Citation for published version (APA):
Shahbazi, M. N., Scialdone, A., Skorupska, N., Weberling, A., Recher, G., Zhu, M., Jedrusik, A., Devito, L. G., Noli, L., MacAulay, I. C., Buecker, C., Khalaf, Y., Illic, D., Voet, T., Marioni, J. C., & Zernicka-Goetz, M. (2017). Pluripotent state transitions coordinate morphogenesis in mouse and human embryos. NATURE, 552, 239-243. [7684]. https://doi.org/10.1038/nature24675
Pluripotent state transitions coordinate morphogenesis in mouse and human embryos

Marta N. Shahbazi\textsuperscript{1}, Antonio Scialdone\textsuperscript{2,10}, Natalia Skorupska\textsuperscript{1}, Antonia Weberling\textsuperscript{1}, Gaelle Recher\textsuperscript{1,3}, Meng Zhu\textsuperscript{1}, Agnieszka Jedrusik\textsuperscript{1}, Liani G. Devito\textsuperscript{4}, Laila Noli\textsuperscript{4}, Iain C. Macaulay\textsuperscript{5}, Christa Buecker\textsuperscript{6}, Yakoub Khalaf\textsuperscript{4}, Dusko Ilie\textsuperscript{4}, Thierry Voet\textsuperscript{7,8}, John C. Marioni\textsuperscript{2,7,9}, & Magdalena Zernicka-Goetz\textsuperscript{1*}.

\textsuperscript{1} Mammalian Embryo and Stem Cell Group, University of Cambridge, Department of Physiology, Development and Neuroscience; Downing Street, Cambridge, CB2 3DY, UK.
\textsuperscript{2} EMBL-European Bioinformatics Institute (EMBL-EBI), Wellcome Genome Campus, Cambridge CB10 1SD, UK. Present address: Institute of Epigenetics and Stem Cells, Helmholtz Zentrum München, München, Germany.
\textsuperscript{3} Present address: Bioimaging and Optofluidics group, IOGS, CNRS & University of Bordeaux. Rue Francois Mitterrand, 33400 Talence, France.
\textsuperscript{4} Faculty of Life Sciences and Medicine, King's College London, Women's Health Academic Centre, Assisted Conception Unit, Guy's Hospital, Great Maze Pond, London SE1 9RT, UK.
\textsuperscript{5} Present address: Earlham Institute, Norwich Research Park, Norwich, NR4 7UG.
\textsuperscript{6} Present address: Max F. Perutz Laboratories, Vienna Biocenter (VBC), Dr. Bohr-Gasse 9, 1030 Vienna, Austria.
\textsuperscript{7} Wellcome Trust Sanger Institute, Wellcome Genome Campus, Cambridge CB10 1SA, UK.
\textsuperscript{8} Laboratory of Reproductive Genomics, Department of Human Genetics, KU Leuven, Herestraat 49, 3000 Leuven, Belgium.
\textsuperscript{9} Cancer Research UK Cambridge Institute, University of Cambridge, Cambridge CB2 0RE, UK.
\textsuperscript{10} Present address: Institute of Epigenetics and Stem Cells, Helmholtz Zentrum München, München, Germany.

* Corresponding author: mz205@cam.ac.uk

**Keywords:** Pluripotency; Epiblast; Embryonic Stem cells; Epithelial tissue.
The foundations of mammalian development lie in a cluster of embryonic epiblast stem cells that, in response to extracellular matrix signaling (ECM), undergo epithelialization creating an apical surface in contact with a cavity\textsuperscript{1,2}, a fundamental event for all subsequent development. Concomitantly, epiblast cells transit through distinct pluripotent states\textsuperscript{3,4}, prior to lineage commitment at gastrulation. These pluripotent states have been characterized at the molecular level\textsuperscript{5}, but their biological significance remains unclear. Here, we show that exit from an unrestricted naïve pluripotent state is required for epiblast epithelialization and generation of the pro-amniotic cavity in mouse embryos. Embryonic stem cells locked in the naïve state are able to initiate polarization but fail to undertake lumenogenesis. Mechanistically, exit from naïve pluripotency activates an Oct4-governed transcriptional program resulting in expression of sialomucins and the vesicle tethering and fusion events of lumenogenesis. Similarly, culture of human embryos beyond implantation reveals that exit from naïve pluripotency triggers amniotic cavity formation and developmental progression. Our results add tissue-level architecture as a new criterion for the characterization of different pluripotent states, and unveil the relevance of transitions between these states during development of the mammalian embryo.

We wished to determine the relationship between morphogenesis of the epiblast, meaning its polarization and lumenogenesis, and the transitions between distinct pluripotency states, both of which are initiated upon embryo implantation. When we examined the temporal correlation between these post-implantation events, we found that as the naïve pluripotency factor Nanog became down-regulated\textsuperscript{6}, the anti-adhesive sialomucin protein Podocalyxin\textsuperscript{7} (Podxl) first became polarized apically and then secreted as the lumen appeared (Fig. 1a). Deep-sequencing analysis at successive implantation stages revealed the kinetics of pluripotent transitions. We found that at E4.5 and E4.75 epiblasts grouped together and expressed naïve pluripotency genes at similar levels, whereas gene expression in the epiblast at E5.0 was quite distinct, with the naïve pluripotency genes already downregulated (although Nanog
transcription was becoming already low at E4.5-E4.75; Fig. 1b, c, Extended Data Fig. 1a-g, Supplementary Table 1). When considered together with previous datasets\(^8\), our findings reveal two distinct groups: pre-implantation (E3.5-E4.75) and post-implantation epiblast (E5.0-E5.5), in which naïve genes are downregulated to a similar extent at the latter. We also noted the upregulation of characteristic post-implantation gene expression at E5.5 compared to E5.0 (Extended Data Fig. 1h, i). Therefore, the naïve gene expression network is dismantled at lumenogenesis. To determine if there is a causal relationship between these events, we cultured E4.5 embryos in IVC1\(^9\) supplemented with 2i/LIF (MEK inhibitor, GSK3 inhibitor and Leukemia Inhibitory Factor, LIF), which maintains mouse embryonic stem cells (mESCs) in the naïve state\(^10\). We found that 2i/LIF preserved expression of Nanog and inhibited Podxl expression and lumenogenesis (Fig. 1d-f). We confirmed that the naïve state was maintained in ESCs derived from embryos cultured in IVC1 containing 2i/LIF (Extended Data Fig. 1j, k). Embryos in IVC1 containing 2i/LIF did not resume development upon 2i/LIF removal (Extended Data Fig. 1l-n), indicating that the kinetics of naïve pluripotency exit need to be tightly coordinated with morphogenesis.

We next examined the kinetics of polarization and lumenogenesis in relation to naïve pluripotency exit using mESCs cultured in matrigel as a model system for embryogenesis\(^5\) (Extended Data Fig. 2a). Upon 2i/LIF removal and after the first cell division, all polarity markers we examined exhibited polarized localisation. Upon subsequent divisions, mESCs organized into polarized rosettes that opened to form lumens 36 hours after 2i/LIF removal (Extended Data Fig. 2b-d). This coincided with the loss of naïve pluripotency gene expression (Extended Data Fig. 2e-l; Supplementary Videos 1-3) whereas expression of the core pluripotency markers Oct4 and Sox2 was maintained. Expression of early post-implantation genes including Otx2 was induced, but primed genes were not expressed (Extended Data Fig. 2m-q) as in the E5.5 epiblast\(^4,8\). When 2i/LIF was removed before cell plating in matrigel, lumens formed after 24 hours without a rosette (Extended Data Fig. 3a-c), similar to post-implantation-like primed human embryonic stem cells (hESCs)\(^11,12\).
To address whether naïve pluripotency exit is required for polarization, we cultured mESCs in matrigel with 2i/LIF (Fig. 2a). Surprisingly, cells displayed polarisation of subcellular components despite the maintenance of Nanog levels (Fig. 2b-e; Extended Data Fig. 3d-g), and the expression of pluripotency genes was indistinguishable from mESCs cultured in gelatin (apolar) (Extended Data Fig. 2g-o, 3h-m). Moreover, mESCs recovered from matrigel lost their polarized organization, grew as canonical mESCs and contributed to post-implantation development in chimeras, indicating that their naïve state was intact (Fig. 2f-h; Extended Data Fig. 3n-p). As expected, mESCs cultured without 2i/LIF progressively lost the ability to generate colonies (Fig. 2g). In accord with the above results, Dgcr8 KO mESCs, which are blocked in the naïve state, underwent polarization; moreover, preservation of naïve pluripotency with a PKC inhibitor did not impair polarization (Fig. 2i, j; Extended Data Fig. 3q-v). Therefore, mESCs can reversibly initiate polarization without losing their naïve character.

By the time mESCs cultured without 2i/LIF formed lumens, mESCs cultured with 2i/LIF remained as closed rosettes and expressed the naïve markers Nanog and Rex1, while Oct4 was expressed at similar levels in both conditions (Fig. 3a-b; Extended Data Fig. 4a-c). Electron microscopy revealed that while mESCs formed expanded lumens in the absence of 2i/LIF, mESCs cultured with 2i/LIF displayed either no, or only rudimentary, lumens despite having apically localized tight junctions (TJs) and Golgi (Extended Data Fig. 4d, e). With time, rosettes of mESCs in 2i/LIF lost polarization and became disorganized (Fig. 3c-d; Extended Data Fig. 4f-h). As we observed in embryos, mESCs cultured in matrigel containing 2i/LIF did not resume morphogenesis upon 2i/LIF removal (Extended Data Fig. 4i-l). To test whether the lumenogenesis defect was a direct consequence of the pluripotent status, we cultured mESCs with combinations of two supplements (2i, MEKi/LIF and GSK3i/LIF) sufficient to maintain naïve pluripotency. All combinations inhibited lumenogenesis and preserved Nanog expression (Extended Data Fig. 4m-o). Given a single supplement, mESCs
forming lumens showed decreased Nanog expression (Extended Data Fig. 4p, q). Dgcr8 KO mESCs expressing high Nanog levels also showed a lumenogenesis defect (Fig. 3e-g; Extended Data Fig. 5a-d). Likewise, PKC inhibition preserved Rex1 and Nanog expression, and impaired lumenogenesis (Extended Data Fig. 5e-h). Notably, when we first induced naïve pluripotency exit and then cultured cells in matrigel containing 2i/LIF, those cells expressing Podxl but lacking Nanog formed lumens (Extended Data Fig. 5i-m). Next, we sought to identify the transcription factors that regulate lumenogenesis. Constitutive expression of Nanog enhances self-renewal\textsuperscript{17}. However, we found that Nanog overexpression in the absence of 2i/LIF and serum was insufficient to block lumenogenesis and to preserve the naïve network (Extended Data Fig. 5n-r). Similarly, Nanog downregulation was insufficient to promote lumenogenesis with 2i/LIF, and Rex1 levels remained constant (Extended Data Fig. 5s-v). We next focused on Oct4 as, together with Otx2, drives enhancer activation upon naïve pluripotency exit\textsuperscript{18,19}. Decreasing Oct4 expression led to defective lumenogenesis and reduced Otx2 levels (Fig. 3h-i; Extended Data Fig. 5w, x). These results indicate that the pluripotency network directs lumen formation and epithelialization via Oct4.

For further mechanistic insight, we tested whether the fusion of apical vesicles\textsuperscript{20} mediates lumenogenesis. Cells expressing a dominant-negative form of Rab11a (Rab11aS25N)\textsuperscript{21} failed to form lumens (Fig. 4a-b). Apical vesicles carry components involved in lumenogenesis, such as Podxl\textsuperscript{22}. Since Podxl was not expressed in naïve conditions (Fig. 1a; Extended Data Fig. 6a-d), we evaluated its role in lumenogenesis by RNAi. mESCs lacking Podxl showed a lumen formation defect at 48 hours, without alterations in naïve pluripotency exit or polarization (Extended Data Fig. 6e-k). To determine the long-term consequences of Podxl deficiency, we generated Podxl KO mESCs. Podxl KO mESCs showed defective lumenogenesis after 48 hours, but formed lumens by 72 hours (Fig. 4c-d; Extended Data Fig. 6l-m), in agreement with the lack of lethality in Podxl KO embryos\textsuperscript{23}. This lumenogenesis delay was rescued by overexpressing Cd34, a sialomucin expressed at very low levels at E5.5\textsuperscript{8} (Extended Data Fig. 6n, p). Moreover, treatment with protamine sulfate, which
neutralises the negative charges of sialomucins\textsuperscript{24}, blocked lumen formation at 72 hours (Fig. 4e-f), indicating that sialomucins mediate lumenogenesis via cell repulsion. Next, we analyzed whether Podxl expression was regulated by Oct4-Otx2. We found that Otx2 KO mESCs expressed low levels of Podxl and displayed a lumenogenesis delay (Extended Data Fig. 7a-d), which was rescued by Otx2 overexpression using a doxycycline (DOX) inducible system (Extended Data Fig. 7e-f). In agreement with previous results\textsuperscript{6,18}, Otx2 overexpression in the presence of 2i/LIF was able to induce naïve pluripotency exit in 50% of WT and 35% of Otx2 KO mESCs. These cells formed lumens despite the presence of 2i/LIF (Extended Data Fig. 7g-j). Although Otx2 KO mESCs showed a delay in naïve pluripotency exit, the lumenogenesis defect was rescued by over-expression of GFP-Podxl (Extended Data Fig. 7k-m). Analysis of Oct4 and Otx2 Chip-seq data\textsuperscript{18} revealed the presence of an intronic enhancer approximately 3.5Kbs downstream of the Podxl transcription start site. In naïve conditions this enhancer was bound by Oct4 and devoid of the activating H3K27ac mark. Naïve pluripotency exit led to Otx2 binding and increased H3K27ac (Extended Data Fig. 7n). However, Podxl overexpression was insufficient to rescue lumen formation in naïve cells; instead the exogenous Podxl co-localized with Rab11 (Fig. 4g; Extended Data Fig. 7o, p). Analysis of the expression of genes involved in the fusion of Podxl-containing vesicles\textsuperscript{25} in E4.5-E5.5 epiblasts\textsuperscript{8} revealed that the TJ protein Cingulin (Cgn) was induced upon naïve pluripotency exit. We confirmed this in embryos and mESCs (Fig. 4h; Extended Data Fig. 8a-b). In MDCK cells Cgn mediates lumenogenesis by tethering Rab11 vesicles through association with the Rab11 interacting protein Fip5\textsuperscript{26}. Indeed, we found that Fip5 showed polarized localisation and associated with Podxl upon 2i/LIF removal (Extended Data Fig. 8c). Depletion of Cgn led to a lumenogenesis defect and accumulation of Podxl in the cytoplasm (Fig. 4i-j), without affecting naïve pluripotency exit (Extended Data Fig. 8d-e). Thus, in mESCs Cgn mediates the apical fusion of Podxl-containing Rab11 vesicles.

The human epiblast also transforms into an epithelium enclosing a lumen (amniotic cavity) at implantation\textsuperscript{2}. However, whether this occurs concomitant with a pluripotency change remains
unknown. We found that at E6-7, epiblast cells (GATA6-) expressed the naïve factor KLF17\textsuperscript{27} and lacked PODXL, whereas 4 out of 9 human embryos cultured until E9-10\textsuperscript{11,28}, had a PODXL-coated lumen (Fig. 5a-b). Upon addition of inhibitors and growth factors (5i/LAF) that preserve naïve pluripotency in hESCs\textsuperscript{29}, embryos failed to form a lumen, displayed high levels of KLF17, NANOG and the naïve marker CD130\textsuperscript{30}, and showed a mild increase in apoptosis (Fig. 5b-e; Extended Data Fig. 9a-f).

Finally, we determined the tissue-level characteristics of distinctive human pluripotent states in different naïve cultures. hESCs overexpressing NANOG and KLF2 with 2i/LIF are in a naïve-like state\textsuperscript{29,31}. We found that presence of DOX upregulated naïve markers and abolished lumenogenesis, whereas DOX removal or switching to primed conditions induced naïve pluripotency exit and lumenogenesis (Extended Data Fig. 10a-e). In transgene-independent naïve conditions (RSet and 5i/LAF\textsuperscript{29}) we found that although NANOG and KLF2 were downregulated, naïve markers were highly expressed and lumenogenesis was abolished without alterations in the initial polarization (Fig. 5f, g; Extended Data Fig. 10f-m). Switching to primed conditions led to both rosette and lumen formation (Extended Data Fig. 10n, o). Therefore, in human embryos and hESCs a pluripotent transition is required for amniotic cavity formation.

Our data thus indicates an evolutionary conserved mechanism that unifies transcriptional and architectural changes of the epiblast. Failure to exit from naïve pluripotency leads to impaired cavity formation and this in turn could contribute to high rates of embryo loss at implantation.

**Author contributions**

M.N.S. designed, performed and analyzed most of the experiments. A.S. analyzed the sequencing data. N.S. and A.W. performed experiments in Fig. 4 and Extended Data Fig. 5-7. M.Z. and G. R. helped with embryo experiments and image analysis. A.J., L.D.G. and L.N. helped with human embryo cultures. I.C.M. prepared cDNA libraries. C.B. generated and analyzed Chip-seq data. D.I. and Y.K. supervised the human embryo experiments. T.V.
supervised the cDNA library preparation. J.C.M. supervised the computational analyses of the sequencing data. M.Z-G. supervised the study. M.N.S. and M.Z-G. conceived the project and wrote the manuscript.

Author information

The authors declare no competing financial interests.

Acknowledgements

We are grateful to K. McNagny, J. Hanna and R. Jaenisch for reagents and discussions; F. Martin-Belmonte, D. Glover, C. Lynch, M. Serrano, A. Hupalowska, F. Antonica and M. Petruzzelli for feedback; J.N. Skepper for help with electron microscopy; W. Mansfield for help with embryo transfer. This work was supported by Wellcome Trust (098287/Z/12/Z) and ERC (669198) grants to M.Z-G. Work in the T.V laboratory was supported by Wellcome Trust and KU Leuven (SymBioSys PFV/10/016). Work in the J.C.M. laboratory was supported by EMBL and Cancer Research UK. M.N.S was supported by Ramon Areces and EMBO postdoctoral fellowships; A.S. by a Wellcome Trust strategic award (105031/D/14/Z) and G.R. by a Newton fellowship.

References

1 Bedzhov, I. & Zernicka-Goetz, M. Self-organizing properties of mouse pluripotent cells initiate morphogenesis upon implantation. *Cell* **156**, 1032-1044, doi:10.1016/j.cell.2014.01.023 (2014).
2 Hertig, A. T., Rock, J. & Adams, E. C. A description of 34 human ova within the first 17 days of development. *Am J Anat* **98**, 435-493 (1956).
3 De Los Angeles, A. *et al.* Hallmarks of pluripotency. *Nature* **525**, 469-478, doi:10.1038/nature15515 (2015).
4 Kalkan, T. & Smith, A. Mapping the route from naive pluripotency to lineage specification. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **369**, doi:10.1098/rstb.2013.0540 (2014).
5 Weinberger, L., Ayyash, M., Novershtern, N. & Hanna, J. H. Dynamic stem cell states: naive to primed pluripotency in rodents and humans. *Nature reviews. Molecular cell biology* **17**, 155-169, doi:10.1038/nrm.2015.28 (2016).
6 Acampora, D., Di Giovannantonio, L. G. & Simeone, A. Otx2 is an intrinsic determinant of the embryonic stem cell state and is required for transition to a stable epiblast stem cell condition. *Development* **140**, 43-55, doi:10.1242/dev.085290 (2013).
Nielsen, J. S. & McNagny, K. M. The role of podocalyxin in health and disease. *J Am Soc Nephrol* **20**, 1669-1676, doi:10.1681/ASN.2008070782 (2009).

Boroviak, T. *et al.* Lineage-Specific Profiling Delineates the Emergence and Progression of Naive Pluripotency in Mammalian Embryogenesis. *Developmental cell* **35**, 366-382, doi:10.1016/j.devcel.2015.10.011 (2015).

Bedzhov, I., Leung, C. Y., Bialecka, M. & Zernicka-Goetz, M. In vitro culture of mouse blastocysts beyond the implantation stages. *Nature protocols* **9**, 2732-2739, doi:10.1038/nprot.2014.186 (2014).

Ying, Q. L. *et al.* The ground state of embryonic stem cell self-renewal. *Nature* **453**, 519-523, doi:10.1038/nature06968 (2008).

Shahbazi, M. N. *et al.* Self-organization of the human embryo in the absence of maternal tissues. *Nat Cell Biol* **18**, 700-708, doi:10.1038/ncb3347 (2016).

Bedzhov, I., Leung, C. Y., Bialecka, M. & Zernicka-Goetz, M. In vitro culture of mouse blastocysts beyond the implantation stages. *Nature protocols* **9**, 2732-2739, doi:10.1038/nprot.2014.186 (2014).

Betschinger, J. *et al.* Exit from pluripotency is gated by intracellular redistribution of the bHLH transcription factor Tfe3. *Cell* **153**, 335-347, doi:10.1016/j.cell.2013.03.012 (2013).

Dutta, D. *et al.* Self-renewal versus lineage commitment of embryonic stem cells: protein kinase C signaling shifts the balance. *Stem cells* **29**, 618-628, doi:10.1002/stem.605 (2011).

Chambers, I. *et al.* Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643-655 (2003).

Buecker, C. *et al.* Reorganization of enhancer patterns in transition from naive to primed pluripotency. *Cell stem cell* **14**, 838-853, doi:10.1016/j.stem.2014.04.003 (2014).

Yang, S. H. *et al.* Otx2 and Oct4 drive early enhancer activation during embryonic stem cell transition from naive pluripotency. *Cell reports* **7**, 1968-1981, doi:10.1016/j.celrep.2014.05.037 (2014).

Bryant, D. M. *et al.* A molecular network for de novo generation of the apical surface and lumen. *Nature cell biology* **12**, 1035-1045, doi:10.1038/ncb2106 (2010).

Ullrich, O., Reinsch, S., Urbe, S., Zerial, M. & Parton, R. G. Rab11 regulates recycling through the pericentriolar recycling endosome. *The Journal of cell biology* **135**, 913-924 (1996).

Blasky, A. J., Mangan, A. & Prekeris, R. Polarized protein transport and lumen formation during epithelial tissue morphogenesis. *Annual review of cell and developmental biology* **31**, 575-591, doi:10.1146/annurev-cellbio-100814-125323 (2015).
Mangan, A. J. et al. Cingulin and actin mediate midbody-dependent apical lumen formation during polarization of epithelial cells. *Nature communications* 7, 12426, doi:10.1038/ncomms12426 (2016).

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

Deglincerti, A. et al. Self-organization of the in vitro attached human embryo. *Nature* 533, 251-254, doi:10.1038/nature17948 (2016).

Theunissen, T. W. et al. Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. *Cell stem cell* 15, 471-487, doi:10.1016/j.stem.2014.07.002 (2014).

Collier, A. J. et al. Comprehensive Cell Surface Protein Profiling Identifies Specific Markers of Human Naive and Primed Pluripotent States. *Cell Stem Cell* 20, 874-890 e877, doi:10.1016/j.stem.2017.02.014 (2017).

Takashima, Y. et al. Resetting Transcription Factor Control Circuitry toward Ground-State Pluripotency in Human. *Cell* 158, 1254-1269, doi:10.1016/j.cell.2014.08.029 (2014).

**Figure legends**

**Figure 1: Epiblast gene expression at peri-implantation.**

a, Immunostaining of mouse embryos. Dotted lines indicate the epiblast; arrowheads polarized Podxl; asterisks Podxl in primitive endoderm; white long arrows positions used to plot intensity profiles (below). b, Principal Component Analysis (PCA) of the gene expression levels of all samples. Numbers indicate percentage of variance. c, Heatmap showing expression of core (black), naïve (green) and post-implantation (purple) genes. Asterisk/hash symbols indicate genes significantly highly/lowly expressed in E4.5-E4.75 compared to E5.0. d, Lumen formation in embryos from panel f (n=19 (IVC1) and 15 (IVC1+2i/LIF) embryos) Chi-square test, ***p=0.0007. e, Nanog intensity in embryos from panel f (n=19 (IVC1) and 15 (IVC1+2i/LIF) embryos). Unpaired Student’s t test, ***p<0.0001. f, Immunostaining of in vitro cultured mouse embryos. Dotted lines indicate the epiblast. Arrows indicate lumens. All scale bars 20 µm.

**Figure 2: Naïve mESCs initiate polarization in matrigel.**

a, Experimental set-up. A: analysis. b, Immunostaining of mESCs cultured as indicated in a. Scale bars, 5 µm. c, Centrosome positions in cells from panel b (n=60 (+2i/LIF) and 62 (-2i/LIF) centrosomes). Unpaired Student’s t test, ns: non-significant. d, Nanog intensity in cells from panel b (n=30 (+2i/LIF) and 31 (-2i/LIF) spheroids). Unpaired Student’s t test, ***p<0.0001. e, Immunostaining of mESCs cultured as indicated in a. Squares denote magnified regions.
Scale bars, 10 µm. **f**, Experimental set-up. **g**, Immunostaining of mESCs cultured as indicated in f. Scale bars, 50 µm. **h**, mESC-embryo chimeras generated using H2B-GFP mESCs cultured as indicated in f. Scale bars, 40 µm. **i**, Immunostaining of mESCs. Scale bars, 5 µm. **j**, Centrosome positions in cells from panel i (n=52 (+Gö6983) and 56 (-Gö6983) centrosomes). Mann Whitney U test, ns: non-significant. All panels: arrows denote polarization; gelatin (G), matrigel (M).

**Figure 3: Naïve pluripotency exit is required for lumenogenesis.** **a**, Experimental set-up. A: analysis. **b-c**, Immunostaining of mESCs cultured as indicated in a. Squares denote magnified regions and arrows non-polarized cells. **d**, Lumen formation in cells from panels b-c. (n=40 (+2i/LIF 48h), 40 (-2i/LIF 48h), 21 (+2i/LIF 72h) and 22 (-2i/LIF 72h) spheroids). Chi-square test, ***p<0.0001. **e**, Immunostaining of control and Dgcr8 KO mESCs. Arrows indicate lumens. **f**, Lumen formation in cells from panel e (n=29 (control +2i/LIF), 31 (control -2i/LIF), 31 (Dgcr8 KO +2i/LIF) and 31 (Dgcr8 KO -2i/LIF) spheroids). Chi-square test, ns: non-significant. **g**, Nanog intensity in cells from panel e (n=20 (control +2i/LIF), 20 (control -2i/LIF), 19 (Dgcr8 KO +2i/LIF) and 21 (Dgcr8 KO -2i/LIF)). Kruskal-Wallis test, *** p<0.0001, *p<0.05. **h**, Immunostaining of control and Oct4 knock-down mESCs. Arrows indicate lumens. **i**, Lumen formation in cells from panel h (n=39 (control), 53 (Oct4 siRNA w/ Oct4) and 32 (Oct4 siRNA w/o Oct4) spheroids). Chi-square test, ***p<0.0001. All panels: scale bars, 10 µm; matrigel (M).

**Figure 4: Vesicle fusion downstream of naïve pluripotency exit.** **a**, Immunostaining of GFP-Rab11a and GFP-Rab11aS25N overexpressing mESCs. Arrows indicate lumens. Scale bars, 10 µm. **b**, Lumen formation in cells from panel a (n=35 (Rab11a WT 48h), 60 (Rab11a WT 72h), 39 (Rab11aS25N 48h) and 29 (Rab11aS25N 72h) spheroids). Chi-square test, ***p<0.0001. **c**, Immunostaining of heterozygous (HET) and Podxl KO mESCs. Arrows indicate lumens. Scale bars, 10 µm. **d**, Lumen formation in cells from panel c (n=34 (HET), 32 (clone 1), 30 (clone 2) and 21 (clone 3) spheroids). Chi-square test, *p=0.0245,
**p=0.0019, ***p<0.0001. e, Immunostaining of control and protamine sulfate-treated mESCs. Arrows point to lumens. Scale bars, 10 µm. f, Lumen formation in cells from panel e (n=41 (control) and 40 (protamine sulfate)). Chi-square test, ***p<0.0001. g, Immunostaining of GFP-Podxl overexpressing mESCs. Squares denote magnified regions. Scale bars, 10 µm. h, Immunostaining of mouse embryos. Dotted lines indicate the epiblast, arrows polarized Cgn. Scale bars, 20 µm. i, Immunostaining of control and Cgn knock-down mESCs. Arrows point to lumens. Scale bars, 10 µm. j, Lumen formation in cells from panel i (n=40 (control) and 39 (Cgn siRNA) spheroids). Chi-square test, ***p<0.0001. All panels: matrigel (M). 

Figure 5: Naïve pluripotency exit direct lumenogenesis in human embryos and hESCs.

a, Immunostaining of day 6-7 human embryos. Squares denote magnified regions, yellow arrowheads epiblast and white arrowheads primitive endoderm. Scale bars, 30 µm. b, Immunostaining of day 9-10 human embryos. Dotted lines indicate the epiblast; arrowheads the amniotic cavity; white long arrows the position used to plot intensity profiles. Scale bars, 50 µm and 10 µm (magnified areas). c, Lumen formation in embryos from panels b. Chi-square test, *p=0.0134. d, PODXL intensity profiles in embryos from panel b. e, KLF17 intensity in embryos from panel b (n=4 (control w/ lumen), 5 (control w/o lumen) and 11 (IVC1+5i/LAF) embryos). Unpaired Student’s t test, *p=0.0366. f, Immunostaining of hESCs. Arrows indicate lumens. Scale bars, 10 µm. g, Lumen formation in cells from panel f (n=31 (2iL/DOX), 30 (5i/LAF), 30 (RSet) and 30 (mTESR) spheroids). Chi-square test, ***p<0.0001. h, Model summarizing the findings of the study.
Methods

Human embryos: all human embryo experiments were done in accordance with Human Fertilization and Embryology Authority (HFEA) regulations (license reference R0075). Informed consent was obtained from all participants, who were informed about the conditions and regulations that apply within the HFEA code of practice. The project has also a local approval by a Research Ethics Committee (UK National Health Service Research Ethics Committee reference 06/Q0702/90). All experimental work conformed to the principles of the WMA Declaration of Helsinki. All experimental work was performed under the HFEA Codes of Practice and the HFEA Act 1990 practices.

Slow-frozen human embryos (day 5-6) were thawed using Quinn’s Advantage Thaw Kit (ART-8016, LifeGlobal Group), and vitrified human embryos (day 5-6) were thawed using Kitazato Thawing Solutions (VT802-0, Hunter Scientific) following the manufacturer’s instructions. Embryos were cultured in Human embryo culture media SAGE 1-Step (67010060A, LifeGlobal Group) for 24 hours before Zona pellucida removal with Acidic Tyrode’s solution (T1788, Sigma). Embryos were washed in SAGE 1-Step medium and subsequently cultured in a pre- to post-implantation culture method, as previously described. Briefly, embryos were plated in IbiTreat µ-plates (IB-80826, Ibidi GmbH) in IVC1 (control) or IVC1 supplemented with 5i/LAF and cultured at 37°C in 21% O₂, 5% CO₂, 24 hours after plating half of the medium was replaced with fresh IVC1. 48 hours after plating half of the medium was replaced with fresh IVC2. 72 hours after plating embryos were fixed with 4% PFA (15710, Electron Microscopy Sciences). The exact composition of the medium was as follows:

IVC1 medium: Advanced DMEM/F12 (12634010, ThermoFisher Scientific), 20% v/v heat-inactivated FBS (Stem Cell Institute, Cambridge), GlutaMAX (35050061, ThermoFisher Scientific), 25 units/ml Penicillin/25 µg/ml Streptomycin (15140122, ThermoFisher Scientific), 1X ITS-X (10 mg/ml insulin, 5.5 mg/L transferrin, 0.0067 mg/L sodium selenite, 2 mg/L etholamine) (51500056, ThermoFisher Scientific), 1X Penicillin/25 µg/ml Streptomycin (15140122, ThermoFisher Scientific), 1X ITS-X (10 mg/ml insulin, 5.5 mg/L transferrin, 0.0067 mg/L sodium selenite, 2 mg/L etholamine) (51500056, ThermoFisher Scientific), 8 nM β-estradiol (E8875, Sigma), 200 ng/ml Progesterone (P0130, Sigma), and 25 µM N-acetyl-L-cysteine (A7250, Sigma).

IVC2 medium: 20% FBS is substituted for 30% KnockOut Serum Replacement (10828010, ThermoFisher Scientific). 5i/LAF: IVC1 and IVC2 were supplemented with 20 ng/mL recombinant human LIF (300-05, PeproTech), 20 ng/mL Activin-A (Stem Cell Institute, Cambridge), 8 ng/mL bFGF2 (Stem Cell Institute, Cambridge), 1 µM MEK inhibitor PD0325901 (Stem Cell Institute, Cambridge), 0.5 µM GSK3 inhibitor IM-12 (BML-WN102-0005, Enzo Life Sciences), 0.5 µM RAF inhibitor SB-590885 (S0459, LKT Labs), 1 µM Src inhibitor WH-4-023 (5413, Tocris) and 10 µM ROCK inhibitor Y-27632.

Embryos that attached to the plates and preserved the epiblast lineage were considered for statistical analyses. From a total of 87 embryos thawed, 8 were fixed at the blastocyst stage (Fig. 5a). Out of these 8 embryos, 4 had epiblast cells as determined by immunofluorescence. The remaining 79 human embryos were cultured in the IVC system. Out of these 79 embryos, 34 survived the thawing procedure, attached to the plates and survived in the in vitro culture system. Out of these 34, the epiblast lineage was preserved in 21 embryos (Fig. 5 and Extended Data Fig. 9).

Mouse embryos: Mice were kept in the Animal House in accordance with national and international guidelines. All experiments have been regulated by the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB), were approved by the Home Office. Animals were inspected daily and those that showed health concerns were culled by cervical dislocation.

Mouse embryos were recovered at embryonic days E4.5 (9am) and E4.75 (4pm) from naturally mated F1 (C57Bl6xCBA) or MF1 females by flushing the uterus with M2 medium. Embryos recovered at E5.0 (12pm) and E5.5 were manually dissected from the decidua. Females for natural matings were used at 3 ± 1 month of age.

For mESC-embryo chimera experiments embryos were obtained from F1 females superovulated by injection of 7.5 IU of pregnant mares’ serum gonadotropin (PMSG, Intervet), followed by injection of 7.5 IU of human chorionic gonadotropin (hCG, Intervet) and mating with F1 males. Chimeric embryos were transferred to F1 females mated with vasectomized CD1 males. Superovulated females were used at 6 ± 1 week of age.

To culture embryos through the pre- to post-implantation transition, the mural trophoderm of E4.5 mouse embryos was removed as previously described. Embryos were subsequently plated in 8 well µ-Slides (80826, Ibidi) for 24 hours in IVC1 medium.
Epiblast dissection at peri-implantation stages: CAG-GFP expressing mouse embryos were recovered at E4.5, E4.75 and E5.0. The mural trophectoderm (E4.5 and E4.75 embryos) and the extraembryonic ectoderm (E5.0 embryos) were removed with a finely pulled glass pipette. Embryos were then treated for 15 min at 4°C with Cell Dissociation Buffer (enzyme-free) (13150016, ThermoFisher Scientific) followed by pipetting with a narrow glass pipette. This treatment removed most of the primitive endoderm/visceral endoderm and polar trophectoderm cells. If remaining GFP-negative cells were observed, these were separated with a finely pulled glass pipette.

mESC culture: mESCs were routinely cultured in gelatin-coated plates in N2B27-2i-LIF at 37°C, 5% CO₂, 21% O₂. N2B27 medium comprised a 1/1 mix of DMEM/F12 (21331-020, ThermoFisher Scientific) and Neurobasal A (10888-022, ThermoFisher Scientific) supplemented with 1% v/v B-27 (10899-038, ThermoFisher Scientific), 0.5% v/v N-2 (homemade), 100 µM β-mercaptoethanol (31350-010, ThermoFisher Scientific), Penicillin-Streptomycin (15140122, ThermoFisher Scientific) and GlutaMAX (35050061, ThermoFisher Scientific). N-2 supplement contained DMEM/F12 medium (21331-020, ThermoFisher Scientific), 2.5 mg/mL Insulin (I9287, Sigma), 10 mg/mL Aprotinin (T1147, Sigma), 0.75% Bovine Albumin Fraction V (15260037, ThermoFisher Scientific), Sodium Pyruvate (11360070, ThermoFisher Scientific) and 100 µM β-mercaptoethanol (31350-010, ThermoFisher Scientific).

As an alternative way to preserve naïve pluripotency, mESCs were cultured on Mitomycin C (M4287, Sigma) - treated Mouse Embryonic Fibroblasts (MEFs) in Fc medium supplemented with 5 µM Gö6983 (G9198, Sigma). Fc medium contained DMEM (41966, ThermoFisher Scientific), 15% Fetal Bovine Serum (Stem Cell Institute, Cambridge), Penicillin-Streptomycin (15140122, ThermoFisher Scientific), GlutaMAX (35050061, ThermoFisher Scientific), MEM Non-Essential Amino Acids (11410035, ThermoFisher Scientific), Sodium Pyruvate (11360070, ThermoFisher Scientific) and 100 µM β-mercaptoethanol (31350-010, ThermoFisher Scientific).

Cells were routinely passaged with Trypsin EDTA (25300054, ThermoFisher Scientific). Fc medium was subsequently added to neutralize the trypsin and cells were centrifuged at 1000 rpm for 5 minutes. The following mESC lines were used: E14 WT mESCs (kindly provided by Prof. Austin Smith, Stem Cell Institute, Cambridge, UK), 129 WT mESCs (kindly provided by Dr. Jacob Hanna, Weizmann Institute of Science, Israel), ΔPE-Oct3/4-GFP mESCs (kindly provided by Prof. Azim Surani, The Gurdon Institute, Cambridge, UK), Rex1::GFPd2 mESCs (kindly provided by Prof. Austin Smith, Stem Cell Institute, Cambridge, UK), Nanog-YFP mESCs, Dicer8 KO mESCs (kindly provided by Dr. Jacob Hanna, Weizmann Institute of Science, Israel), Otx2 KO mESCs and KH2 dox-inducible Nanog mESCs (kindly provided by Dr. Konrad Hochedlinger, Harvard Stem Cell Institute, Boston, US).

mESC-embryo chimeras: Chimeras were generated by aggregation of H2B-GFP expressing mESCs with 8-cell stage mouse embryos. Briefly, 4-cell stage embryos were recovered by flushing the oviducts with M2 medium. Embryos were cultured in KSOM medium (MR-020P-5F, Millipore) until the 8-cell stage, at which point zona pellicuda was removed by brief treatment with Acidic Tyrode’s solution (T1788, Sigma). H2B-GFP expressing mESCs were cultured in 3D matrigel for 24 or 72 hours. To obtain a single cell suspension, the 3D cultures were treated with Cell Recovery Solution (354253, Corning) for 15 min at 4°C. The matrigel was broken into small pieces with a scraper, the resulting suspension was centrifuged and the cell pellet was gently resuspended in Trypsin EDTA (25300054, ThermoFisher Scientific). After trypsin inactivation, cells were plated in gelatin-coated plates. The resulting ESC colonies were gently dissociated into small clumps and aggregated with the 8-cell embryos in M2 medium. The resulting chimeras were cultured in KSOM medium until E3.5, when they were transferred into pseudo-pregnant females. Chimeras were recovered at E6.5.

mESC derivation: For Nanog-YFP mESC derivation, 8-cell stage mouse embryos were recovered from the oviducts of pregnant females and cultured in KSOM (MR-020P-5F, Millipore) in the presence of 2i. 24 h later, the medium was changed to N2B27-2i-LIF for 48 h. Hatched blastocysts were
subsequently plated on Mitomycin C - treated MEFs in Fe medium containing 2i-LIF. 2 days later, blastocysts outgrowths were trypsinized and plated to obtain mESC colonies. Positive colonies were identified by immunofluorescence and PCR using two different sets of primers: Nanog-YFP PCR1 FW1: AGCTTGGAAATGTGCTCCGC Nanog-YFP PCR1 RV1: CAATTAGAGCTATGCGAGAA Nanog-YFP PCR1 RV2: CCACCCCCGTAACAGCCTCC Nanog-YFP PCR2 FW1: AGCTTGGAAATGTGCTCCGC Nanog-YFP PCR2 RV1: CTTGCTCGCAGACTAGTTC Nanog-YFP PCR2 RV2: CCACCCCCGTAGAAACAGCCTCC For derivation of mESCs from implantation cultures, 24 hours after culture in IVC1 or IVC1 +2i-LIF embryos were plated on Mitomycin C – treated MEFs in Fe medium containing 2i-LIF. 2 days later the outgrowths were trypsinized and re-plated, and the appearance of dome-shaped mESC colonies was monitored.

**hESC culture:** All hESC experiments were approved by the UK Stem Cell Bank Steering Committee and comply with the regulations of the UK Code of Practice for the Use of Human Stem Cell Lines. The WIBR3 ΔPE-OCT4-GFP dox-inducible NANOG KLF2 hESC line\(^9\) (kindly provided by Prof. Rudolf Jaenisch, MIT, Boston US) was routinely cultured in irradiated CF-1 MEFs (GSC-6101G, Amsbio) in N2B27-2i-LIF-dox at 37°C, 5% CO\(_2\), 21% O\(_2\); N2B27 medium comprised a 1/1 mix of DMEM/F12 (21331-020, ThermoFisher Scientific) and Neurobasal A (10888-022, ThermoFisher Scientific) supplemented with 2% v/v B-27 (10889-038, ThermoFisher Scientific), 1% v/v N-2 (17502-048, ThermoFisher Scientific), 100 µM β-mercaptoethanol (31530-010, ThermoFisher Scientific), Penicillin-Streptomycin (15140122, ThermoFisher Scientific), GlutaMAX (35050061, ThermoFisher Scientific), MEM Non-Essential Amino Acids (1140035, ThermoFisher Scientific), 50 µg/mL BSA (A3311, Sigma) and 0.5% v/v KnockOut Serum Replacement (10828010, ThermoFisher Scientific). To preserve naïve pluripotency 2i-LIF, hESC culture: 2 days after plating the medium was switched to transgene independent naïve hESC media. Two days later the outgrowths were trypsinized and plated, and the appearance of dome-shaped mESC colonies was monitored.

To convert naïve hESCs to primed conditions, two days after plating the medium was changed to DMEM/F12 (21331-020, ThermoFisher Scientific) supplemented with 15% FBS (10082139, ThermoFisher Scientific), 5% KnockOut Serum Replacement (10828010, ThermoFisher Scientific), GlutaMAX (35050061, ThermoFisher Scientific), MEM Non-Essential Amino Acids (1140035, ThermoFisher Scientific), Penicillin-Streptomycin (15140122, ThermoFisher Scientific), 100 µM β-mercaptoethanol (31530-010, ThermoFisher Scientific) and 4 ng/mL bFGF2 (Stem Cell Institute, Cambridge). 4 to 5 days later, and when clear primed colonies were identified, cells were passaged using Collagenase/dispase (000000010269638001, Sigma) for 15 min, followed by addition of Fc medium and centrifugation for 3 min at 1000 rpm. Cells were routinely tested for mycoplasma contamination by PCR.

Naïve WIBR3 ΔPE-OCT4-GFP dox-inducible NANOG KLF2 hESCs were plated on CF-1 MEFs and two days after plating the medium was switched to transgene independent naïve hESC media. Two different formulations were used:

- 5i/LIF\(^9\): N2B27 medium was supplemented with 20 ng/mL recombinant human LIF (300-05, PeproTech), 20 ng/mL Activin-A (Stem Cell Institute, Cambridge), 1 µM MEK inhibitor PD0325901 (Stem Cell Institute, Cambridge), 1 µM MEK inhibitor CHIR99021 (Stem Cell Institute, Cambridge), 1 µM β-mercaptoethanol, 100 µM β-mercaptoethanol, 2 µg/mL BSA (A3311, Sigma), and 0.5% v/v KnockOut Serum Replacement (10828010, ThermoFisher Scientific). To preserve naïve pluripotency 5i, hESCs were plated on CF-1 MEFs (GSC-6101G, Amsbio) in N2B27 medium comprised a 1/1 mix of DMEM/F12 (21331-020, ThermoFisher Scientific) and Neurobasal A (10888-022, ThermoFisher Scientific) supplemented with 2% v/v B-27 (10889-038, ThermoFisher Scientific), 1% v/v N-2 (17502-048, ThermoFisher Scientific), 100 µM β-mercaptoethanol (31530-010, ThermoFisher Scientific), Penicillin-Streptomycin (15140122, ThermoFisher Scientific), GlutaMAX (35050061, ThermoFisher Scientific), MEM Non-Essential Amino Acids (1140035, ThermoFisher Scientific), 50 µg/mL BSA (A3311, Sigma) and 0.5% v/v KnockOut Serum Replacement (10828010, ThermoFisher Scientific). To preserve naïve pluripotency 5i-LIF dox was added to the medium (1 µM MEK inhibitor PD0325901 (Stem Cell Institute, Cambridge), 3 µM GSK3 inhibitor CHIR99021 (Stem Cell Institute, Cambridge), 20 ng/mL recombinant human LIF (300-05, PeproTech) and 1 µg/mL doxycycline hyclate (D9891, Sigma). Cells were routinely passaged once/twice per week by treatment with StemPro Accutase reagent (A1110501, ThermoFisher Scientific) for 3 min, followed by addition of Fe medium and centrifugation for 3 min at 1000 rpm. Cells were routinely tested for mycoplasma contamination by PCR.

To convert naïve hESCs to primed conditions, two days after plating the medium was changed to DMEM/F12 (21331-020, ThermoFisher Scientific) supplemented with 15% FBS (10082139, ThermoFisher Scientific), 5% KnockOut Serum Replacement (10828010, ThermoFisher Scientific), GlutaMAX (35050061, ThermoFisher Scientific), MEM Non-Essential Amino Acids (1140035, ThermoFisher Scientific), Penicillin-Streptomycin (15140122, ThermoFisher Scientific), 100 µM β-mercaptoethanol (31530-010, ThermoFisher Scientific) and 4 ng/mL bFGF2 (Stem Cell Institute, Cambridge). 4 to 5 days later, and when clear primed colonies were identified, cells were passaged using Collagenase/dispase (000000010269638001, Sigma) for 15 min, followed by addition of Fe medium, centrifugation for 3 min at 1000 rpm and plating in the presence of 10 µM ROCK inhibitor Y-27632 (72304, Stemcell Technologies) for 24 h. Primed hESCs were switched to a MEF-independent culture by using geltrex (A1569601, ThermoFisher Scientific) - coated plates and mTESR1 medium (05850, Stemcell Technologies). Primed hESCs cultured in mTESR1 were routinely passaged using StemPro Accutase reagent (A1110501, ThermoFisher Scientific). During the first 24 h after passing 10 µM ROCK inhibitor Y-27632 (72304, Stemcell Technologies) was added to the medium.

Naïve WIBR3 ΔPE-OCT4-GFP dox-inducible NANOG KLF2 hESCs were plated on CF-1 MEFs and two days after plating the medium was switched to transgene independent naïve hESC media. Two different formulations were used:

- 5i/LIF\(^9\): N2B27 medium was supplemented with 20 ng/mL recombinant human LIF (300-05, PeproTech), 20 ng/mL Activin-A (Stem Cell Institute, Cambridge), 8 ng/mL bFGF2 (Stem Cell Institute, Cambridge), 1 µM MEK inhibitor PD0325901 (Stem Cell Institute, Cambridge), 0.5 µM GSK3 inhibitor IM-12 (BML-WN102-0005, Enzo Life Sciences), 0.5 µM RAF inhibitor SB-590885 (S0459, LKT Labs), 1 µM Src inhibitor WH-4-023 (5413, Tocris) and 10 µM ROCK inhibitor Y-27632.
- RSet medium (05970, Stemcell Technologies).

Transgene-independent hESCs were routinely cultured on CF-1 MEFs (GSC-6101G, Amsbio) and passaged using StemPro Accutase reagent (A1110501, ThermoFisher Scientific).
mESC spheroid formation: to induce polarization and lumenogenesis of mESCs we used matrigel, as it can mimic in vitro the basement membrane that surrounds the epiblast at implantation in vivo. Two different protocols were used:

- Embedded: pellets containing 20000 mESCs were resuspended in 20 µL of ice-cold Growth Factor Reduced Matrigel (356230, BD Biosciences). The solution was placed as a drop in a well of a µ-Slide 8 well ibiTreat (IB-80826, Ibidi) and incubated for 2 min at 37°C to allow the matrigel to solidify. Next, 300 µL of medium (N2B27 or Fc depending on the experiment) were added to the well.

- 3D on top: a well of a µ-Slide 8 well ibiTreat (IB-80826, Ibidi) was covered with 35 µL of ice-cold Growth Factor Reduced Matrigel (356230, BD Biosciences) and incubated for 2 min at 37°C to allow the matrigel to solidify. In the meantime, 20000 mESCs were resuspended in N2B27 and the cell suspension was carefully plated on the matrigel-coated well. When approximately 80% of the cells had attached to the matrigel (5-10 min after plating) the medium was removed and replaced with N2B27 containing 5% matrigel. Where indicated mESCs were treated with 50 µg/mL of protamine sulfate (1101230005, Merck Millipore).

hESC spheroid formation: hESCs were induced to polarize and form lumens using the 3D on top protocol as previously described. Briefly, a single cell suspension of hESCs was plated on matrigel-coated µ-Slide 8 well ibiTreat (IB-80826, Ibidi) as detailed above. Upon attachment to the matrigel-coated surface, the medium was replaced with hESC medium (mTESR or naïve hESC medium depending on the experiment) containing 5% matrigel. To avoid single cell-induced primed hESC death 10 µM ROCK inhibitor Y-27632 (72304, Stemcell Technologies) was added to the medium for 24 h. To monitor de dynamics of naïve pluripotency exit and morphogenesis, naïve hESCs were plated in 3D matrigel in mTESR medium.

siRNA treatments: mESCs were transfected with Lipofectamine RNAiMAX Transfection Reagent (13778030, ThermoFisher Scientific) using a reverse transfection protocol. Briefly, 100000 cells were plated on gelatin-coated 12 well plates together with the Lipofectamine RNAiMAX-siRNA mix. 0.5 µL of a 20 µM siRNA solution were used per well. AllStars negative control siRNAs (1027280, Qiagen) were used as control.

For downregulation of Podxl expression an equimolar mix of the following siRNAs (Quiagen) was used:

- SI01383123: AAGAAUGUAAGUCUAAUUA
- SI01383130: CUGGAAUUAUUGAGAGAUUA
- SI01383137: CCCAUUUUCAUCCUAUAUU

For downregulation of Cgn expression an equimolar mix of the following stealth siRNAs (ThermoFisher Scientific) was used:

- CgnMSS230393: CCCUCAUCCAUUGCAUCACUGCUUA
- CgnMSS230394: GAAAGACAGUUCUGCAGUCCACAAU
- CgnMSS230395: GGCUUGCCUUUAUGAGUCCUAGUAA

For downregulation of Nanog expression an equimolar mix of the following siRNAs (Qiagen) was used:

- SI01323357: AGCCTTGGAATTATTCCTGA
- SI04460869: TGCCAGTGATTTGGAGGTGA
- SI04460883: CAGGTTTCAGAAGCAGAAGTA

For downregulation of Oct4 expression the following stealth siRNA (ThermoFisher Scientific) was used:

- Pou5f1MSS237605: ACCUUCUCAACUCAGCCAUUG

Cloning: Cloning procedures were done using Gateway technology (ThermoFisher Scientific). Briefly, the fragment of interest was amplified by PCR to introduce attB sites. This was cloned into a pDONR221 vector (kind gift of Jose Silva, Stem Cell Institute, Cambridge, UK) using the BP clonase II (11789020, ThermoFisher Scientific).

For cloning GFP-Rab11aS25N (dominant negative): a Rab11aS25N-pEGFP plasmid was used as a template for cloning (kindly provided by Prof. Jon Clarke, King’s College London, UK). The fragment corresponding to GFP-Rab11aS25N was amplified by PCR using the following primers:
Rab11a FW:
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGTGAGCAAGGGCGAGGAG
Rab11a RV:
GGGGACCACTTTGTACAAAAAAGCAGGCTTCACCATGGTGACAGCACTGC
- GFP-Podxl: a GFP-Podxl-pEGFP plasmid was used as a template for cloning (kindly provided by Dr. Arnaud Echard, Institut Pasteur, Paris, France). The fragment corresponding to CD8 tag – VSV-g tag – EGFP – Rhodopsin – rabbit Podxl was amplified by PCR using the following primers:
FW: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGCCTTACCAGTGACCGC
RV: GGGGACCACTTTGTACAAAAAAGCAGGCTTCACCATGGTGACAGCACTGC

- GFP-Cd34: Mouse Cd34 was amplified from cDNA using the following primers:
Cd34 FW: ACCACGGAGACTTCTACACAAGG
Cd34 RV: TCACAGTTCTGTGTCAGCCAC

Using the GFP-Podxl-pEGFP plasmid as a template, the N-terminal region corresponding to CD8-VSV-G-GFP-Rhodopsin was amplified by PCR using the following primers:
GFP FW: ATGGCCTTACCAGTGACCGC
GFP RV: GAATTCCGTCGCATTGGAGAA

The two fragments were joined by overlap PCR and attB sites were added for cloning into the pBlastidicin vector.

- H2B-GFP: An H2B-GFP expressing plasmid was used as a template for cloning (kindly provided by Dr. Mirna Perez-Moreno, CNIO, Madrid, Spain). The fragment corresponding to H2B-GFP was amplified by PCR using the following primers:
FW: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGCCAGAGCCAGAAGT
RV: GGGGACCACTTTGTACAAAAAAGCAGGCTTCACCATGCCAGAGCCAGAAGT

To generate a WT GFP-Rab11a construct we performed site-directed mutagenesis using the GFP-Rab11aS25N-pEGFP plasmid as a template. A 4-primer PCR strategy was designed to correct the mutated site. The following primers were used:
Rab11a FW (external):
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGGTGAGCAAGGGCGAGGAG
Rab11a RV (external):
GGGGACCACTTTGTACAAAAAAGCAGGCTTCACCATGGTGACAGCACTGC
Rab11a-WT FW (internal):
CTGGTGTTGGAAAGTCTAATCTCTTGTCTCGATTTACTCGAAATGAGTTTAATCTCGAAAG
Rab11a-WT RV (internal):
GAGACAAGAGATTAGACTTTCCAACAGAAAAGCTGGTGCTTACTTCGATTTACTCGAAAG

The two fragments obtained were joined by overlap PCR.

CRISPR/Cas9: genome editing was performed using the CRISPR-Cas9 system as previously described. gRNAs were designed using the MIT CRISPR Design Tool (http://crispr.mit.edu:8079/). A two-gRNA strategy was designed in order to remove the initiation ATG. The following gRNAs were used:
gRNA1: CGAACTCCGGAGTCGCGATC
gRNA2: CTGCAAGCGGTCCGACCACG

The gRNA templates were assembled and ligated into the PX459 vector. The following primers were used:
gRNA1 FW: CACCGCTAAGGATCCGCGATCCCGATCGC

Three KO clones were selected for further analyses by RT-PCR (data not shown) and immunofluorescence.
**mESC transfection:** mESCs were transfected using Lipofectamine 3000 (L3000001, ThermoFisher Scientific) following the manufacturer’s instructions. Briefly, the day before transfection 50000 cells were plated on 24 well gelatin-coated plates in N2B27-2i-LIF without antibiotics. For transfection 0.5 μg of a PiggyBac transposon vector (kind gift of Dr. Jose Silva, Stem Cell Institute, Cambridge, UK) and 0.5 μg of either GFP-Podxl-pHygro, GFP-Cd34-pLasticidin, tetO-Otx2-puro18, H2B-GFP-pHygro, GFP-Rab11a-pHygro or GFP-Rab11aS25N-pHygro were used. Transfected cells were selected with 200 μg/mL of Hygromycin B (10687010, ThermoFisher Scientific), 10 μg/mL of Blastidin (R210-01, ThermoFisher Scientific) or 2 μg/mL of Puromycin (ant-pr-1, Invivogen) and the resulting colonies were manually picked and expanded.

**Immunofluorescence:** Cells and embryos were fixed in 4% PBS/paraformaldehyde (PFA) (15710, Electron Microscopy Sciences) for 20 min at RT and subsequently washed twice with 0.1% Tween/PBS. To stain centrosomes, cells and embryos were fixed in ice-cold methanol for 5 min at 4°C. Permeabilization was done in PBS containing 0.3% Triton X-100 and 0.1 M glycine for 30 min at RT. Cells were incubated with primary antibodies (Supplementary Table 2) at 4°C ON, followed by incubation with fluorescently-conjugated Alexa Fluor secondary antibodies (ThermoFisher Scientific) for 2 h at RT (Supplementary Table 2). Both primary and secondary antibodies were diluted in 1% BSA, 0.1% Tween/PBS.

Images were acquired in an inverted SP5 confocal microscope (Leica Microsystems) with a Leica HC PL APO 1.4 NA 63X oil objective or in an inverted SP8 confocal microscope (Leica Microsystems) with a Leica HC PL APO CS2 1.4 NA 63x oil objective.

**Time-lapse microscopy:** Time-lapse images of mESCs were acquired in an inverted SP5 confocal microscope (Leica Microsystems) with a Leica HC PL FLUOTAR 0.5 NA 20.0X dry objective. Cells were imaged in a humidified chamber with 21% O2 and 5% CO2. Images were taken at intervals of 30 min with a Z step of 4 μm.

**Fixation and staining for serial block face imaging:** Samples were fixed by immersion with 2% formaldehyde (made from PFA) and 2% vacuum distilled glutaraldehyde, containing 2mmol/L CaCl2, in 0.05M sodium cacodylate buffer at 4°C and pH 7.4 for 8 hours at 4°C. They were rinsed for 5 x 3 minutes in cold cacodylate buffer containing 2mM calcium chloride. After buffer washes, they were incubated in 1% osmium ferricyanide for 18 hours at 4°C and rinsed 5 times in Distilled-Deionized Water (DIW). This was followed by 30 minutes incubation in 1% thioacetohydrazide at room temperature and 5 more rinses in DIW. Samples were then incubated in 1% uranyl acetate in 0.05 maleate buffer at pH 5.5 and at 4°C for 48 hours. They were again rinsed with DIW at room temperature 5 x 3 minutes, and dehydrated through 2 times each of 50%, 70%, 90%, and 100% ethanol, dry ethanol, dry acetone and dry acetonitrile. The dehydrated samples were infiltrated with Quetol 651 epoxy resin over a period of 5 days. The resin was cured for 48 hours at 65°C. Thin sections were prepared with a Leica Ultracut S mounted on 200 mesh copper grids and viewed with a Tecnica G2 operated at 200kV.

**Image analysis:** All microscopy data were analyzed using Fiji software38 (http://fiji.sc). For 3D reconstructions images were cropped and filter in Fiji and the rendering was done using Chimera (v.1.8.1) (https://www.cgl.ucsf.edu/chimera/). For all quantifications, laser power and detector gain were maintained constant to quantitatively compare different experimental conditions within a single experiment

- Quantification of nuclear fluorescence intensity in ESCs: DAPI images were binarized and a mask was created to segment in 2D the nuclei. These were saved as Regions Of Interest (ROIs), which were used to delineate nuclei in the channel of interest, and nuclear fluorescence intensity was measured within each ROI. For every ESC spheroid a unique averaged fluorescence intensity value was generated. Error bars represent the variability (s.e.m.) across different spheroids from different independent experiments. To compare fluorescence intensity values across different experiments, raw fluorescence intensity values were normalized to the average fluorescence intensity of the corresponding 2iLIF experimental condition. Using this approach, mESCs were considered to be negative for Oct4 (in the Oct4 siRNA group) or negative for Nanog (in the Nanog siRNA group) when the average fluorescence of the spheroid was below the 25% percentile of the corresponding control group.

- Quantification of Podxl and Cgn: to determine whether a spheroid was negative for Podxl (in the Podxl siRNA group) or negative for Cgn (in the Cgn siRNA group) the Podxl or Cgn channels in the
control group were used to set a threshold value of intensity. This threshold was applied to the experimental group and the images were binarized. mESC spheroids that did not show any signal based on the threshold value of intensity were considered to be negative.

- Quantification of nuclear fluorescence intensity in embryos: a representative Z plane was chosen for every embryo and the fluorescence intensity of epiblast pluripotency genes was measured using the DAPI channel to segment the nuclei as mentioned above. To account for changes in fluorescence in the Z-axis, the fluorescence intensity of the gene of interest in the epiblast was normalized to the fluorescence intensity of DAPI in the epiblast. Error bars represent the variability (s.e.m.) across different embryos.

- Quantification of fluorescence intensity in time-lapse experiments: to quantify the dynamics of naive pluripotency gene expression, the brightfield channel was used to create a mask, and applied to the channel of interest as mentioned above. For every time step a single fluorescence value corresponding to the average population was measured. Error bars represent the variability (s.e.m.) across different fields of view. To account for photobleaching during imaging in Extended Data Fig. 2e the raw fluorescence intensity values of the -2i/LIF experimental group were normalized to the raw fluorescence intensity values of the 2i/LIF control group (Extended Data Fig. 3k-m) throughout the time lapse. All experimental conditions shown in Extended Data Fig. 2e and Extended Data Fig. 3k-m were imaged simultaneously.

- Analysis of centrosome positions: to analyze the position of centrosomes the nucleus-nucleus axis and the nucleus-centrosome axis were determined. The angle between both vectors was calculated and the XY positions were normalized to the internuclear distance.

- Analysis of lumen and rosette formation: to quantitatively determine whether rosettes and lumens form in embryos and ESC spheroids we immunostained components of the Par polarity complex (either aPKC or Par6) and/or Podxl, as they are known to localize to the lumen in 3D in vitro cultures\textsuperscript{20} and are used as bona-fide luminal markers in different model systems\textsuperscript{39,40}. Phalloidin staining was used to reveal cell shapes. Lumens and rosettes displayed a polarized organization, whereas disorganized structures did not show a polarized localization of Par proteins and/or Podxl. In addition, the fluorescence intensity profile was plotted from a line drawn through the location of interest (rosettes or lumens). One peak of fluorescence intensity corresponds to a rosette (a closed spot of fluorescence) whereas two peaks depict a lumen (two walls and a central depression) (Fig. 1a, Fig. 5d and Extended Data Fig. 2b are shown as examples).

In Extended Data Fig. 9a the F-actin channel was separated from the nanog channel by applying a subtract function in Fiji.

**RNA extraction and RT-PCR:** For RNA extraction of mESCs cultured in matrigel (3D on top protocol), the matrigel was first removed by treatment with Cell Recovery Solution (354253, Corning) for 15 min at 4°C. To facilitate the depolimerization, the matrigel was manually broken into small pieces. Cells were subsequently centrifuged and pellets were washed once with PBS.

RNA was extracted using TRIzol Reagent (15596010, ThermoFisher Scientific) following the manufacturer’s instructions. 1 µg of RNA was used to perform a reverse transcriptase reaction in the presence of random primers (C1181, Promega), dNTPs (N0447S, New England BioLabs), RNase inhibitor (M0314L, New England BioLabs) and M-MuLV reverse transcriptase (M0253L, New England BioLabs). RT-PCR reactions were carried out using Power SYBR Green PCR Master Mix (4368708, ThermoFisher Scientific) in a Step One Plus Real-Time PCR machiner (Applied Biosystems). The following program was used: 10 min 95°C followed by 40 cycles of 15 sec 95°C (denaturing) and 1 min 60°C (annealing and extension). The primers used are listed on Supplementary Table 3.

Mouse gene expression data were normalized to gapdh, and human gene expression data were normalized to HPRT. Error bars represent variability (s.e.m.) across multiple independent experiments.

**Library preparation, RNA sequencing and mapping of reads**

The SMARTSeq2 protocol\textsuperscript{11} was used to amplify mRNA with the addition of ERCC spike-in control (1µl of 1:1,000,000 dilution of mix 1 (4456740, Ambion) per sample).

Nextera XT (Illumina) was used to generate multiplex sequencing libraries from amplified cDNA. Libraries were sequenced on a HiSeq 2500 running in rapid mode. The paired-end reads were then mapped to both the M. musculus genome (Ensembl v.38.77) as well as to ERCC sequences using the default settings on GSNAP (v.2014-10-07). Hiseq-count\textsuperscript{22} (v.0.6.1p1 default options) was used to count the number of reads mapped to each gene. All the samples were analysed in a single experiment to avoid batch effects.
Sample quality assessment

Three metrics were used to assess data quality: the fraction of reads that were mapped, the number of genes that had more than 10 reads per million (RPM) as well as the fraction of reads that were mapped to mitochondrial genes. Extended Data Fig. 1b-e displays these metrics, with each being shown as a function of the total read number per sample. A principal component analysis was run and outliers were defined as performing worse than average on all three of these metrics (Extended Data Fig. 1f). These analyses uncovered one clear outlier (sample name “E4.5_Sample1” – highlighted in grey), which was then excluded from all further downstream analyses.

We also controlled for possible contamination with cells from the primitive endoderm. We verified that the E5.0 samples do not show any expression of primitive endoderm markers like Gata6, Gata4, Sox17, Sox7 and Pdgfra. As for samples from E4.5 and E4.75 embryos, they all cluster with the epiblast samples previously published by Boroviak et al (see Extended Data Fig. 1g), except for one sample (“E4.5_Sample3”) that was found in the primitive endoderm cluster and was therefore excluded from further analysis (see below for details on the clustering algorithm).

The samples that passed the quality check were normalized for sequencing depth using size factors calculated on endogenous genes.

Principal component analysis and hierarchical clustering

The principal component analyses (PCAs) plots shown in Fig. 1b and Extended Data Fig. 1f and h were generated using the log10-transformed read counts (by summing 1 to avoid infinities) of the union of the 5,000 most highly expressed genes in each sample.

The hierarchical clustering shown in Fig. 1c and Extended Data Fig. 1g was based on the dissimilarity matrix defined as (1-ρ)/2, where ρ is the Spearman correlation coefficient between pairs of samples or genes. Once the dissimilarity matrix was calculated, the hierarchical tree was generated with the R function “hclust” with the “average” agglomeration method.

Differential Expression Analysis

The Bioconductor package DESeq2 was used to find differentially expressed genes between E5.0 samples and E4.5-E4.75 samples at a 0.1 false discovery rate. Before running DESeq2, genes with an average expression level of less than 1 normalized read counts across all samples were excluded.

Comparing our dataset to data in Boroviak et al

In Extended Data Fig. 1h, before selecting the genes for the PCA, we pooled together our data with the ICM and epiblast samples previously published in Boroviak et al (FPKM normalized read-counts available from the cited paper) and performed a quantile normalization.

The log-fold-changes plotted in Extended Data Fig. 1i were computed after adding a pseudo-count of 0.1. The genes used for the clustering in Supplementary Extended Data Fig. 1g are those differentially expressed between epiblast and primitive endoderm at E4.5 as reported by Boroviak et al.

Statistical analyses: Statistical analyses were performed using GraphPad Prism (excluding the analysis of the sequencing data). Embryos were randomly allocated to control and experimental groups. Sample size was determined based on previous experimental experience. Investigators were not blinded to group allocation. Quantitative data is presented as a contingency table and was analyzed with a Chi-square test. Quantitative data is presented as mean ± s.e.m. and was analyzed for normality using a D’Agostino-Pearson omnibus normality test. Data that presented a Gaussian distribution was analyzed using a two-tailed unpaired Student’s test (two groups) or an ANOVA test (multiple groups) with a Tukey’s multiple comparison test. Significant differences in the variance were taken into account using a Welch’s correction. Data that did not present a Gaussian distribution was analyzed using a Mann-Whitney test (two groups) or a Kruskal-Wallis test (multiple groups) with a Dunn’s multiple comparison test. For all quantifications a minimum of two independent experiments was performed. Sequencing data was analyzed with standard programs and packages. Code is available on request.

Data availability statement: RNA sequencing data is available at Array Express under accession number E-MTAB-5147. The immunofluorescence and time-lapse data that support the findings of this study are available from the corresponding author upon reasonable request. Source data for RT-PCR experiments and quantifications of the immunofluorescence data are provided with the paper.
Rhee, J. M. et al. In vivo imaging and differential localization of lipid-modified GFP-variant fusions in embryonic stem cells and mice. *Genesis* 44, 202-218, doi:10.1002/dvg.20203 (2006).

Yeom, Y. I. et al. Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. *Development* 122, 881-894 (1996).

Kalkan, T. et al. Tracking the embryonic stem cell transition from ground state pluripotency. *Development*, doi:10.1242/dev.142711 (2017).

Panamarova, M. et al. The BAF chromatin remodelling complex is an epigenetic regulator of lineage specification in the early mouse embryo. *Development* 143, 1271-1283, doi:10.1242/dev.131961 (2016).

Lee, G. Y., Kenny, P. A., Lee, E. H. & Bissell, M. J. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nat Methods* 4, 359-365, doi:10.1038/nmeth1015 (2007).

Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. *Nature protocols* 8, 2281-2308, doi:10.1038/nprot.2013.143 (2013).

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nature methods* 9, 671-675 (2012).

Martin-Belmonte, F. et al. Cell-polarity dynamics controls the mechanism of lumen formation in epithelial morphogenesis. *Current biology : CB* 18, 507-513, doi:10.1016/j.cub.2008.02.076 (2008).

Yang, Z. et al. De novo lumen formation and elongation in the developing nephron: a central role for afadin in apical polarity. *Development* 140, 1774-1784, doi:10.1242/dev.087957 (2013).

Picelli, S. et al. Full-length RNA-seq from single cells using Smart-seq2. *Nature protocols* 9, 171-181, doi:10.1038/nprot.2014.006 (2014).

Anders, S., Pyl, P. T. & Huber, W. HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166-169, doi:10.1093/bioinformatics/btu638 (2015).

Anders, S. & Huber, W. Differential expression analysis for sequence count data. *Genome Biol* 11, R106, doi:10.1186/gb-2010-11-10-r106 (2010).

Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15, 550, doi:10.1186/s13059-014-0550-8 (2014).
Extended Data Figure 1: Epiblast gene expression patterns at peri-implantation stages. a, CAG-GFP positive embryos recovered at peri-implantation stages, before and after dissection of the epiblast. (n=4 E4.5, 4 E4.75 and 4 E5.0 embryos) b-f, For all the collected samples we computed the total number of read counts (b), the fraction of reads mapped to endogenous genes (c), the number of genes with more than 10 reads per million (d) and the fraction of reads mapped to mitochondrial genes (e). A PCA of these metrics is shown in f. The sample coloured in grey (E4.5_Sample1) is characterised by a very low fraction of mapped reads and number of genes detected and was therefore removed from all downstream analysis. g, Hierarchical clustering of the E4.5-E4.75 samples that passed the quality check (black) and samples from the epiblast (red) and primitive endoderm (blue) at E4.5 from Boroviak et al. Only one sample (E4.5_Sample3, grey) clusters with the primitive endoderm, while all the others cluster together with epiblast. This sample was excluded from further analysis. h, PCA of our samples and samples from Boroviak et al. i, Log-fold-change of pluripotency marker genes between E5.0 and E4.5-E4.75 samples in our dataset (x-axis) and log-fold-change between E5.5 and E4.5 epiblast samples from Boroviak et al (y-axis). Squares, circles and triangles mark genes differentially expressed in both datasets, only in our dataset or only in Boroviak et al dataset respectively. The naïve pluripotency genes significantly downregulated at E5.5 were highly enriched among genes downregulated at E5.0 (p-value 7x10^{-8}, Fisher’s exact test), whereas the enrichment of upregulated early post-implantation factors was only marginally significant (p-value=0.03, Fisher’s exact test). j, Brightfield images of cells derived from embryos cultured in IVC1 (0/6 mESC colonies from IVC1 embryos and 4/6 mESC colonies from IVC1 +2i/LIF embryos). Scale bars, 50 µm. k, Immunostaining of mESCs derived from embryos cultured in IVC1 +2i/LIF for 24 hours. Scale bars, 10 µm. l, Experimental set-up. A: analysis. m, Immunostaining of mouse embryos cultured as indicated in panel l. Dotted lines denote the epiblast and the arrow points to the pro-angiomatic cavity. Scale bars, 20 µm. n, Lumen formation in embryos rom panel k (n=12 IVC1 control and 15 IVC1 experimental embrios). Chi-square test, *p=0.0137.

Extended Data Figure 2: Dynamics of polarization, lumenogenesis and naïve pluripotency exit in mESCs. a, Experimental set-up. A: analysis. b-c, Immunostaining of mESCs in 3D matrigel. Red long arrows indicate the position used to plot intensity profiles (below). Yellow arrows indicate polarization and white arrows lumen formation. Scale bars, 10 µm. d, Quantification of lumen formation in cells from panels b and c (n=21 (2 cells), 22 (4 cells) , 20 (8 cells) and 21 (>10 cells) spheroids per group). Chi-square test, ***p<0.0001. e, Fluorescence intensity in cells from panel f. Raw fluorescence intensity values were normalized to the fluorescence intensity of reporter cells cultured in gelatin 2i/LIF throughout the movie (n=55 Nanog-YFP, 92 ΔPE-Oct4-GFP and 63 Rex1::GFPd2 spheroids). f, Time-lapse frames of naïve reporter mESCs cultured in matrigel without 2i/LIF. mt: membrane. tomato. Time is indicated as hours:minutes. Dotted lines indicate the outline of the spheroids at the end of the experiment. Scale bars, 20 µm. g-o, Expression of pluripotency genes in mESCs cultured in gelatin or matrigel, with or without 2i/LIF. (n=5 (24 h), 3 (36 h), 3 (48 h) independent samples for Nanog, Esrrb, and Otx2; n=5 (24 h), 3 (36 h), 4 (48 h) independent samples for Fgf5; n=4 (24 h), 3 (36 h), 3 (48 h) independent samples for Klf4, and Klf2; n=4 (24 h), 3 (36 h), 4 (48 h) independent samples for Zfp42; and n=3 (24 h), 3 (36 h), 4 (48 h) independent samples for Tbx3 and Pou5f1). Unpaired Student’s t test, ns: non-significant, nd: non-detected. p, Immunostaining of mESCs cultured in 3D matrigel. Scale bars, 10 µm. All panels: gelatin (G), matrigel (M).

Extended Data Figure 3: mESCs initiate polarisation in naïve conditions in a 3D matrigel culture. a, Experimental set-up. A: analysis. b, Immunostaining of mESCs cultured as indicated in panel a. Arrows point to lumen. Scale bars, 5 µm. c, Lumen formation in cells from panel b (n=31 (2 cells, G +2i/LIF), 30 (4 cells, G +2i/LIF), 31 (2 cells, G -2i/LIF) and 31 (4 cells, G -2i/LIF) spheroids per condition). Chi-square test, ***p<0.0001. d, Internuclear distance in cells from Figure 2b (n=31 (+2i/LIF) and 30 (-2i/LIF) spheroids per condition). Mann Whitney U test, ns: non-significant. e-g, Immunostaining of mESCs cultured in matrigel with or without 2i/LIF. Squares denote magnified regions. Scale bars, 10 µm (panels e and f) and 5 µm (panel g). h-j, Representative frames from time-lapse experiments using different naïve reporter mESC lines, cultured in either gelatin or matrigel in the presence of 2i/LIF. Time is indicated as hours:minutes. Scale bars, 50 µm. k-m, Intensity of Nanog-YFP, ΔPE-Oct4-GFP and Rex1::GFPd2 mESCs analyzed by time-lapse microscopy (panels h-j) (n=65 Nanog-YFP, 55 ΔPE-Oct4-GFP and 43 Rex1::GFPd2 mESC colonies -gelatin; and n=61 Nanog-YFP, 84 ΔPE-Oct4-GFP and 61 Rex1::GFPd2 mESC spheroids -matrigel). n, Experimental set-up. o, Immunostaining of mESCs cultured as indicated in panel n. Squares denote magnified
regions. Dotted lines demarcate the border of the colonies. The nucleus-centrosome angle ($\alpha$) measured in p is indicated. Scale bars, 10 µm. p, Histogram of the angle between the centrosome-nucleus axis and the basal-apical axis of cells in the border of the colonies (panel o). Data is shown as relative frequencies (%) (n=76 (M +2i/LIF cultured in gelatin), 61 (M -2i/LIF cultured in gelatin), 62 (M +2i/LIF) and 60 (M -2i/LIF) centrosomes per condition). Kruskal-Wallis test, ***p=0.0001, ns: non-significant. q, Immunostaining of control and Dgcr8 KO mESCs (with or without 2i/LIF). Scale bars, 5 µm. r, Angle between the nucleus-centrosome axis and the nuclear-nuclear axis in cells from panel q. Each dot represents an individual centrosome (n=60 control +2i/LIF, 62 control -2i/LIF, 56 Dgcr8 KO -2i/LIF and 58 Dgcr8 KO -2i/LIF centrosomes per condition). Kruskal-Wallis test, ns: non-significant. s, Internuclear distance in cells from panel q (n=30 control +2i/LIF, 31 control -2i/LIF, 28 Dgcr8 KO +2i/LIF and 29 Dgcr8 KO -2i/LIF spheroids per condition). Kruskal-Wallis test, ns: non-significant. t, Nanog intensity in cells from panel q (n=30 control +2i/LIF, 31 control -2i/LIF, 28 Dgcr8 KO +2i/LIF and 30 Dgcr8 KO -2i/LIF spheroids per condition). Kruskal-Wallis test, ***p<0.0001, ns: non-significant. u, Immunostaining of mESCs cultured in matrigel with or without G06983. Scale bars, 10 µm. v, Internuclear distance in cells from Figure 2i (n=26 (+G06983) and 28 (-G06983) spheroids per condition). Mann Whitney U test, ns: non-significant. All panels: matrigel (M).

**Extended Data Figure 4: 2i/LIF inhibits lumen formation in mESCs.** a, Immunostaining of mESCs cultured in matrigel. Scale bars, 10 µm. b, Immunostaining of Rex1::GFPd2 mESCs cultured in matrigel. Scale bars, 10 µm. c, Oct4, Nanog and Rex1::GFPd2 fluorescence intensity in cells from panels a and b (n=30 spheroids per condition (Nanog); n=21 (+2i/LIF) and 20 (-2i/LIF) spheroids (Oct4); and 21 (+2i/LIF) and 20 (-2i/LIF) spheroids (Rex1::GFPd2)). Unpaired Student’s t test, ***p<0.0001. d-e, Electron microscopy images of mESCs cultured in matrigel with or without 2i/LIF. Arrowheads point to lumens. a: Golgi apparatus, b: basal cell-cell adhesion sites, c: Tight Junctions. Scale bars, 2 µm (panel d) and 500 nm (panel e). f-g, Immunostaining of mESCs cultured in matrigel. Squares denote magnified regions. Arrows point to inner non-polarized cells. Scale bars, 10 µm. h, Polarization in cells from panel g and Figure 3c (n=27 (+2i/LIF 48 h), 26 (-2i/LIF 48 h), 24 (+2i/LIF 72 h) and 24 (-2i/LIF 72 h) spheroids per condition). Chi-square test, ***p<0.0001. i, Experimental set-up. A: analysis j, Immunostaining of mESCs cultured as indicated in panel i. Arrows point to lumens. Scale bars, 10 µm. k, Lumen formation in cells from panel j (n=20 spheroids per condition). Chi-square test, ns: non-significant. l, Nanog intensity in cells from panel j (n=20 spheroids per condition). ANOVA test, ***p<0.0001. m, Immunostaining of mESCs cultured in matrigel with different combinations of inhibitors. Arrows point to lumens. Scale bars, 10 µm. n, Lumen formation in cells from panel m (n=20 spheroids per condition). Chi-square test, ***p<0.0001. o, Nanog intensity in cells from panel m (n=20 spheroids per condition). ANOVA test, ***p<0.0001, ns: non-significant. p, Immunostaining of mESCs cultured in matrigel with a single inhibitor/supplement. Arrows point to lumens. Scale bars, 10 µm. q, Nanog intensity in cells from panel p as a function of their ability to undergo lumenogenesis (n=30 (+2i/LIF control), n=50 (+LIF), n=48 (+GSK3i), n=51 (+MEKi) and n=39 (-2i/LIF) spheroids per condition). Mann Whitney U test, **p=0.0055, ***p<0.0001, ns: non-significant. All panels: matrigel (M).

**Extended Data Figure 5: Exit from naïve pluripotency is required for lumenogenesis in mESCs.** a, Immunostaining of control and Dgcr8 KO mESCs (with or without 2i/LIF). Scale bars, 10 µm. b, Lumen formation in cells from panel a (n=20 (control +2i/LIF), 20 (control -2i/LIF), 20 (Dgcr8 KO +2i/LIF) and 27 (Dgcr8 KO -2i/LIF) spheroids per condition). Chi-square test, *p=0.024. c, Nanog intensity in cells from a (n=20 (control +2i/LIF), 20 (control -2i/LIF), 20 (Dgcr8 KO +2i/LIF) and 27 (Dgcr8 KO -2i/LIF) spheroids per condition). ANOVA test, ***p<0.0001. d, Nanog intensity in Dgcr8 KO mESCs after 72 hours of 3D matrigel culture (panel a), as function of their ability to undergo lumenogenesis (n=6 (lumen) and 21 (no lumen) spheroids per condition). Unpaired Student’s t test, *p=0.0208. e, Immunostaining of mESCs cultured in matrigel with or without G06983. Scale bars, 10 µm. f, Lumen formation in cells from panel e (n=30 spheroids per condition). Chi-square test, ***p<0.0001. g, Immunostaining of Rex1::GFPd2 mESCs cultured in matrigel with or without G06983. Scale bars, 10 µm. h, Nanog, Rex1::GFPd2 and Oct4 intensity in cells from panels e and g (n=30 spheroids per condition (Nanog); 41 (+G06983) and 40 (-G06983) spheroids (Oct4 and Rex1::GFPd2)). Mann Whitney U test, **p=0.0002, ***p<0.0001. i, Experimental set-up. A: analysis. j, Immunostaining of mESCs cultured as indicated in panel i. The percentage of structures showing the representative phenotype is indicated. Scale bars, 5 µm. k, Nanog intensity in cells from panel j as a function of their ability to undergo lumenogenesis (n=13 (no lumen) and 20 (lumen) spheroids per condition). Unpaired Student’s t test, ***p<0.0001. l, Immunostaining of mESCs cultured as indicated in panel i. The percentage of structures showing the representative phenotype is indicated. Scale bars,
10 µm. **m**, Nanog intensity in cells from panel i as a function of their ability to undergo lumenogenesis (n=14 (no lumen) and 17 (lumen) spheroids per condition). Unpaired Student’s t test, ***p<0.0001. **n**, Immunostaining of DOX-inducible Nanog mESCs. Scale bars, 10 µm. **o**, Lumen formation in cells from panel n (n=30 (+2i/LIF/DOX), 30 (+2i/LIF), 31 (+DOX) and 28 (-2i/LIF) spheroids per condition). Chi-square test, ns: non-significant. **p**, Nanog intensity in cells from n (n=30 (+2i/LIF/DOX), 30 (+2i/LIF), 31 (+DOX) and 28 (-2i/LIF) spheroids per condition). Kruskal-Wallis test, ***p<0.0001. **q**, mRNA levels of Nanog and Otx2 in DOX-inducible Nanog mESCs (n=5 (Nanog) and 3 (Otx2) independent samples per group). Unpaired Student’s t test, **p=0.0044, ns: non-significant. **r**, mRNA levels of naïve pluripotency genes in DOX-inducible Nanog mESCs (n=4 independent samples per group). Unpaired Student’s t test, **p=0.0075, ns: non-significant. **s**, Immunostaining of control and Nanog knock-down mESCs. Scale bars, 10 µm. **t**, Lumen formation in cells from panel s (n=20 (control siRNA) and 42 (Nanog siRNA) spheroids per condition). Chi-square test, ns: non-significant. **u**, Nanog and Rex1::GFPd2 intensity in cells from panel s (n=20 (control siRNA) and 42 (Nanog siRNA) spheroids per condition). **v**, Correlation between Nanog and Rex1::GFPd2 intensity in cells from panel s (n=20 (control siRNA) and 42 (Nanog siRNA) spheroids per condition). **w**, Otx2 and Oct4 fluorescence intensity in Oct4 knock-down mESCs (n=19 (lumen) and 28 (no lumen) spheroids per condition). **x**, Immunostaining of control and Otx4 knock-down mESCs. Scale bars, 10 µm. All panels: arrows point to lumens; matrigel (M).

Extended Data Figure 6: Sialomucins are required for mESC lumenogenesis. **a**, mRNA levels of Podxl in mESCs (n=5 (24 h), 3 (36 h) and 4 (48 h) independent samples per time point). Unpaired Student’s t test, *p=0.0395 (gelatin 24 h), *p=0.0481 (matrigel 24 h), **p=0.0038 (gelatin 36 h), *p=0.0126 (matrigel 36 h), **p=0.0075 (gelatin 48 h), *p=0.0139 (matrigel 48 h). **b**, Immunostaining of mESCs cultured in 3D matrigel. Arrows indicate lumens. Scale bars, 10 µm. **c**, Immunostaining of control and Dgcr8 KO mESCs cultured in matrigel with or without 2i/LIF. Arrows indicate lumens. Scale bars, 10 µm. **d**, Immunostaining of mESCs cultured in matrigel with or without G66983. Arrows indicate lumens. Scale bars, 10 µm. **e**, Immunostaining of control and Podxl knock-down mESCs. A binarized image of the Podxl channel was used to determine presence or absence of Podxl. The percentages indicate the proportion of spheroids with (w) or without (w/o) Podxl in the Podxl siRNA group. Arrows indicate lumens. Scale bars, 10 µm. **f**, Lumen formation in cells from panel e (n=43 (control)), 45 (Podxl siRNA w/Podxl) and 35 (Podxl siRNA w/o Podxl) spheroids per condition). Chi-square test, ***p<0.0001. **g**, mRNA levels of early post-implantation factors in control and Podxl knock-down mESCs 24 hours after removal of 2i/LIF (n=3 independent samples per group). Student’s t test, ns: non-significant. **h**, mRNA levels of naïve pluripotency genes in control and Podxl knock-down mESCs 24 hours after removal of 2i/LIF (n=3 independent samples per group). Student’s t test, ns: non-significant. **i**, Immunostaining of control and Podxl knock-down mESCs cultured in matrigel. Arrows indicate polarization. Scale bars, 5 µm. **j**, Centrosome positions in cells from panel i. Each dot represents an individual centrosome (n=40 (control) and 36 (Podxl siRNA) centrosomes per condition). Mann Whitney U test, ns: non-significant. **k**, Intracellular distance in cells from panel i (n=20 (control siRNA) and 18 (Podxl siRNA) spheroids per condition). Unpaired Student’s t test, ns: non-significant. **l**, Immunostaining of Podxl heterozygous (HET) and KO mESCs. One KO clone is shown as an example. Arrows indicate lumens. Scale bars, 10 µm. **m**, Lumen formation in cells from panel l (n=30 (HET), 33 (clone 1) and 20 (clone 2) spheroids per condition) Chi-square test, ns: non-significant. **n**, Immunostaining of control and Podxl knock-down mESCs with or without GFP-Cd34 overexpression. Arrows point to lumens. Scale bars, 10 µm. **o**, Lumen formation in cells from panel n (n=37 (control siRNA), 29 (Podxl siRNA), 33 (control siRNA +GFP-Cd34) and 31 (Podxl siRNA +GFP-Cd34) spheroids per condition). Chi-square test, ***p<0.0001, ns: non-significant. **p**, mRNA levels of sialomucin proteins in control and Podxl knock-down mESCs (with or without GFP-Cd34 overexpression) 48 hours after removal of 2i/LIF (n=4 (without GFP-Podxl) and 6 (with GFP-Podxl) independent samples). Unpaired Student’s t test, *p=0.0145, ***p<0.0001, ns: non-significant. All panels: matrigel (M), gelatin (G).

Extended Data Figure 7: Otx2 and Oct4 induce Podxl expression upon naïve pluripotency exit. **a**, mRNA levels of Fgf5 and Podxl in wild-type (WT) and Otx2 KO mESCs at different time points after removal of 2i/LIF (n=2 independent samples per group). ANOVA test, ***p=0.0092, **p<0.0001. **b-c**, Immunostaining of WT and Otx2 KO mESCs cultured in matrigel. **d**, Lumen formation in cells from panel b and c (n=46 (WT 48 h), 34 (WT 72 h), 36 (Otx2 KO 48 h) and 35 (Otx2 KO 72 h) spheroids per condition). Chi-square test, ***p<0.0001. **e**, Immunostaining of Otx2 KO mESCs with or without overexpression of Otx2 (addition of DOX). **f**, Lumen formation in cells from panel e and in WT mESCs cultured without 2i/LIF and with/without Otx2 overexpression (n=19 (WT), 20 (WT Otx2), 20
Extended Data Figure 8: Cgn is induced upon naïve pluripotency exit and mediates Rab11 vesicle tethering to the apical membrane. a, mRNA levels of Cgn in mESCs (n=5 (24 h), 3 (36 h) and 4 (48 h) independent samples per group). Unpaired Student’s t test, *p=0.0431 (gelatin 24 h), *p=0.0347 (matrigel 24 h), ***p=0.0003 (gelatin 36 h), *p=0.0126 (matrigel 36 h), **p=0.0075 (gelatin 48 h), *p=0.0139 (matrigel 48 h). b–c, Immunostaining of mESCs cultured in matrigel with or without 2i/LIF. Squares denote magnified regions. d, mRNA levels of naïve pluripotency genes and early post-implantation factors in control and Cgn knock-down mESCs (n=3 independent samples per group). Unpaired Student’s t test, ***p=0.0001, ns: non-significant. e, Immunostaining of Rex1::GFpD2 mESCs in control and Cgn knock-down mESCs. All panels: arrows indicate lumens; matrigel (M); scale bars, 10 µm.

Extended Data Figure 9: Characterization of pluripotency gene expression and epiblast morphogenesis in post-implantation human embryos. a, Immunostaining of day 9-10 human embryos. Dotted lines indicate the epiblast and arrows the amniotic cavity. Scale bars, 50 µm and 10 µm (magnified areas). b, Nanog intensity in embryos from panel a (n=6 embryos per condition). Mann Whitney U test, *p=0.0381. c, 3D reconstruction of the epiblast (based on the KLF17 staining) and the amniotic cavity (based on the PODXL staining) of embryos from Figure 6b. d, Immunostaining of day 9-10 human embryos. (n=2 embryos per group). Scale bars, 50 µm. e, Immunostaining of day 9-10 human embryos. Scale bars, 50 µm. f, Number of apoptotic cells per embryo in embryos from panel d (n=3 and 5 embryos per condition). Mann Whitney U test, *p=0.0179.

Extended Data Figure 10: Exit from naïve pluripotency is required for lumenogenesis in hESCs. a, Immunostaining of WIBR3 ΔPE-OCT4-GFP cultured in primed FBS/KSR/bFGF2 medium or N2B27 2i/LIF with or without DOX. DOX addition induces the expression of NANO and KLF2. Scale bars, 20 µm. b–c, mRNA levels of pluripotency genes in cells from panel a (n=3 independent samples per condition). ANOVA test, ***p<0.0001 (NANO and KLF2), ***p<0.0001 (TFCP2L1), *p=0.0013 (DNMT3L). d, Immunostaining of WIBR3 ΔPE-OCT4-GFP cultured in matrigel in primed or naïve conditions. Arrows indicate lumens. Scale bars, 20 µm. e, Nanog intensity in cells from panel d (n=38 (2i/L/Dox), 33 (2iL -DOX), 34 (FBS/KSR/bFGF2) and 20 (mTESR) spheroids per condition). Chi-square test, ***p<0.0001. f–h, mRNA levels of pluripotency genes and early post-implantation factors in cells from panel i (n=4 (NANO, KLF2, TFCP2L1, DNMT3L, KLF4 and PODXL) and 3 (KLF17) independent samples per group). Kruskal Wallis test, ***p=0.0002 (NANO), ***p<0.0001 (KLF2, DNMT3L, KLF4, PODXL), *p=0.0058 (TFCP2L1), *p=0.0205 (KLF17). i, Immunostaining of WIBR3 ΔPE-OCT4-GFP cultured in primed mTESR medium or naïve conditions (2i/L/DOX, 5i/LAF and RSet). Scale bars, 10 µm. j, Immunostaining of hESCs cultured in 3D matrigel under different naïve and primed conditions. Arrows indicate lumens. Scale bars, 10 µm. k, Lumen formation In cells from panel j (n=31 (2i/L/DOX), 27 (5i/LAF), 33 (RSet) and 20 (mTESR) spheroids per condition). Chi-square test, ***p=0.0001, ***p<0.0001. l, Immunostaining of hESCs cultured in 3D matrigel under different naïve and primed conditions. Arrows point to polarized centrosomes. Scale bars, 5 µm. m, Angle between the nucleus-centrosome axis and the nuclear-nuclear axis in cells from panel l. Each dot represents an individual centrosome (n=53 (2i/L/DOX), 51 (5i/LAF), 40 (RSet) and 42 (mTESR) centrosomes per condition). Kruskal Wallis test, ns: non-significant. n, Immunostaining of...
naïve hESCs cultured in 3D matrigel with mTESR. The initial naïve conditions in which the cells were cultured are indicated. Arrows indicate lumens. Scale bars, 10 µm. α, Lumen formation in cells from panel n (n=30 (2iL/DOX 48 h), 30 (2iL/DOX 72 h), 30 (5i/LAF 48 h), 29 (5i/LAF 72 h) 30 (RSet 48 h) and 30 (RSet 72 h) spheroids per condition and time point). All panels: matrigel (M).
GFP (GFP-Rab11a)/Podxl/F-actin/DAPI

GFP-Rab11a WT  GFP-Rab11a S25N

% mESC spheroids

48 h 72 h 48 h 72 h

Rab11a S25N

Lumen Rosette Disorganized

HET Podxl KO clone 1

Podxl/F-actin/Par6/DAPI

Cgn/Podxl/F-actin/DAPI

E4.5  E5.0  E5.5

HET Clone 1 Clone 2 Clone 3

Podxl KO

Control siRNA  Cgn siRNA

% mESC spheroids

Control Protamine sulfate

Protamine sulfate

Cgn

Lumen Rosette Disorganized

Control Protamine sulfate

Protamine sulfate

Cgn

Lumen Rosette Disorganized

Rab11/GFP (GFP-Podxl)/DAPI
**a** KLF17/GATA6-F-actin
/PODXL/DAPI

**Day 6-7 human embryos**

**b** Day 9-10 human embryos

**c**

Number of embryos

| Distance (µm) | IVC control | IVC +5i/LAF |
|--------------|-------------|-------------|
| 0            | 5           | *           |
| 10           | 6           |             |
| 20           | 8           |             |
| 30           | 10          |             |
| 40           | 12          |             |

**d**

PODXL IF intensity (a.u.)

| Distance (µm) | IVC control | IVC +5i/LAF |
|--------------|-------------|-------------|
| 0            | 10          | *           |
| 10           | 12          |             |
| 20           | 14          |             |
| 30           | 16          |             |
| 40           | 18          |             |

**e**

KLF17 IF intensity (a.u.)

| w/ lumen | w/o lumen | IVC control | IVC +5i/LAF |
|----------|-----------|-------------|-------------|
| 0        | 10        | *           |             |
| 20       | 22        |             |             |
| 40       | 42        |             |             |

**f**

| w/ lumen | w/o lumen | IVC control | IVC +5i/LAF |
|----------|-----------|-------------|-------------|
| 0        | 10        | *           |             |
| 20       | 22        |             |             |
| 40       | 42        |             |             |

**g**

% mESC spheroids

| 2i/DOX  | 5i/LAF  | RSet mTESR |
|---------|---------|------------|
| 0       | 100     | ***        |
| 20      | 80      | ***        |
| 40      | 60      | ***        |

**h**

Exit from naive pluripotency

- Rab11
- Rab11Fip5
- Cingulin
- Podxl

Exit from naive pluripotency

- Rab11
- Rab11Fip5
- Vesicle fusion
- Lumen formation

Naive ECM

- Rab11
- Rab11Fip5

Lumen formation

- Rab11
- Rab11Fip5

Lumen formation

- Rab11
- Rab11Fip5
M +2i/LIF 48 h
M -2i/LIF 48 h

Nanog
ECad/DAPI

Oct4/Rex1::GFPd2/DAPI

IF intensity (a.u.)

Oct4   Nanog  Rex1::GFPd2

M +2i/LIF 48 h
M -2i/LIF

ns

***

M +2i/LIF 24 h
M -2i/LIF

100
80
60
40
20
0

% mESC spheroids

Lumen
Rosette
Disorganized

M +2i/LIF 48 h
M -2i/LIF

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0

Nanog IF intensity (a.u.)

M +2i/LIF
M +GSK3i/LIF
M +MEKi/LIF
M -2i/LIF

M +2i/LIF 72 h
M -2i/LIF 72 h

F-actin/DAPI

M +2i/LIF 48 h
M -2i/LIF 48 h

Nanog/aPKC/F-actin/DAPI

M +2i/LIF 24 h
M -2i/LIF 24 h

Nanog/aPKC/F-actin/DAPI

M +2i/LIF 24 h
M -2i/LIF

M +2i/LIF
M +GSK3i/LIF
M +MEKi/LIF
M -2i/LIF

M +2i/LIF
M +GSK3i/LIF
M +MEKi/LIF
M -2i/LIF

M +LIF
M +GSK3i
M +MEKi

Nanog/aPKC/F-actin/DAPI

M +2i/LIF
M +2i
M +GSK3i/LIF
M +MEKi/LIF
M -2i/LIF

Nanog

M +2i/LIF
M -2i/LIF

48 h
72 h

M +2i/LIF
M -2i/LIF

Par3/ZO1/DAPI

Matrigel 0 h
24 h
48 h

M +2i/LIF 48 h
M -2i/LIF 48 h

M +2i/LIF
M +GSK3i
M +MEKi

Nanog

M +2i/LIF
M -2i/LIF

48 h
72 h

M +2i/LIF
M -2i/LIF
**M -2i/LIF 48 h**

**Nanog/Podxl/Otx2/DAPI**

Otx2 KO Otx2 KO + Otx2 Otx2 KO + Otx2/DOX

---

**Relative mRNA levels**

**[(Tbx3, Klf4)]** **(Esrrb)** *(Zfp42)*

---

**Fgf5** **(Podxl)**

---

**Par6/F-actin/Otx2/DAPI**

Podxl/F-actin/DAPI

---

**% mESC spheroids**

---

**Nanog/Podxl/Otx2/DAPI**

---

**% mESC spheroids**

---

**Nanog IF intensity (a.u.)**

---

**Otx2 IF intensity (a.u.)**

---

**GFP-Podxl**

---

**F-actin/GFP (GFP-Podxl)/DAPI**

---

**GFP-Podxl**

---

**EpiLCs**

---

**Gene expression**

---

**Lumen**

---

**Rosette**

---

**Disorganized**

---

**Clone 1**

---

**Clone 2**

---
**Graph a**

- "Cgn relative mRNA levels"
- "Time after plating (h)"

**Graph b**

- "M +2i/LIF"
- "M -2i/LIF"
- "Podxl/Cgn/DAPI/F-actin"

**Graph c**

- "M +2i/LIF"
- "M -2i/LIF"
- "Podxl/Rab11Fip5/DAPI/F-actin"

**Graph d**

- "Relative mRNA levels"
- "Cgn"
- "Podxl"
- "Otx2"
- "Nanog"
- "Klf4"
- "Control siRNA"
- "Cgn siRNA"

**Graph e**

- "Control siRNA"
- "Cgn siRNA"
- "Cgn/GFP (Rex1::GFPd2)/DAPI/F-actin"
Day 9-10 human embryos

**a**

Podxl/Nanog/F-actin/DAPI

**b**

Nanog IF intensity (a.u.)

**c**

Epiblast/PODXL

**d**

CD130/DAPI

**e**

Oct4/Active Caspase 3/DAPI

**f**

Number of apoptotic cells per embryo
Supplemental Table and Video Legends

**Supplementary Table 1:** Genes expressed in the mouse epiblast at peri-implantation stages.

**Supplementary Table 2:** Antibodies used in this study.

**Supplementary Table 3:** List of RT-PCR primers used in this study.

**Supplementary Video 1:** ΔPE-Oct4-GFP mESCs cultured in 3D matrigel without 2i/LIF and imaged every 30 minutes. The arrow points to the cell shown in Extended Data Fig. 2e. The maximum projection is shown throughout the movie. Scale bars, 50 µm.

**Supplementary Video 2:** Rex1::GFPd2 mESCs cultured in 3D matrigel without 2i/LIF and imaged every 30 minutes. The arrow points to the cell shown in Extended Data Fig. 2e. The maximum projection is shown throughout the movie. Scale bars, 50 µm.

**Supplementary Video 3:** Nanog-YFP mTmG mESCs cultured in 3D matrigel without 2i/LIF and imaged every 30 minutes. The arrow points to the cell shown in Extended Data Fig. 2e. The maximum projection is shown throughout the movie. Scale bars, 50 µm.