner cells YAP1 is retained in the cytoplasm and GATA3 is absent, consistent with previous findings[15,16] (**Extended Data Fig. 2b-e**). We observed nuclear YAP1 and GATA3 expression in cow and human embryos at late compaction and their expression was not detectable prior to compaction (**Fig. 1g** and **Extended Data Fig. 2f** and **4a**). At the morula stage, outer cells show co-localized nuclear expression of YAP1 and GATA3, while inner cells do not have detectable expression (**Fig. 1d-i** and **Extended Data Fig. 4a**). In expanded cow and human blastocysts, YAP1 and GATA3 expression is maintained in the nuclei of TE cells and is not detected in ICM cells (**Fig. 1d, g** and **Extended Data Fig. 4a**). Similar to the mouse[16], we observed WWTR1 and YAP1 overlapping nuclear expression in outer and TE cells in human morula and blastocyst stages, respectively (**Extended Data Fig. 5a, b**). GATA2 is considered a TE marker in human blastocysts[3-5,28]. Importantly, at the morula stage

GATA2 is not detected (**Extended Data Fig. 2g**), despite its restriction to TE cells at the blastocyst stage (**Extended Data Fig. 2h**). By contrast, GATA3 is expressed at both morula and blastocyst stages in outer and in TE cells, respectively (**Extended Data Fig. 2g, h**), indicating that expression of GATA3, and not GATA2, at the morula stage may distinguish cells that are initiating a TE program.

To find additional genes associated with TE initiation in human embryos, we initially identified genes that were co-expressed with *GATA3* in TE cells at the blastocyst stage using the aforementioned scRNA-seq datasets[4,5,22] and analyzed the expression of these genes earlier at the morula stage. Among these genes, 22 showed a positive correlation (Pearson's $r > 0.25$) with *GATA3* when comparing all human morula cells (**Extended Data Table 2**). Genes related to epithelial cell formation (*KRT18*, *CLDN4, RAB20, RAB25*), and placenta morphogenesis (*PTGES*, *PLAC8*) and genes encoding transporter subunits (*ATP6V1B1*, *ATP6V1C2*, *FXYD4*, *ATP6V0A4*, *SLC7A2*) positively correlate with *GATA3* (**Fig. 1k** and **Extended Data Fig. 6a-c, 8** and **Extended Data Table 3**). Interestingly, we observed that Vestigial-like protein 4 (*VGLL4),* a transcriptional co-factor and regulator of TEAD transcriptional activity[29], also shows a positive correlation with *GATA3* (**Extended Data Fig. 6d, 8** and **Extended Data Table 3**). Immunofluorescence analysis confirmed the specific expression of KRT18 in outer and TE cells in human morula and blastocyst stage embryos, respectively (**Fig. 1j**, **Extended Data Fig. 4a** and **Extended Data Fig. 5c**), as previously described[30]. Interestingly, the chromatin accessibility data indicates enrichment of GATA binding motifs at the *KRT8* and *KRT18* loci (**Fig. 1l**), suggesting that *KRT8* and *KRT18* may be candidate target genes of GATA3 in human embryos. In the positively correlated gene list, we also detected Grainyhead-like transcription factor 2 (*GRHL2*) (**Extended Data Fig. 6a, 8** and **Extended Data Table 3**), a gene important for epithelial morphogenesis and trophoblast branching in mouse embryos[31,32]. We also observed an enrichment of GATA binding motifs upstream the *GRHL2* locus (**Extended Data Fig. 5d**). By immunofluorescence analysis, we observed that GRHL2 is expressed in both outer and inner cells at the morula stage (**Extended Data Fig. 5e**). Upon blastocyst expansion, GRHL2 is specifically expressed in TE cells and no longer present in ICM cells (**Extended Data Fig. 5f**). Next, we analyzed scRNA-seq datasets to identify genes that exhibited an anti-correlated expression pattern to GATA3 in human morula cells (**Fig. 1a**) and that could be putative inner cell-associated markers. Interestingly, genes involved in embryonic stem cell pluripotency and/or genes enriched in the EPI/ICM, such as *DPPA3*[33], *KLF17*[4,34] and *ARGFX*[4,28,35] were transcriptionally negatively correlated with GATA3 (**Fig. 2e-g**, **Extended Data Tables 4, 5** and **Extended Data Fig. 7, 8**). Altogether, our analysis of existing scRNA-seq data suggests transcriptional differences between inner and outer cells at the morula stage.

In the mouse morula, the transcription factor SOX2 is specifically restricted to inner cells and is considered the first marker of ICM pluripotency[36,37] (**Extended Data Fig. 9a, b**). At the 8-cell stage, SOX2 was detected in a few blastomeres in cow embryos, while in human embryos SOX2 was expressed in all nuclei (**Fig. 2c, Extended Data Fig. 4b** and **9c**). In late compacting embryos, SOX2 was detected in all nuclei in both species (**Fig. 2c, Extended Data Fig. 4b** and **9c**). At the morula stage, when GATA3 begins to be differentially expressed between outer and inner cells, SOX2 remains expressed in all cells in human and cow embryos, in contrast to the mouse (**Fig. 2a-d** and **Extended Data Fig. 4b** and **9a, b**). SOX2 becomes restricted to ICM cells only in expanded blastocysts (**Fig. 2a, c** and **Extended Data Fig. 4b** and **9d**), thus confirming previously published data in human embryos[30]. Altogether, these data indicate that SOX2, the specific inner cell marker in the mouse, displays a different expression pattern in cow and human embryos, thus suggesting it may be regulated differently in these species.

Since we observed expression of YAP1 and GATA3 specifically in the nuclei of outer cells at the morula stage in these three species, we next sought to investigate upstream regulators of this pathway in cow and human embryos. We analyzed the expression pattern of aPKC and AMOT, which influence YAP1 cellular localization in mouse outer cells[13]. We confirmed that in the mouse morula, aPKC and AMOT are expressed at the apical membrane of outer cells, while in inner cells AMOT and E-CADHERIN are enriched at the cell-cell contact sites (**Fig. 3a, d**, **Extended Data Fig. 4c** and **10a**). We detected a similar expression pattern in cow morula stage embryos with aPKC and AMOT strongly co-localized at the apical domain of outer cells and β-CATENIN at the basolateral domain (**Fig. 3b, e**, **Extended Data Fig. 4c** and **10b**). We identified expression of aPKC and AMOT at the apical domain of human morula stage embryos, which opposed β-CATENIN and E-CADHERIN at the basolateral domain (**Fig. 3c, f, Extended Data Fig. 4c** and **10c**). Moreover, we could observe co-localization of aPKC and its partner, PARD6B, at the apical domain of cells in both human morula and blastocyst stage embryos (**Extended Data Fig. 10d, e**). These data reveal differential cell polarization between outer and inner cells in cow and human embryos, with apical and basolateral proteins showing a similar expression pattern to that of the mouse[10,38].

Our data suggest a possible functional link between cell polarity and TE lineage initiation at the morula stage in cow and human embryos. In order to test this hypothesis, we used a potent aPKC inhibitor, CRT0276121, a derivative of CRT0103390, which has previously been shown to specifically inhibit aPKC in various biological and cellular contexts[39-41]. Initially, we performed a dose-response experiment with treatment from the 4-cell stage to determine the effective concentration of aPKC inhibitor (**Extended Data Fig. 11a, b** and **Extended Data Table 6**). Following aPKC inhibition, we observed that YAP1 was restricted to the cytoplasm in outer cells (**Extended Data Fig. 12a, b**), consistent with previous descriptions in aPKC knockdown and

knockout studies[9,13,42]. We also observed that inhibition of aPKC led to reduced GATA3 expression at the morula stage (**Extended Data Fig. 12a, c**), indicating that aPKC is required to initiate TE-associated gene expression in outer cells. Moreover, SOX2 was ectopically expressed in outer cells at the morula stage following aPKC inhibition in the mouse (**Extended Data Fig. 13a-c**). These data phenocopy the *Yap1*$^{-/-}$*;Wwtr1*$^{-/-}$ phenotype and the effects of ROCK inhibition in mouse embryos[18], thus indicating specificity of the aPKC inhibitor. In addition, in our morphokinetic analysis, we could not detect differences in cleavage rate between control and treated embryos (**Extended Data Fig. 14a, b**). In addition, while DMSO-treated control mouse embryos developed to the blastocyst stage, aPKC inhibitor-treated embryos failed to cavitate and underwent developmental arrest at the morula stage (**Extended Data Fig. 14c, d**), phenocopying aPKC null mutant embryos[13,42].

We next analyzed the effects of aPKC inhibition on cow and human embryos by treating from pre-compaction until the morula stage, following dose-response experiments (**Extended Data Fig. 11c-f** and **Extended Data Tables 7, 8**). Inhibition of aPKC in cow embryos resulted in reduction of nuclear YAP1 and GATA3 expression in outer cells at the morula stage (**Fig. 4a-c**). Similarly, human embryos at the morula stage exhibited reduced expression of YAP1 and GATA3 in outer cells following aPKC inhibition (**Fig. 4d-f**). Moreover, when both cow and human embryos were allowed to develop to the blastocyst stage, the DMSO-treated embryos were able to form expanded blastocysts, while a significant number of aPKC-inhibitor treated embryos arrested at cavitation (**Extended Data Fig. 14e-h**). Interestingly, in aPKC inhibitor-treated cow and human embryos, SOX2 expression was retained in all cells similar to control embryos (**Extended Fig. 13d, e**), which is in striking contrast to the mouse. As expected, TEAD4 expression was unchanged in both mouse and human aPKC-inhibitor treated embryos (**Extended Data Fig. 12d, e**), thus further corroborating the specificity of the aPKC inhibitor.

In order to further confirm our results, we sought to test our hypothesis applying TRIM-Away protein depletion method in embryos. TRIM-Away has been recently reported to induce rapid and efficient degradation of proteins of interest in mouse oocytes[43]. Firstly, we optimized electroporation of *mCherry-TRIM21* mRNA with an antibody against aPKC in mouse embryos at the 4-cell stage (**Extended Data Fig. 15** and **Extended Table 9**). Our analysis of mouse morula stage embryos showed that the expression of endogenous aPKC was reduced following *mCherry-TRIM21* mRNA and aPKC antibody electroporation compared to the control embryos where only *mCherry-TRIM21* mRNA was provided (**Extended Data Fig. 16a, b**). Moreover, we observed a reduction of YAP1 and GATA3 protein expression in embryos electroporated with *mCherry-TRIM21* mRNA and aPKC antibody (**Extended Data Fig. 16c-d**). Electroporation of 4-cell stage human embryos led to a similar reduction of YAP1 and GATA3 protein expression compared to control embryos (**Fig. 4g-k**), confirming the effect seen with the aPKC inhibitor.

Despite attempts to optimize electroporation in cow embryos, we were unable to identify a concentration of TRIM-Away components that affected YAP1 and GATA3 protein expression without affecting embryo viability (**Extended Data Fig. 17** and **Extended Table 10**), suggesting that further refinement of the method is needed in this species.

Altogether, these data provide novel insights into cow and human preimplantation development and TE specification. With protein expression, morphokinetic, transcriptomic and functional analyses, we propose that cell polarity, through aPKC activity, initiates a TE program at the morula stage in the outer cells of human and cow embryos (**Fig. 4l**), similar to the mouse[9,13,42]. We propose that in cow and human morula stage embryos, outer cells acquire cell polarization, which triggers a molecular cascade influencing cell fate. Our data suggest that similar to the mouse, in human and cow embryos, aPKC sequesters AMOT at the apical domain, thus keeping the Hippo signaling pathway in an inactive state. YAP1 subsequently translocates to the nucleus, where together with TEAD4, it promotes the transcriptional activation of a TE program. Additional molecular characterization is needed in human and cow embryos to elucidate how differences in cell polarity leads to differential Hippo signaling in outer and inner cells, and whether it involves mechanisms that are conserved or divergent compared to the mouse. We observed that GATA3 is the earliest TE-associated transcription factor detected so far in human and cow morula stage embryos. It will be interesting to determine whether and how GATA3 drives this early human placental program.

Besides compaction and cell polarization, another morphological change occurring during preimplantation development is cavitation. During this process, outer cells pump fluid into the embryo to form a blastocoel cavity, causing disruption of the radial symmetry of the embryo[21]. To support the formation of a cavity, outer cells assemble functional tight junctions, in order to form a seal and prevent fluid leakage[1,21]. Interestingly, our re-analysis of scRNA-seq datasets shows that genes involved in cell epithelialization and genes encoding transporter subunits are positively correlated with *GATA3* expression in human morula cells. scRNA-seq datasets lack positional information of the cells collected, but our protein expression analysis shows that GATA3 is detected only in outer cells at the morula stage. In addition, re-analysis of chromatin accessibility profiles of the human morula stage allowed us to identify enrichment of GATA motifs near genes involved in cell epithelialization, such as *KRT8*, *KRT18* and *GRHL2*. Therefore, we propose that in human preimplantation embryos, outer cells at the morula stage initiate a TE program in order to support cavitation and formation of a blastocyst (**Fig. 4l**). While our data reveal a molecular cascade that leads to the initiation of a TE program, cells are unlikely to be committed at this stage, which is supported by studies suggesting that cell fate determination occurs later[5,44].

Interestingly, CDX2, a TE lineage-associated transcription factor that is detectable in the mouse morula, is expressed in human and cow embryos only at the blastocyst stage[2,6]. This suggests that despite a high degree of conservation in the link between cell polarity and initiation of a TE program, not all factors associated with an early TE gene regulatory network may be conserved across species. Consistently, by immunofluorescence analysis, we were not able to detect cytoplasmic retention of YAP1 in the inner cell population in cow and human morula, while we clearly detected this pattern in mouse embryos. This observation suggests a difference in the regulation of YAP1 in inner cells. It is unclear whether this relates to the different expression pattern we observed for SOX2 in the mouse compared to cow and human embryos. It has recently been shown that SOX2 is repressed by YAP1/WWTR1/TEAD4 in the inner cell population until LATS1/2 become expressed at the 16-cell stage[18,45]. It would be interesting to understand whether SOX2 expression is modulated by YAP1/WWTR1/TEAD4 in cow and human embryos at later stages when SOX2 is restricted to the ICM, or if it is regulated by alternative mechanisms. Additional functional analysis such as dominant negative mutations or CRISPR/Cas9-mediated genome editing will help to address these questions and to further understand the factors that function in parallel, downstream or upstream of YAP1 and GATA3 to drive a placental progenitor program.

Data Availability

The datasets analyzed during the current study were previously published and are available at the GEO repository GSE36552, at EMBL-EBI ArrayExpress: E-MTAB-3929 and at EMBL-EBI ENA: PRJNA494280.

Code availability

The data processing and analysis pipelines are publicly available at https://github.com/galanisl/TE_differentiation.

References

1 Cockburn, K. & Rossant, J. Making the blastocyst: lessons from the mouse. *The Journal of Clinical Investigation* **120**, 995-1003, doi:10.1172/JCI41229 (2010).

2 Niakan, K. K. & Eggan, K. Analysis of human embryos from zygote to blastocyst reveals distinct gene expression patterns relative to the mouse. *Developmental Biology* **375**, 54-64, doi:https://doi.org/10.1016/j.ydbio.2012.12.008 (2013).

3 Fogarty, N. M. E. *et al.* Genome editing reveals a role for OCT4 in human embryogenesis. *Nature* **550**, 67, doi:10.1038/nature24033 https://www.nature.com/articles/nature24033#supplementary-information (2017).

4 Blakeley, P. *et al.* Defining the three cell lineages of the human blastocyst by single-cell RNA-seq. *Development (Cambridge, England)* **142**, 3151-3165, doi:10.1242/dev.123547 (2015).

5 Petropoulos, S. *et al.* Single-Cell RNA-Seq Reveals Lineage and X Chromosome Dynamics in Human Preimplantation Embryos. *Cell* **165**, 1012-1026, doi:https://doi.org/10.1016/j.cell.2016.03.023 (2016).

6 Goissis, M. D. & Cibelli, J. B. Functional characterization of CDX2 during bovine preimplantation development in vitro. *Molecular Reproduction and Development* **81**, 962-970, doi:doi:10.1002/mrd.22415 (2014).

7 Berg, D. K. *et al.* Trophectoderm Lineage Determination in Cattle. *Developmental cell* **20**, 244-255, doi:10.1016/j.devcel.2011.01.003 (2011).

8 Ducibella, T. & Anderson, E. Cell shape and membrane changes in the eight-cell mouse embryo: Prerequisites for morphogenesis of the blastocyst. *Developmental Biology* **47**, 45-58, doi:https://doi.org/10.1016/0012-1606(75)90262-6 (1975).

9 Plusa, B. *et al.* Downregulation of Par3 and aPKC function directs cells towards the ICM in the preimplantation mouse embryo. *Journal of Cell Science* **118**, 505-515, doi:10.1242/jcs.01666 (2005).

10 Vinot, S. *et al.* Asymmetric distribution of PAR proteins in the mouse embryo begins at the 8-cell stage during compaction. *Developmental Biology* **282**, 307-319, doi:https://doi.org/10.1016/j.ydbio.2005.03.001 (2005).

11 Ohsugi, M. *et al.* Expression and cell membrane localization of catenins during mouse preimplantation development. *Developmental dynamics : an official publication of the American Association of Anatomists* **206**, 391-402, doi:10.1002/(sici)1097-0177(199608)206:4<391::Aid-aja5>3.0.Co;2-d (1996).

12 Alarcon, V. B. Cell polarity regulator PARD6B is essential for trophectoderm formation in the preimplantation mouse embryo. *Biol Reprod* **83**, 347-358, doi:10.1095/biolreprod.110.084400 (2010).

13 Hirate, Y. *et al.* Polarity-dependent distribution of angiomotin localizes Hippo signaling in preimplantation embryos. *Current biology : CB* **23**, 1181-1194, doi:10.1016/j.cub.2013.05.014 (2013).

14 Strumpf, D. *et al.* Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development (Cambridge, England)* **132**, 2093-2102, doi:10.1242/dev.01801 (2005).

15 Ralston, A. *et al.* Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2. *Development (Cambridge, England)* **137**, 395-403, doi:10.1242/dev.038828 (2010).

16 Nishioka, N. *et al.* The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Developmental cell* **16**, 398-410, doi:10.1016/j.devcel.2009.02.003 (2009).

17 Cockburn, K., Biechele, S., Garner, J. & Rossant, J. The Hippo pathway member Nf2 is required for inner cell mass specification. *Current biology : CB* **23**, 1195-1201, doi:10.1016/j.cub.2013.05.044 (2013).

18 Frum, T., Murphy, T. M. & Ralston, A. HIPPO signaling resolves embryonic cell fate conflicts during establishment of pluripotency in vivo. *eLife* **7**, doi:10.7554/eLife.42298 (2018).

19 Bou, G. *et al.* CDX2 is essential for cell proliferation and polarity in porcine blastocysts. *Development (Cambridge, England)* **144**, 1296-1306, doi:10.1242/dev.141085 (2017).

20 Marikawa, Y. & Alarcon, V. B. Creation of trophectoderm, the first epithelium, in mouse preimplantation development. *Results and problems in cell differentiation* **55**, 165-184, doi:10.1007/978-3-642-30406-4_9 (2012).

21 Maitre, J. L. Mechanics of blastocyst morphogenesis. *Biology of the cell* **109**, 323-338, doi:10.1111/boc.201700029 (2017).

22 Yan, L. *et al.* Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. *Nature Structural &Amp; Molecular Biology* **20**, 1131, doi:10.1038/nsmb.2660
https://www.nature.com/articles/nsmb.2660#supplementary-information (2013).

23 Liu, L. *et al.* An integrated chromatin accessibility and transcriptome landscape of human pre-implantation embryos. *Nature Communications* **10**, 364, doi:10.1038/s41467-018-08244-0 (2019).

24 Hendrickson, P. G. *et al.* Conserved roles of mouse DUX and human DUX4 in activating cleavage-stage genes and MERVL/HERVL retrotransposons. *Nature Genetics* **49**, 925-934, doi:10.1038/ng.3844 (2017).

25 Madissoon, E. *et al.* Characterization and target genes of nine human PRD-like homeobox domain genes expressed exclusively in early embryos. *Scientific Reports* **6**, 28995, doi:10.1038/srep28995 (2016).

26 Hirate, Y., Cockburn, K., Rossant, J. & Sasaki, H. Tead4 is constitutively nuclear, while nuclear vs. cytoplasmic Yap distribution is regulated in preimplantation mouse embryos. *Proceedings of the National Academy of Sciences* **109**, E3389-E3390, doi:10.1073/pnas.1211810109 (2012).

27 Nishioka, N. *et al.* Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. *Mechanisms of Development* **125**, 270-283, doi:https://doi.org/10.1016/j.mod.2007.11.002 (2008).

28 Kilens, S. *et al.* Parallel derivation of isogenic human primed and naive induced pluripotent stem cells. *Nature Communications* **9**, 360, doi:10.1038/s41467-017-02107-w (2018).

29 Lin, K. C., Park, H. W. & Guan, K.-L. Regulation of the Hippo Pathway Transcription Factor TEAD. *Trends in Biochemical Sciences* **42**, 862-872, doi:https://doi.org/10.1016/j.tibs.2017.09.003 (2017).

30 Cauffman, G., De Rycke, M., Sermon, K., Liebaers, I. & Van de Velde, H. Markers that define stemness in ESC are unable to identify the totipotent cells in human preimplantation embryos. *Human Reproduction* **24**, 63-70, doi:10.1093/humrep/den351 (2008).

31 Senga, K., Mostov, K. E., Mitaka, T., Miyajima, A. & Tanimizu, N. Grainyhead-like 2 regulates epithelial morphogenesis by establishing functional tight junctions through the organization of a molecular network among claudin3, claudin4, and Rab25. *Molecular biology of the cell* **23**, 2845-2855, doi:10.1091/mbc.E12-02-0097 (2012).

32 Walentin, K. *et al.* A Grhl2-dependent gene network controls trophoblast branching morphogenesis. *Development (Cambridge, England)* **142**, 1125-1136, doi:10.1242/dev.113829 (2015).

33 Bowles, J., Teasdale, R. P., James, K. & Koopman, P. Dppa3 is a marker of pluripotency and has a human homologue that is expressed in germ cell tumours. *Cytogenetic and genome research* **101**, 261-265, doi:10.1159/000074346 (2003).

34 Guo, G. *et al.* Epigenetic resetting of human pluripotency. *Development (Cambridge, England)* **144**, 2748-2763, doi:10.1242/dev.146811 (2017).

35 Stirparo, G. G. *et al.* Integrated analysis of single-cell embryo data yields a unified transcriptome signature for the human pre-implantation epiblast. *Development (Cambridge, England)* **145**, doi:10.1242/dev.158501 (2018).

36 Wicklow, E. *et al.* HIPPO Pathway Members Restrict SOX2 to the Inner Cell Mass Where It Promotes ICM Fates in the Mouse Blastocyst. *PLOS Genetics* **10**, e1004618, doi:10.1371/journal.pgen.1004618 (2014).

37 Guo, G. *et al.* Resolution of Cell Fate Decisions Revealed by Single-Cell Gene Expression Analysis from Zygote to Blastocyst. *Developmental cell* **18**, 675-685, doi:https://doi.org/10.1016/j.devcel.2010.02.012 (2010).

38 Hirate, Y. *et al.* Par-aPKC-dependent and -independent mechanisms cooperatively control cell polarity, Hippo signaling, and cell positioning in 16-cell stage mouse embryos. *Development, Growth & Differentiation* **57**, 544-556, doi:10.1111/dgd.12235 (2015).

39 Kjaer, S. *et al.* Adenosine-binding motif mimicry and cellular effects of a thieno[2,3-d]pyrimidine-based chemical inhibitor of atypical protein kinase C isoenzymes. *The Biochemical journal* **451**, 329-342, doi:10.1042/bj20121871 (2013).

40 Rodriguez, J. *et al.* aPKC Cycles between Functionally Distinct PAR Protein Assemblies to Drive Cell Polarity. *Developmental cell* **42**, 400-415.e409, doi:10.1016/j.devcel.2017.07.007 (2017).
41 Aguilar-Aragon, M. *et al.* Pak1 Kinase Maintains Apical Membrane Identity in Epithelia. *Cell Reports* **22**, 1639-1646, doi:10.1016/j.celrep.2018.01.060 (2018).
42 Korotkevich, E. *et al.* The Apical Domain Is Required and Sufficient for the First Lineage Segregation in the Mouse Embryo. *Developmental cell* **40**, 235-247.e237, doi:10.1016/j.devcel.2017.01.006 (2017).
43 Clift, D. *et al.* A Method for the Acute and Rapid Degradation of Endogenous Proteins. *Cell* **171**, 1692-1706.e1618, doi:10.1016/j.cell.2017.10.033 (2017).
44 De Paepe, C. *et al.* Human trophectoderm cells are not yet committed. *Human reproduction (Oxford, England)* **28**, 740-749, doi:10.1093/humrep/des432 (2013).
45 Frum, T., Watts, J. L. & Ralston, A. TEAD4, YAP1 and WWTR1 prevent the premature onset of pluripotency prior to the 16-cell stage. *Development (Cambridge, England)* **146**, doi:10.1242/dev.179861 (2019).

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Author contributions

C.G. and K.K.N. conceived the study; K.K.N. supervised the project; C.G., K.K.N. and A.M. designed the experiments; C.G., K.K.N., A.M. and N.M.E.F. performed experiments; G.A-L. performed the bioinformatic analysis of scRNA-seq and ATAC-seq datasets; C.G., K.K.N., A.M. and G.A-L. analyzed data. S.L. managed human embryos donated to research in Nantes; C.G. and A.D. performed experiments on human embryos in Brussels; A.B. and S.L. performed experiments on human embryos in Nantes; K.E., P.S. and L.C. coordinated donation of embryos to the research project in London; L.D. supervised experiments on human embryos in Nantes; H.V.d.V. supervised experiments on human embryos in Brussels; A.F-N. and D.H. provided cow ovaries; A.F-N. provided techniques for cow embryo generation, helped with conceptualization and design of experiments on cow embryos, and hosted C.G. in his lab; C.G. and K.K.N. wrote the manuscript with help from all the authors.

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Figure legends.

Fig. 1. Transcriptional and protein expression differences between cells at the morula stage in cow and human embryos.

a, Violin plot showing log-transformed size-factor-normalized expression of *GATA3* in human morula cells. *n* = 197 cells. Black line corresponds to the median. Highlighted in red are the 10% of the human morula cell samples with lowest levels of *GATA3* expression and in blue are the members of the cluster with high levels of *GATA3* expression in panel b. **b,** griph dimensionality reduction analysis of the human morula cells. Single cells have been colored with the log-transformed size-factor-normalized expression of *GATA3*. **c,** ATAC-seq chromatin accessibility in human embryos at the morula stage compared to the 8-cell stage. Transcription factors with a significant change in activity score (p-value < 0.05) are highlighted in purple in the morula and in cyan in the 8-cell stage. **d,** Time-course immunofluorescence analysis of GATA3 (green), β-CATENIN (red), YAP1 (magenta) and DAPI nuclear staining (blue) in cow embryos at different developmental stages: morula (*n* = 11) and expanded blastocyst (*n* = 5). **e, f,** Quantification of YAP1 (**e**) and GATA3 (**f**) fluorescence intensity, normalized to DAPI intensity, in either inner or outer cells in cow morula stage embryos (*n* = 97 cells from 11 embryos). *t*-test, ****$p < 0.0001$. **g,** Time-course immunofluorescence analysis of GATA3 (green), F-ACTIN (red), YAP1 (magenta) and HOECHST-33342 nuclear staining (blue) in human embryos at different developmental stages: pre-compaction (*n* = 5), late compaction (*n* = 5), morula (*n* = 10), expanded blastocyst (*n* = 4). **h, i,** Quantification of YAP1 (**h**) and GATA3 (**i**) fluorescence intensity, normalized to HOECHST-33342 intensity, in either inner or outer cells in human morula embryos (*n* = 95 cells for YAP1 and *n* = 79 for GATA3 from 10 embryos). *t*-test for YAP1 distribution, ****$p < 0.0001$; Mann-Whitney U test for GATA3 distribution, ****$p < 0.0001$. Yellow arrowheads point to outer cells expressing YAP1 and GATA3, while cyan arrows mark inner cells devoid of YAP1 and GATA3 expression. **j,** Immunofluorescence analysis of GATA3 (green), KRT18 (magenta) and DAPI nuclear staining (blue) in human morula stage embryos. *n* = 3. **k,** Scatter plots showing positive correlation of *GATA3* expression profile with *KRT18* expression profile in human morula cells. *n* = cells considered. r = Pearson correlation coefficient. Values are displayed as log-transformed size-factor-normalized counts. The black line corresponds to a linear regression model fitted to the data with 95% confidence bands. **l,** Genome browser view of ATAC-seq signal at the *KRT8* and *KRT18* loci. High confidence peaks (FDR < 0.001) were used to identify transcription factor motifs. Representative binding motifs associated with the footprints are highlighted. Scale bars, as displayed in figures.

Fig. 2. SOX2 is an inner cell-specific marker in mouse, but not in cow and human morula stage embryos.

a, Time-course immunofluorescence analysis of SOX2 (green), β-CATENIN (red), GATA3 (magenta) and DAPI nuclear staining (blue) in cow embryos at different developmental stages: morula (*n* = 9) and expanded blastocyst (*n* = 5). **b**, Quantification of SOX2 fluorescence intensity, normalized to DAPI intensity, in either inner or outer cells in cow morula stage embryos (*n* = 136 cells from 9 embryos). **c,** Time-course immunofluorescence analysis of SOX2 (green), β-CATENIN (red), GATA3 (magenta) and DAPI nuclear staining (blue) in human embryos at different developmental stages: pre-compaction (*n* = 5), late compaction (*n* = 5), morula (*n* = 6), expanded blastocyst (*n* = 5). **d**, Quantification of SOX2 fluorescence intensity, normalized to DAPI intensity, in either inner or outer cells in human morula stage embryos (*n* = 68 cells from 6 embryos). Yellow arrowheads point to outer cells expressing SOX2 and GATA3. *t*-test, ****$p < 0.0001$, ns = not significant. **e-g,** Scatter plots showing negative correlation of *GATA3* expression profile with *DUXA* (**e**), *KLF17* (**f**) and *DPPA3* (**g**) expression profiles in human morula cells. *n* = cells considered. r = Pearson correlation coefficient. Values are displayed as log-transformed size-factor-normalized counts. The black line corresponds to a linear regression model fitted to the data with 95% confidence bands. Scale bars, as displayed in figures.

Fig. 3. Apical expression of aPKC and AMOT in outer cells in mouse, cow and human morula stage embryos.

a, Immunofluorescence analysis of aPKC (green), E-CADHERIN (red), AMOT (magenta) and DAPI nuclear staining (blue) in mouse morula stage embryos (*n* = 10). **b,** Immunofluorescence analysis of aPKC (green), β-CATENIN (red), AMOT (magenta) and DAPI nuclear staining (blue) in cow morula stage embryos (*n* = 10). **c,** Immunofluorescence analysis of aPKC (green), β-CATENIN (red), AMOT (magenta) and DAPI nuclear staining (blue) in human morula stage embryos (*n* = 10). **d-f,** Fluorescence intensity profile of aPKC and AMOT shown along the yellow arrows in mouse (**d**), cow (**e**) and human (**f**) morula stage embryos. Scale bars, as displayed in figures.

Fig. 4. aPKC depletion leads to reduced nuclear YAP1 and GATA3 expression in outer cells in mouse, cow and human embryos.

a, Immunofluorescence analysis of GATA3 (green), β-CATENIN (red), YAP1 (magenta) and DAPI nuclear staining (blue) in control and aPKC inhibitor-treated cow morula stage embryos. *n* = 3 biological experiments. **b, c,** Quantification of YAP1 (**b**) and GATA3 (**c**) fluorescence intensity, normalized to DAPI intensity, in outer cells in control and aPKC-inhibitor treated cow morula stage embryos (*n* = 209 cells for YAP1 from 19 embryos, and *n* = 218 cells for GATA3 from 21 embryos). Mann-Whitney U test for YAP1 distribution, ****$p < 0.0001$. **d,** Immunofluorescence analysis of GATA3 (green), β-CATENIN (red), YAP1 (magenta) and DAPI nuclear staining (blue) in control and aPKC inhibitor-treated human morula stage embryos. *n* = 3 biological experiments. **e, f,** Quantification of YAP1 (**e**) and GATA3 (**f**) fluorescence intensity,

normalized to DAPI intensity, in outer cells in control and aPKC-inhibitor treated human morula stage embryos (*n* = 406 cells for YAP1 from 37 embryos, and *n* = 218 cells for GATA3 from 21 embryos). Mann-Whitney U test, ****$p < 0.0001$. **g,** Schematic of the TRIM-Away approach. **h,** Schematic representation of the TRIM-Away experiment. **i,** Immunofluorescence analysis of anti-mouse secondary antibody (to detect the aPKC antibody electroporated in) (green), YAP1 (red), GATA3 (magenta) and DAPI nuclear staining (blue) at the morula stage in human control embryos and embryos electroporated with *mCherry-TRIM21* mRNA and anti-aPKC antibody. Yellow arrowheads point to decrease YAP1 and GATA3 expression in the TRIM-Away experiment. *n* = 3 biological experiments. **j, k,** Quantification of YAP1 (**j**) and GATA3 (**k**) fluorescence intensity, normalized to DAPI intensity, in outer cells at the morula stage in human control embryos and embryos electroporated with *mCherry-TRIM21* mRNA and anti-aPKC antibody (*n* = 91 cells from 8 embryos). Mann-Whitney U test, *$p < 0.05$, ****$p < 0.0001$. Scale bars as displayed in figures. **l,** Proposed model for human early lineage specification. EPI, epiblast; PrE, primitive endoderm; TE, trophectoderm. E-CAD, E-CADHERIN; β-CAT, β-CATENIN.