Lineage specificity of primary cilia in the mouse embryo

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Primary cilia are required for vertebrate cells to respond to specific intercellular signals. Here we define when and where primary cilia appear in the mouse embryo using a transgenic line that expresses ARL13B–mCherry in cilia and Centrin 2–GFP in centrosomes. Primary cilia first appear on cells of the epiblast at E6.0 and are subsequently present on all derivatives of the epiblast. In contrast, extraembryonic cells of the visceral endoderm and trophectoderm lineages have centrosomes but no cilia. Stem cell lines derived from embryonic lineages recapitulate the epiblast. In contrast, extraembryonic cells of the visceral endoderm and trophectoderm lineages have centrosomes but no cilia.

Primary cilia are microtubule-based organelles templated by centrioles that project from the surface of most vertebrate cells and are required for the responses to specific intercellular signals¹. The regulation of cillum formation during the cell cycle has been studied in cultured cell lines, and anatomical surveys of adult tissues have shown that many cells are ciliated, but some cells, such as acinar cells of the pancreas, are not ciliated, and cilia are frequently absent from tumours²–⁴. Despite the importance of cilia for mammalian biology, the rules and mechanisms that determine whether a particular cell type will have primary cilia in vivo are unknown.

ARL13B is a small GTPase that is strongly and specifically localized to the cillum membrane⁵,⁶. To study the temporal and spatial regulation of cillum formation in vivo, we generated a double transgenic mouse line that expressed both ARL13B fused to the monomeric red fluorescent protein mCherry⁷ and Centrin 2, a centriolar protein, fused to GFP (ref. 8). Homozygous ARL13B–mCherry transgenic animals had fluorescently labelled cilia and were viable and fertile, indicating that the transgene did not interfere with normal cillum function (Supplementary Fig. 1A–L).

Primary cilia appear after implantation only on epiblast cells

Using the ARL13B–mCherry Centrin 2–GFP double transgenics, we examined the temporal and spatial pattern of centrosomes and cilia during embryonic development. Centrin 2–GFP+ centrioles were readily detected in both inner cell mass (ICM) and trophectoderm cells in 32 cell blastocysts (Fig. 1a,b and Supplementary Video 1), consistent with previous observations⁸,¹⁰. In contrast, no ARL13B–mCherry expression was observed in either the ICM (0/46 cells) or trophoderm cells (0/86 cells) of these embryos (Fig. 1a,b). Small puncta of ARL13B–mCherry were detected adjacent to the centrosomes in about 2% of ICM cells in blastocysts with 64–100 cells (Fig. 1c,d, arrow; two puncta/107 cells scored from nine embryos; Supplementary Video 2), but these puncta did not show cillum morphology and did not express the cillum marker acetylated α-tubulin (Supplementary Fig. 1M). No ARL13B–mCherry expression was detected in trophectoderm cells at this stage (Fig. 1c; 0/255 cells from nine embryos).

Cilia were first detected after implantation on epiblast cells, at the time of cavitation (embryonic day 5.5, E5.5). At this stage, elongated ARL13B–mCherry cilia adjacent to Centrin 2–GFP centrioles were detected in a small fraction (<1%) of epiblast cells (Fig. 1e,f; 3/654 epiblast cells from 10 embryos; Supplementary Video 3). By E6.0, elongated ARL13B–mCherry+ primary cilia were present on 32.7±7.3% epiblast cells (Fig. 1g,h, arrows; 388/1039 epiblast cells from six embryos; Supplementary Video 4). At E8.0, mCherry+ primary cilia were detected on all non-dividing epiblast-derived cells of the three germ layers, ectoderm, mesoderm and definitive endoderm (Fig. 1i,j and Supplementary Video 5), including cells of the embryonic node (Fig. 1k). At mid-gestation (e10.5), mCherry-expressing cilia projected from Centrin 2–GFP+ centrioles in cells throughout the embryo, including limb mesenchyme (Fig. 1l) and neural epithelial cells (Fig. 1m).

In contrast to the presence of primary cilia on epiblast cells at E6.0, cilia were never detected on cells of extraembryonic lineages, the visceral endoderm (Fig. 1h, asterisk) or extraembryonic ectoderm (0/837 cells from six embryos, ~140 cells analysed/embryo). Antibody staining confirmed that primary cilia positive for ARL13B and IFT88 (intraflagellar transport 88) were present only on epiblast cells and

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not cells of the visceral endoderm in E6.0 embryos (Supplementary Fig. 1N;O; 0/659 cells from five embryos, ~130 cells per embryo).

The presence of primary cilia on epiblast cells and not on extraembryonic lineages was particularly striking in the E7.5 extraembryonic visceral endoderm cells and these cells were ciliated, whereas the AFP–GFP-expressing cells in the endoderm layer still lacked ARL13B–mCherry+ primary cilia (Fig. 2g–i and Supplementary Video 6). Thus the presence of primary cilia in cells of the endoderm
Figure 2 Primary cilia are restricted to embryonic lineages in late gastrulation stage embryos. (a–c) Primary cilia labelled with ARL13B–mCherry (red) are present on epiblast cells but are absent from cells of the visceral endoderm marked with AFP–GFP (green). (a) Distal view of pre-streak stage embryo (E6.5; representative image selected from four embryos) expressing AFP–GFP and ARL13B–mCherry. (b) Transverse optical section of embryo in a. (c) Magnification of dashed box in b shows primary cilia labelled with ARL13B–mCherry (red) on epiblast cells (arrow), but absent from visceral endoderm cells (ve), labelled with AFP–GFP (green). The dotted line denotes the boundary between epiblast (above) and visceral endoderm (below). (d–i) As the definitive endoderm intercalates with the visceral endoderm (green), cilia (red) are present on the definitive, but not the visceral, endoderm. (d) Distal view of mid-streak stage embryo (E7.0; representative image selected from three embryos) expressing AFP–GFP and ARL13B–mCherry. (e) Transverse optical section of embryo in d; the primitive streak (ps) is to the right. (f) Magnification of the dashed box in e shows that primary cilia are present on definitive endoderm cells (arrow), but absent from surrounding visceral endoderm cells, labelled with AFP–GFP (green). The dotted line denotes the boundary between epiblast and definitive endoderm. (g) Distal view of early bud stage embryo (E7.5) expressing AFP–GFP and ARL13B–mCherry (representative image selected from three embryos). (h) Transverse optical section of embryo in g; the primitive streak (ps) is to the right. (i) An expanded view of the box in h shows primary cilia on definitive endoderm cells (arrows) that have intercalated between AFP–GFP+ visceral endoderm cells (green). The dotted line denotes the boundary between epiblast (below) and definitive endoderm (above). P, posterior; A, anterior; Pr, proximal; D, distal; L, left; R, right. (j) A schematic representation of an E8.0 embryo in cross-section illustrates the locations of the images shown in k and m. (k) Cells of the ectoplacental cone (derivatives of trophoblast lineage) expressing ARL13B–mCherry and Centrin 2–GFP. (l) Magnification of the dashed box in k shows Centrin 2–GFP-labelled centrioles with no primary cilium on extraembryonic ectoderm cells. (m) Cells of the extraembryonic visceral endoderm expressing ARL13B–mCherry and Centrin 2–GFP are not ciliated, although epiblast cells in the headfolds are ciliated (arrows). (n) Magnification of the dashed box in m shows Centrin 2–GFP-labelled centrioles but no primary cilia on extraembryonic visceral endoderm cells. Nuclei are marked with DAPI (blue). Scale bars: a–f, 30 μm; g–i, 40 μm; k, m, 20 μm; l, n, 5 μm.
Figure 3 Extraembryonic lineages of the placenta and yolk sac lack cilia at E14.5. (a) Schematic representations of the placenta and yolk sac—trophoblast giant cells (dark green), spongiotrophoblast (light green) and labyrinth (red)—and a magnified view of the yolk sac, illustrating a blood vessel surrounded by endothelial cells (dark pink) and the positions of mesothelial cells (light pink) and extraembryonic visceral endoderm derivatives (yellow). (b) Section of the E14.5 labyrinth layer from an ARL13B–mCherry Centrin 2–GFP placenta; dotted lines outline fetal blood vessels (bv). (c) A magnified view of the box in b: non-ciliated trophoblast-derived syncytiotrophoblast cells between fetal blood vessels lack cilia; arrowheads indicate Centrin 2–GFP+ centrioles. (d) A magnified view of the box in b, showing cilia on mesoderm-derived cells surrounding fetal blood vessels (arrows). (e) A section of E14.5 yolk sac expressing ARL13B–mCherry and Centrin 2–GFP; the dashed line demarcates the boundary between mesoderm-derived cells (mesothelial and endothelial cells, above) and extraembryonic-visceral-endoderm-derived cells (below). The arrow indicates a ciliated mesothelial cell. (f) A magnified view of the box in e shows ciliated endothelial cells (arrowheads) whereas extraembryonic visceral endoderm cells, below the dashed line, are not ciliated. (g) Section of E14.5 yolk sac; the dotted line demarcates the boundary between mesoderm-derived cells (mesothelial and endothelial cells, above) and extraembryonic visceral endoderm-derived cells (below). Antibody staining for γ-tubulin (labels centrosomes, green) and ARL13B (labels cilia, red), present on mesothelial cells (arrows) and PECAM1-expressing (magenta) endothelial cells (white arrowheads). Extraembryonic-visceral-endoderm-derived cells have centrosomes (black arrowheads) but no cilia (221/386 mesothelial cells; 95/292 endothelial cells; 0/1032 extraembryonic-visceral-endoderm-derived cells from two embryos). bv = blood vessel. Nuclei are marked with DAPI (blue). Scale bars: b, 30 μm; c,d, 10 μm; e, 30 μm; f, 10 μm; g, 20 μm.
was determined by their cell lineage and not by their position in the embryo. At E8.0, there were still no ARL13B–mCherry+ cilia detectable on cells derived from the trophoblast lineage (ectoplacental cone and extraembryonic ectoderm; Fig. 2j–l) or the extraembryonic visceral endoderm (Fig. 2m,n).

Lineage specific assembly of primary cilia persists through gestation

The absence of cilia from the trophoblast and visceral endoderm lineages persisted later in gestation. In the labyrinth layer of the E14.5 placenta, no ARL13B+ primary cilia were detected on syncytiotrophoblast cells of the trophoblast lineage (Fig. 3a–c, arrowheads), whereas epiblast-derived cells that surround the fetal blood vessels were ciliated (Fig. 3d, arrows). The yolk sac includes cells derived from both the extraembryonic visceral endoderm and the epiblast-derived mesoderm. At E14.5, ARL13B–mCherry+ primary cilia were present on 57.4 ± 8.1% of mesoderm-derived mesothelial cells (Fig. 3e arrow, g arrows; 221/386 cells from two embryos) and 33.4 ± 3.1% of mesoderm-derived endothelial cells (Fig. 3f,g arrowheads; 95/292 cells from two embryos) that surrounded the blood vessels in the yolk sac, labelled with PECAM1 (platelet/endothelial cell adhesion molecule 1) (Fig. 3g). In contrast, no ARL13B–mCherry+ cilia were seen associated with Centrin 2–GFP+ centroosomes in the cells of the extraembryonic-visceral-endoderm-derived layer of yolk sac cells (Fig. 3f,g triangles; 0/1032 cells from two embryos).

Stem cell lines stably recapitulate the in vivo pattern of ciliogenesis

Stem cell lines that stably retain the developmental potential of each of the lineages of the early mouse embryo can be derived and maintained in culture13; we therefore tested whether the lineage dependence of ciliogenesis was reflected in these stem cell lines. We derived mouse embryonic stem cells (mESCs) from double transgenic embryos under 2i+LIF (leukemia inhibitory factor) conditions that promote ground state pluripotency14 and observed that 18% of mESCs were ciliated (Fig. 4a), similar to previous results in standard mESC conditions15. Trophoblast stem cells (TSCs) and extraembryonic endoderm (XEN) stem cells retain the ability to differentiate into the trophoblast derivatives of the placenta16 and extraembryonic endoderm derivatives17, respectively, in chimeras. TSCs, marked by expression of EOMES18, were not ciliated, as shown by lack of acetylated α-tubulin+ structures adjacent to γ-tubulin+ centroosomes (Fig. 4b and Supplementary Fig. 2A–D). In XEN cells derived from ARL13B–mCherry Centrin 2–GFP transgenic embryos, no focal ARL13B–mCherry was detected adjacent to Centrin 2–GFP+ centroosomes (Fig. 4c), even though western blotting showed that ARL13B–mCherry was expressed in these cells (Supplementary Fig. 2l). Antibody staining for ARL13B–mCherry showed that almost every non-mitotic cell in EpiSC colonies was ciliated (Fig. 4e; 87.2 ± 8.4%). Thus there was a correlation between the presence of cilia in the embryo and in the corresponding stem cell line: primary cilia are present on embryonic epiblast and its derivatives and in EpiSCs, whereas cilia are not present on visceral endoderm or trophoderm cells in the embryo or in XEN or TSC lines.

XEN cells are believed to be more similar to embryonic parietal endoderm than visceral endoderm19,20. We therefore added the glycogen synthase kinase 3β (GSK3β) inhibitor CHIR99021 to convert XEN cells into visceral endoderm21. This treatment caused upregulation of FOXA2, a visceral endoderm specific transcription factor (Supplementary Fig. 2l), and caused a transition from a rounded XEN cell morphology to an epithelial morphology and upregulation of E-cadherin, as in the visceral endoderm (Supplementary Fig. 2l). Antibody staining for acetylated α-tubulin and γ-tubulin showed that these cells lacked primary cilia (Supplementary Fig. 2K,M), demonstrating that visceral endoderm cells derived from XEN cells lack primary cilia, similarly to visceral endoderm cells in the embryo.

Removal of serum from many cultured cells triggers exit from the cell cycle and promotes cilium formation2. In contrast, withdrawal of serum from XEN cells did not lead to cilium assembly (Fig. 4d; 0/267 cells from two independent experiments) and the EpiSCs that formed cilia were grown in the presence of serum. Thus mechanisms that have been described in cell culture may not regulate cilium formation in embryo-derived stem cell lines.

XEN cell basal bodies are mature but do not nucleate cilia

The absence of cilia in XEN cell lines made it possible to test why cilia fail to form in this lineage. Transcription factors of the RFX family promote the expression of genes required for ciliogenesis22, and RFX3 is required for the formation of normal primary cilia in the mouse embryo23. RFX3 was highly expressed in epiblast cells and at lower levels in visceral endoderm cells (Supplementary Fig. 3A,B); however, RFX3 was expressed in all nuclei in both mESCs (Supplementary Fig. 3C,D) and XEN cells (Supplementary Fig. 3E,F), indicating that RFX3 is not the factor that determines which lineages are ciliated.

For ciliogenesis to initiate, the mother centriole must mature into a basal body with distal appendages that dock the mother centriole to the plasma membrane and subdistal appendages that mediate attachment to microtubules; after membrane attachment, proteins of the ciliary transition zone are recruited24. CEP164, a centrosomal distal appendage protein, and Ninein, a subdistal appendage protein, were both localized to the distal end of mother centrosomes in XEN cells (Fig. 4f,h), as in ciliated mouse embryonic fibroblasts (MEFs) and EpiSCs (Fig. 4g,i and Supplementary Fig. 3G,H). In cycling fibroblasts, serum withdrawal triggers the recruitment of positive regulators to the distal tip of the mother centriole, including the kinase TTBK2, which acts at the mother centriole to recruit intraflagellar transport proteins and initiate axoneme assembly25. Antibody staining showed that TTBK2 and IFT88 both localized to the distal end of the mother centriole in XEN cells (Fig. 4j,l), as in ciliated MEFs and EpiSCs, where IFT88 was also present in the axoneme (Fig. 4k,m and Supplementary Fig. 3I,J). In addition, the transition zone proteins NPHP4, MKS1, CEP290 and Inversin were present at centrioles in XEN cells (Fig. 4n,p,r,t), as in ciliated MEFs and EpiSCs, where IFT88 was also present in the axoneme (Fig. 4o,q,s,u). Thus the basal bodies in XEN cells seem to be poised to make cilia.

The initiation of ciliogenesis is associated with removal of negative regulators, including CP110, from the distal tip of the mother...
Figure 4  
Embryo-derived stem cells recapitulate the cilium status of embryonic lineages. (a–e) Presence of primary cilia on embryo-derived stem cells. (a) 18% of asynchronously dividing mESCs derived from ARL13B–mCherry (red) Centrin 2–GFP (green) transgenic embryos grown in 2i medium are ciliated. (b) Antibody staining for γ-tubulin (green) and acetylated α-tubulin (red) shows that TSCs lack primary cilia. (c) No cilia are detected on XEN cells derived from ARL13B–mCherry Centrin 2–GFP transgenic embryos. (d) Antibody staining for ARL13B (red) and acetylated α-tubulin (magenta) shows that serum-starved XEN cells lack cilia (0/267 cells from two independent experiments). (e) Antibody staining of EpiSCs for α-tubulin (green) and ARL13B (red) shows that almost all EpiSCs are ciliated. (f–u) XEN cells have mature basal bodies. Centrioles marked with γ-tubulin (red) are associated with the distal appendage marker CEP164 (green; f) and subdistal appendage marker Ninein (green; h). Positive regulators of ciliogenesis TTBK2 (green; j) and IFT88 (green; l) as well as transition zone proteins (green) NPHP4 (n), MKS1 (p), CEP290 (r) and Inversin (t) are also present at the mother centriole in XEN cells. (g,i,k,m,o,q,s,u) The localization of basal body proteins is the same in ciliated MEFs as in XEN cells, although IFT88 is also present in the axoneme in MEFs marked with acetylated α-tubulin (magenta). (v–x) The negative regulator of ciliogenesis CP110 (green) is present on both centrioles in all XEN cells (v) but is removed from the mother centriole on cilium assembly in MEFs (w). CP110 (red) is also present on both centrioles marked with Centrin 2–GFP (green) in cells of the embryonic visceral endoderm (x, arrows). Nuclei are marked with DAPI (blue). Scale bars: a–e, 7 μm; f–w, 2 μm; x, 3 μm.

In ciliated MEFs and EpiSCs, CP110 was only present on the daughter centriole and was absent from the mother centriole (Fig. 4w and Supplementary Fig. 3K), whereas CP110 localized to both the mother and daughter centrioles in XEN cells (Fig. 4v) and in cells of the embryonic visceral endoderm at E6.5 (Fig. 4x, arrows).

We used short interfering RNA (siRNA) to partially (68%) knock down expression of CP110. In those knockdown cells where CP110 could not be detected at the centriole, cilia were not formed, as no ARL13B+ or IFT88+ axonemes were detected (Supplementary Fig. 3P,R). Instead, as in U2OS osteosarcoma cells,37,28 knockdown of CP110 in XEN cells...
Increased AURKA activity, we cultured XEN cells in the presence of

To test whether lack of primary cilia on XEN cells was caused by

Blocking the cilium disassembly pathway permits cilium

We did not detect any role for these components in

Recently, a number of proteins and factors, including PLK1, APC/C

The cilium disassembly pathway is active in XEN cells

When a cell enters mitosis, the primary cilium is disassembled and

The cilium disassembly pathway is active in XEN cells

Caused formation of elongated centrioles (16.6 ± 7.7%, 65/503 cells),
distinguished by expression of γ-tubulin and Centrin 2–GFP along their

Figure 5 Components of the cilium disassembly pathway are highly expressed in XEN cells. (a) Western blot analysis of activated and total AURKA. (b) Quantification of the western blots for total AURKA (green) and activated AURKA (red) (n = 3 independent experiments, normalized to XEN). (c) Western blot analysis of NEDD9 protein levels. (d) Quantification of western blot analysis (c) shows that NEDD9 protein levels are

The AURKA inhibitor alisertib (MLN8237; ref. 33). Treatment with

Treatment with 5 μM PHA-680632, a second, albeit less specific,

Blocking the cilium disassembly pathway permits cilium

To test whether lack of primary cilia on XEN cells was caused by

The mitotic regulatory kinase Aurora kinase A (AURKA)

We therefore
distinguished by expression of CP110 on centrioles does not account for the lack of cilia in XEN cells.

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After 12 h of 5 μM tubacin treatment, 3.9 ± 2.3% of XEN cells were ciliated (22/573 cells, n = 573 cells pooled from three independent experiments) formed a cilium (Supplementary Fig. 5O,P), significantly different from 0% in DMSO controls (0/433 cells, n = 433 cells pooled from three independent experiments; P < 0.0001 from t-test). To test whether the AURKA/HDAC6 pathway regulates cilium formation in the visceral endoderm in vivo, we cultured embryos

Figure 6 XEN and visceral endoderm cells form cilia when the cilium disassembly pathway is inhibited. (a) Western blot shows that treatment for 16 h with 250 nM alisertib reduces levels of phosphorylated AURKA by 32.5-fold when compared with DMSO controls. Phosphorylated AURKA was not detected in cells treated with 500 nM alisertib, whereas total AURKA levels are unaffected (n = 2 independent experiments). (b) Western blot analysis shows that siRNA knockdown of AURKA reduces levels of total AURKA to about 15% of control levels (n = 2 independent experiments). (c,d) Treatment of XEN cells with 250 nM AURKA inhibitor alisertib for 16 h causes formation of primary cilia marked with IFT88 (c, green), ARL13B (d, green) and an acetylated α-tubulin (magenta)-labelled axoneme projecting from a centrosome marked with γ-tubulin (red; 20/328 cells). (e,f) Treatment of XEN cells with 5 μM AURKA inhibitor PHA-680632 for 72 h causes formation of primary cilia marked with IFT88 (e, green), ARL13B (f, green) and acetylated tubulin (magenta) projecting from a centrosome marked with γ-tubulin (red; 15/302 cells). (g) siRNA knockdown of AURKA causes formation of primary cilium on XEN cells marked with ARL13B (green) and acetylated α-tubulin (magenta) projecting from a centrosome marked with γ-tubulin (red; 7/610 cells from two independent experiments). (h,i) Treatment of XEN cells with 5 μM HDAC6 inhibitor tubacin for 72 h causes formation of primary cilia on XEN cells marked with IFT88 (h), ARL13B (i) and acetylated α-tubulin (magenta), projecting from a centrosome marked with γ-tubulin (red; 8/717 cells from three independent experiments). (j,k) Culture of E7.5 embryos for 12 h in 5 μM tubacin causes cilium formation (red) on visceral endoderm cells (green). (j) Visceral endoderm cells in an E7.5 embryo expressing AFP–GFP (green) are never ciliated in DMSO-treated control embryos (0/1,263 cells counted across six embryos). (k) Primary cilia, labelled with ARL13B–mCherry (red), are observed on AFP–GFP (green) visceral endoderm cells in embryos cultured with 5 μM tubacin for 12 h (30/1,313 cells counted across seven embryos). The arrow indicates a primary cilium present on this AFP–GFP+ cell. The side panels show the yz view. Nuclei are stained with DAPI (blue). Scale bars: c–i, 2 μm; j,k, 20 μm. Uncropped images of blots/gels are shown in Supplementary Fig. 6.
that expressed both AFP–GFP, to mark the visceral endoderm\(^1\), and ARL13B–mCherry, to mark cilia, in 5 μM tubacin for 12 h. This treatment effectively increased cytoplasmic microtubule acetylation (Supplementary Fig. 5Q–T; pixel intensity: DMSO, 1; tubacin, 4.6). In tubacin-treated embryos 2.6 ± 1.9% of visceral endoderm cells were ciliated (Fig. 6k, arrow, 30/1,313 cells, \(n = 1,313\) cells counted across seven embryos), which was significantly different from 0% in DMSO controls (Supplementary Fig. 6j; 0/1,263 cells, \(n = 1,263\) cells counted across six embryos; \(P = 0.0065\) from \(t\)-test). These experiments demonstrate that mother centrioles in XEN and visceral endoderm cells can template cilia in extraembryonic lineages in vivo when the cilium disassembly pathway is blocked.

In cystic renal disease, loss of VHL (Von Hippel–Lindau tumor suppressor) leads to loss of cilia through activation of AURKA and NEDD9\(^3\), when GSK3β is inactive\(^4\). Western blot analysis showed that VHL protein was present at equivalent levels in XEN cells and EpiSCs (Supplementary Fig. 4A, \(n = 3\) from two independent experiments). Thus VHL protein is not limiting for cillum formation in XEN cells. VHL regulates the stability of the α-subunit of hypoxia inducible factor (HIF)-1α and HIF-2α, which in turn can activate NEDD9 and AURKA (ref. 35). 24 h treatment of XEN cells with 1 μM FM19G11, which blocks the function of both HIF-1α and HIF-2α (median inhibitory concentration = 80 nM; ref. 37) did not cause cilium formation (Supplementary Fig. 4B,C; 0/284 cells; DMSO 0/278 cells; from two independent experiments). Thus, in XEN cells, NEDD9 and AURKA are activated by an HIF-independent pathway to inhibit cilium formation.

**Discussion**

Our studies show that cilium formation in vivo is controlled by stable lineage-based mechanisms. In contrast to studies in cell culture, which have focused on the formation of cilia in response to serum withdrawal, we find that stem cell lines make stem cells on the basis of their lineage: visceral endoderm and XEN cells fail to form cilia whereas embryonic epiblast and EpiSCs grow cilia, independent of the presence of serum. The basal bodies in XEN cells are mature; they contain all the tested components of the ciliary appendages and transition zone, as well as IFT88 and the ciliun-initiating kinase TTBK2, but fail to grow a ciliary axoneme. The maturity of these basal bodies is demonstrated by the formation of cilia when the AURKA/HDAC6 cilium disassembly pathway is blocked. As only a relatively small fraction of XEN cells are ciliated after withdrawal, we find that stem cell lines make stem cells on the basis of their lineage: visceral endoderm and XEN cells fail to form cilia whereas embryonic epiblast and EpiSCs grow cilia, independent of the presence of serum.

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**METHODS**

Methods and any associated references are available in the online version of the paper.

„Note: Supplementary Information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

E.K.B. and K.V.A. designed the experiments and wrote the article. E.K.B. carried out the experiments. N.S. and A.-K.H. provided advice on stem cell derivation and culture. A.-K.H. provided reagents and mice.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Mouse strains. The species used here was Mus musculus. To generate ARL13B–mCherry transgenics, the coding region of an ARL13B complementary DNA was fused in-frame upstream of mCherry then inserted into pCAGGs, which on a mixed background of FVB and C57BL/6J. Other mouse strains used were ARL13BmCherry (ref. 5), Centrin 2–GFP and ARL13B–mCherry; GFP-GFP were both on a mixed background of FVB and C57BL/6J. Embryos were stained in U-bottomed 96 well plates coated with 1% agar/0.9% NaCl/0.05 mM Tris-HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% Na2CO3 and transferred between wells with a pulled glass pipette. Blastocysts were fixated in 4% PFA for 5 min and washed three times in 0.1% Triton X-100/PBS, the zona pellucida was removed with acid Tyrode’s solution for 1 min, permeabilized in 0.5% Triton X-100/100 mM glycine/PBS for 5 min, washed, and blocked in 2% goat serum/PBS for 1 h, and primary antibody was diluted in blocking solution and incubated at 4°C overnight. Rabbit polyclonal antibody was then washed three times in 0.1% Triton X-100/PBS, incubated with secondary antibody for 1 h, washed, and imaged in 0.1% Triton X-100/PBS on a glass-bottomed MatTek dish.

Postimplantation embryos were fixed for 1 h at room temperature in 4% PFA and washed in 0.1% Triton X-100/PBS. Embryos were blocked overnight in 0.1% Triton-X100/5% goat serum/PBS at 4°C on a rocking platform. Primary antibody was diluted in blocking buffer and incubated overnight at 4°C. Embryos were washed five times for 2 h in 0.1% Triton X-100/PBS, then overnight. Secondary antibody was then washed in blocking buffer and incubated for 2 h at 4°C. Embryos were washed a further five times for 2 h in 0.1% Triton X-100/PBS, then overnight before mounting in 2% low melting point agarose on glass-bottomed MatTek dishes and sealed with VectaSHIELD Mounting Medium (Vector Labs).

For neural tube, placenta and yolk sac, embryos were fixed in 2% PFA at 4°C overnight, embedded in optimal cutting temperature compound (OCT) and cryosectioned at 12 µm. Slides were mounted using VECTASHIELD (Vector Labs).

For immunofluorescence staining, yolk sac sections were boiled for 10 min in 10 mM Na citrate. All sections were washed with PBS and blocked in 1% goat serum/PBS for 30 min. Primary antibody was diluted in blocking buffer and incubated at 4°C overnight. Sections were washed four times in blocking buffer, and incubated in secondary antibody for 1 h at room temperature. Slides were mounted with Prolong Gold mounting media (Life Technologies).

Antibodies used were FOXA2 (1:100 Ab40874), ISLET1 and Pax7 (1:10 DSHB), NX22 (1/2 DSHB), Smo (1:500; ref. 46), Glu2 (1:1000, gift from J. Eggenschwiler10), ARL13B (1:500, gift from T. Caspary4), Nanog (1:500, biovision 14-5761-80), Sox17 (1/1000, R&D AF1924), Eomes (1:500, Abcam 23345), HNF4α (1:1000, Santa Cruz C-19), CP110 (1:100, Proteintech 12780-1-AP), CEP164 (1:500, Sdxs 4533.00.02), Ninein (1:1000, gift from J. Sillibourn and M. Bornens), Tubb2c (1:500, Sigma Prestige HPA011813), Itf88 (1:500, Proteintech 13967-1-AP), Rex3 (1:200, Sigma Prestige HPA035689), E-cadherin (1:200, Sigma U2534), actin-tubulin (1:1000, Sigma T6793), γ-tubulin (1:1000, Sigma T66575), Msks1 (1:1000, Proteintech 16206-1AP), Nphp4 (1:20, Proteintech 13812-1-AP), Cep290 (1:100, Novus Biologicals NB100-86991), Inversin (1:100, T. Yokoyama) and Pecam1 (1:200, BD Pharmingen 500274). Some antibodies were from the DSHB (Developmental Studies Hybridoma Bank), which was created by the NICHD of the NIH and maintained at the University of Iowa, Department of Biology, Iowa City, IA, USA.

Western blot analysis. Cells were lysed in standard RIPA buffer (0.15 mM NaCl/0.05 mM Tris-HCL pH 7.2/1% Triton X-100/0.1% sodium deoxycholate/0.1% SDS) with Phosphatase Inhibitor Cocktail 1 and 2 (Calbiochem) at 4°C for 30 min then centrifuged at 19,000g for 10 min. Lysates were run on 8–12% polyacrylamide gels transfected onto polyvinylidene difluoride either for 1 h at room temperature at 100 V (pVHL) or at 4°C overnight at 30 V. Membranes were blocked in 5% BSA or 5% non-fat milk in TBST buffer. Antibodies: AURKA (1:1,000, BD Biosciences 610938 1AK1), pAURKA (T288) (1:100, Cell Signaling C9398), Hefi (1:1000, Abcam 16663), mCherry (1:1000, Clontech), Vhl (1:1000, Cell Signaling 2738) and GADPH (1:1,000, Santa Cruz 25778).

Imaging. Wholemount embryos were imaged on a Leica TCS SP8 inverted laser scanning confocal microscope and deconvolved using AutoQuantX3. Cells were imaged using an inverted DeltaVision Image Restoration Microscope. Sections and images for quantification were imaged on an upright Zeiss 2 axi imaging microscope.

Reproducibility of experiments and statistical tests. Sample sizes were determined by the nature of the experiment and variability of the output, not by a statistical method. Numbers of embryos and cells measured are indicated in the text and figure legends. For observations in embryos, 3–10 ARL13B–mCherry; Centrin 2–GFP embryos were analysed at each stage. Three or four ARL13B–mCherry; GFP-GFP embryos were analysed at each stage. Given the consistency of the results this was considered sufficient. For ciliogenesis in embryos 46–1,039 cells were counted from two to six embryos. Cilium length was calculated from three wild-type and three transgenic embryos. For cell-based assays 6–23 fields of cells with a total of at least 200 cells were counted from two or three independent biological replicates. Drug treatments that did not result in assembly of cilia were repeated two or three times as indicated in the text and figure legends. All correctly processed and imaged samples were included. Figure 1a,b shows representative data from four embryos, c,d from nine embryos, e,f from 10 embryos, g,h from
six embryos, i–k from two embryos. Figure 2a–c shows representative data from four embryos, d–i from three embryos. Figure 3e–g shows representative data from two embryos. Figure 4a–c shows representative data from three independent experiments, d from two independent experiments, e from three independent experiments, f–w from three independent experiments and x from two independent experiments. Figure 5 shows representative data from three independent experiments. Figure 6a–d shows representative data from two independent experiments, e, f from three independent experiments, g from two independent experiments, h, i from three independent experiments, j from six independent experiments and k from seven independent experiments. Supplementary Fig. 1A–F shows representative data from two independent experiments, H–I from 10 independent experiments, M from nine independent experiments and N, O from five independent experiments. Supplementary Fig. 2A–H shows representative data from three independent experiments, I from one independent experiment and J–M from two independent experiments. Supplementary Fig. 3A–F and G–K shows representative data from two independent experiments and L–S from three independent experiments. Supplementary Fig. 4A–C shows representative data from two independent experiments. Supplementary Fig. 5A–H shows representative data from two independent experiments, I–N from two independent experiments, O, P from three independent experiments, and Q–T from two independent experiments. The experiments were not randomized and the investigators were not blinded to allocation during experiments or when assessing the outcome of experiments. Mean averages are given ± s.d. Student’s t-test was used for statistical analysis.

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Supplementary Figure 1 The ARL13B-mCherry transgene does not affect Shh signaling, rescues the phenotype of Arl13b mutants and reproduces cilia labeling with the ARL13B antibody. Overexpression of ARL13B has been shown to promote formation of long cilia in cultured cells. Primary cilia in mesenchymal cells of ARL13B-mCherry homozygous embryos were ~37% longer (2.43 ± 0.56 μm) than in non-transgenic embryos (1.77 ± 0.34 μm, p≤ 0.0001 from t-test, n= 50 cilia from 3 wild type and 3 transgenic embryos). Despite this, Shh-dependent neural patterning remained normal.

(A-C) Neural patterning in e10.5 ARL13B-mCherry homozygous embryos. Shh-dependent neural progenitor markers were expressed normally, including (A) FoxA2 (red), (B) Islet1 (green), (C) Pax7 (green). D, E) Smo (green) was present in cilia marked with ARL13B (red) on cells of the floor plate and ventral neural tube (D) but was not present in cilia on cells of the roof plate (E).

(F) Gli2 (green) was present at the tips of mesenchymal cilia marked with ARL13B. (G-L) The ARL13B-mCherry transgene rescues the Arl13b mutant phenotype. Transverse sections of e10.5 ARL13B^{hnn/hnn}; Tg/Tg neural tube shows the normal pattern of ventral neural cell types: floor plate cells express FoxA2 (red), V3 interneuron progenitors express Nkx2.2 (green) (G), motor neurons express Islet1 (green) and motor neuron progenitors express Olig2 (red) (J). In contrast, Arl13b^{hnn/hnn} mutant embryos without the transgene had reduced floor plate and V3 progenitors (H) and expanded motor neurons (K), as previously described. Neural pattern was rescued in Arl13b^{hnn/hnn} mutants carrying ARL13B-mCherry transgene (compare I to H and L to K).

(M) 72 cell blastocyst with γ-tubulin labeled centrosomes (green) not associated with acetylated α-tubulin (red) positive axonemes indicating puncta observed with ARL13B-mCherry are not cilia (Representative image selected from 9 embryos). Acetylated α-tubulin stains mid-bodies. (N and O) Antibody staining of ARL13B (red, N) or IFT88 (red, O) in Centrin2-GFP embryos (green) at e6.0 showed primary cilia (arrows) on epiblast cells but not on centrosomes on visceral endoderm (ve) cells (arrow heads; 0/659 cells from 5 embryos). Dotted line delineates epiblast (above) and visceral endoderm (below). High background staining in the visceral endoderm is an inherent technical problem with antibody staining of embryos at this stage due to high levels of endogenous IgG in visceral endoderm cells. Nuclei stained with DAPI (blue). Scale bars: (A-C) 50 μm; (D-F) 5μm; (G-L) 50 μm, (M-O) 10 μm.
Antibody staining confirms that TS cells, XEN cells and XEN-derived visceral endoderm-like cells lack primary cilia. (A-D) Antibody staining for γ-tubulin (green, A) and acetylated tubulin (red, B) showed TS cells, marked by Eomes expression (magenta, C), lack primary cilia. (E-H) Antibody staining for centrosome marker γ-tubulin (green, E) and ARL13B (red, F) show XEN cells, marked by Sox17 expression (magenta, G), lack primary cilia. (I) By Western blot, wild-type XEN cells (Lane 1) and mouse embryonic fibroblasts (MEFs) (lane 2) do not express mCherry; ARL13B transgenic MEFs (lane 3 express an mCherry band (arrow) at ~88kDa, the size of ARL13B (60kDa) plus mCherry (28kDa). This band is also detected in transgenic XEN cells (lane 4 and 5). Higher molecular weight band is non-specific. (J-M) XEN cells differentiated into visceral endoderm lack primary cilia. Addition of CHIR99021 upregulates FoxA2 (Magenta, J) and E-Cadherin (magenta, L), demonstrating XEN cells have differentiated into visceral endoderm, however no cilia, marked by expression of acetylated α-tubulin (red), form; centrosomes marked with γ-tubulin (green) (K, M). DAPI stains nuclei in blue. (A-D) 5 μm; (E-H) 7 μm; (J-M) 7 μm.
Supplementary Figure 3 Lack of cilia on XEN cells is not due to lack of RFX3 expression or negative regulation via CP110. (A, B) RFX3 (green) is expressed at higher levels in epiblast cells than visceral endoderm cells at e6.5. (C, D) RFX3 is expressed in mESC and (E, F) XEN cell nuclei. (G-K) Localization of Cep164, Ninein, TTBK2, IFT88, CP110 (green) in ciliated EpiSCs stained with γ-tubulin (red) to mark the centrosome and acetylated α-tubulin (magenta) to mark the axoneme. (L-S) Knockdown of CP110 (green, L) leads to formation of long centrioles in 16.6 ± 7.7% of cells (65/503 cells from 3 independent experiments), marked with γ-tubulin (red) and acetylated α-tubulin (magenta) and Centrin2-GFP (green, N) in XEN cells. These long structures do not express the cilia markers IFT88 (green, P) or ARL13B (green, R). Centrioles and the localization of IFT88 are unaffected in scramble treated controls (M, O, Q, S). DAPI stains nuclei in blue. Scale bars: (A, B) 20 μm; (C, D) 5 μm; (E, F) 7 μm; (G-K) 2 μm; (L-S) 5 μm.
Supplementary Figure 4 Plk1, APC/C, ceramide, VHL and HIFα, which have been reported to regulate ciliogenesis in specific cell types, do not regulate cilia formation in XEN cells. Polo like kinase 1 (Plk1) is recruited to the centrosome via PCM1 and activated by AurkA, where it activates HDAC6, promoting cilia disassembly. 24h treatment of XEN cells with 1μM Plk1 inhibitor BI2536 did not cause cilia formation in XEN cells (0/308 cells, DMSO controls 0/301 cells, 1 independent experiment), despite formation of monopolar spindles, which are associated with loss of Plk1 activity. N-nervonoyl d-erythro sphingosine (C24:1) ceramide is required for ciliogenesis in MDCK cells and in human ES cell derived neural progenitors to sequester aPKC to the apicolateral cell membrane preventing it from activating AURKA, leading to cilium disassembly. Exposure to 2μM or 20μM of C24:1 ceramide for 24 h did not induce ciliogenesis in XEN cells (0/281 cells, DMSO controls 0/322 cells, 1 independent experiment). APC/C and Cdc20 localize to the basal body in human epithelial cells and are required for cilia disassembly prior to anaphase. This is mediated by APC/C dependent degradation of Nek1, which is essential for maintenance of axonemal microtubule stability and integrity. Treatment of XEN cells for 24 h with 20μM APC inhibitor proTAME did not induce cilia formation in these cells (0/326 cells, DMSO controls 0/345 cells, 1 independent experiment). VHL is required for cilia stability in renal cysts specifically when GSK3β is inactive. Treatment of XEN cells with 1μM FM19G11, which blocks the function of both HIF1α and HIF2α (IC50 = 80nM), does not cause cilia formation (0/284 cells; DMSO 0/278 cells; from 2 independent experiments), marked with IFT88 (green), acetylated α-tubulin (magenta) and centrosomes γ-tubulin (red). NEDD9 and AURKA are therefore activated in XEN cells by a HIF-independent pathway. Nuclei stained with DAPI (blue). Scale bar = (B, C) 5 μm.
**Supplementary Figure 5** AurkA and HDAC6 inhibitors and siRNA controls. (A-F) Phosphorylated AurkA (green) is detected at the spindle pole marked with γ-tubulin (red) in mitotic cells, arrowed (A, C) but is lost upon treatment with Alisertib (B, D). (E, F) Total AurkA (green) is still detected at the centrosome, marked with pericentrin (PCNT, red) in Alisertib treated cells (F). (G, H) Following Alisertib treatment, ciliated XEN cells continue to express XEN cell markers including Sox17 (red). (I-K) siRNA knock down of AurkA reduces levels of AurkA (green) at the centrosome, marked with PCNT (red), compared to control cells treated with scrambled siRNA (L-N). (O, P) Treatment of XEN cells with 5 μM HDAC6 inhibitor Tubacin for 72 h causes formation of primary cilia, marked with IFT88 (O, green), ARL13B (P, green), and acetylated α-tubulin (magenta), that project from a centrosome marked with γ-tubulin (red; 22/573 cells from 3 independent experiments). (Q-T) Culture of e7.5 embryos for 12h in 5 μM Tubacin causes stabilization of microtubules, marked by upregulation of acetylated α-tubulin (red). Average pixel intensity of acetylated α-tubulin was 4.6 times higher in Tubacin-treated embryos than in DMSO controls. Nuclei stained with DAPI (blue). Scale bar = (A-H) 5 μm; (I-N) 5 μm; (O, P) 2 μm (Q-T) 30 μm.
Supplementary Figure 6 Uncropped western blots. (A) Uncropped blot referring to Figure 5A. Blot for activated AURKA (T288), arrow indicates correct band, which correlates to single band on Total AURKA blot. (B) Uncropped blot referring to Figure 5C. Blot for Nedd9, arrow indicates 93kDa band. (C) Uncropped blot referring to Figure 6A. Active AURKA was reduced by 32.5 fold at 250 nM Alisertib, and was undetectable at 500 nM Alisertib. Total AURKA remained unaffected. (D) Uncropped blot referring to Figure 6B. siRNA knock down reduced levels of AURKA to 15% of wild type levels. (E) Uncropped blot referring to Supplementary Figure 2I. Blot for mCherry using a dsRed antibody detects ARL13B-mCherry fusion ~88kDa. Arrow indicates correct band present in transgenic ciliated MEFs, and transgenic XEN cells but absent in wild type MEFs and wild type XEN cells. Two central bands were could not be interpreted due to bubbles in the transfer. (F) Uncropped blot referring to Supplementary Figure 4A. Blot for pVHL, arrow indicates band at 24kDa. Levels of pVHL are equal in XEN cells and EpiSCs. Loading controls GAPDH.
Supplementary Figure 6 continued
Supplementary Videos Legends

**Supplementary video 1:** 3D reconstruction of confocal imaging of a 32 cell blastocyst expressing ARL13B-mCherry. Centrioles marked with Centrin2-GFP (green) are present in ICM and TE cells but primary cilia are not present in the embryo at this stage.

**Supplementary video 2:** 3D reconstruction of confocal imaging of a 72 cell blastocyst expressing ARL13B-mCherry and Centrin2-GFP. On rare occasions (~2% of cells), ARL13B-mCherry expression can be detected adjacent to centrosomes in cells of the ICM but not in trophectoderm cells.

**Supplementary video 3:** 3D reconstruction of confocal imaging of an e5.5 embryo after cavitation, expressing ARL13B-mCherry and Centrin2-GFP. Some ARL13b-mCherry expression can be detected adjacent to centrosomes in the epiblast but not the extraembryonic visceral endoderm or trophoectoderm lineages.

**Supplementary video 4:** 3D reconstruction of confocal imaging of an e6.0 embryo expressing ARL13B-mCherry (red) and Centrin2-GFP. Primary cilia are only present on epiblast cells and not on cells of the visceral endoderm.

**Supplementary video 5:** 3D reconstruction of confocal imaging of the distal region and node of an 8.0 embryo. Primary cilia expressing ARL13B-mCherry (red) are broadly distributed in both the long cilia of the node (the pit at the center of the Video) and in cells of all three germ layers. Centrioles are marked with Centrin2-GFP (green).

**Supplementary video 6:** 3D reconstruction of confocal imaging of an early bud embryo (e7.5), AFP-GFP (green) is expressed in visceral endoderm cells. ARL13B-mCherry (red) labels primary cilia, which are only present on non-GFP expressing definitive endoderm cells.