

## RESEARCH ARTICLE

# Loss of LKB1 leads to impaired epithelial integrity and cell extrusion in the early mouse embryo

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## ABSTRACT

LKB1/PAR-4 is essential for the earliest polarization steps in *Caenorhabditis elegans* embryos and *Drosophila* oocytes. Although LKB1 (also known as STK11) is sufficient to initiate polarity in a single mammalian intestinal epithelial cell, its necessity in the formation and maintenance of mammalian epithelia remains unclear. To address this, we completely remove LKB1 from mouse embryos by generating maternal-zygotic *Lkb1* mutants and find that it is dispensable for polarity and epithelia formation in the early embryo. Instead, loss of *Lkb1* leads to the extrusion of cells from blastocyst epithelia that remain alive and can continue to divide. Chimeric analysis shows that *Lkb1* is cell-autonomously required to prevent these extrusions. Furthermore, heterozygous loss of *Cdh1* exacerbates the number of extrusions per blastocyst, suggesting that LKB1 has a role in regulating adherens junctions in order to prevent extrusion in epithelia.

**KEY WORDS:** Serine threonine kinase 11, STK11, Epithelial polarity, Cell extrusion, E-cadherin, Preimplantation

## INTRODUCTION

Polarity formation is an important step in the development of the embryonic and extraembryonic lineages of the preimplantation mouse embryo. At the eight-cell stage, apical and basolateral polarization occurs simultaneously with compaction and before the earliest lineage markers are detected (Anani et al., 2014; Ziomek and Johnson, 1980), establishing an inner-outer configuration of cells in the developing morula. Polarized outer cells will become the trophectoderm epithelium in the blastocyst, giving rise to the trophoblast and placenta; inner cells will form the inner cell mass (ICM) made up of epiblast (EPI) and polarized primitive endoderm, giving rise to the embryo and yolk sac, respectively (reviewed in Artus and Hadjantonakis, 2011; Lanner, 2014; Yamanaka and Ralston, 2010). Maintenance of epithelial integrity in the trophectoderm and primitive endoderm epithelia is required for blastocoel filling and proper formation of the basement membrane, respectively. Consistent with this, disruption of polarity through experimental manipulation of the apical proteins aPKCz, PARD3 and PARD6

causes failure of proper development into the implantation stages (Alarcon, 2010; Hirate et al., 2013; Plusa et al., 2005).

LKB1 (also known as STK11 in mammals and PAR-4 in invertebrates) is a serine-threonine kinase required for correct polarization of many cell types in both invertebrates and vertebrates (reviewed in Partanen et al., 2013). Invertebrate *par-4* is part of the partitioning-defective group of genes and is required for polarized asymmetrical division in *Caenorhabditis elegans* embryos (Kempthues et al., 1988; Watts et al., 2000) and polarization of *Drosophila* oocytes, follicle cells and retinal cells (Amin et al., 2009; Martin and St Johnston, 2003). In mammals, LKB1 is involved in maintaining or establishing polarity in mammary epithelia (Partanen et al., 2012), pancreatic B cells (Hezel et al., 2008), testes (Tanwar et al., 2012) and neurons (Huang et al., 2014; Shelly et al., 2007; Shelly and Poo, 2011). Strikingly, induction of LKB1 activity by expression of binding partner STRAD can spontaneously polarize intestinal epithelial cells *in vitro* in lieu of cell–cell contacts (Baas et al., 2004). Mouse blastomeres are also able to polarize in the absence of cell–cell contacts (Anani et al., 2014; Ziomek and Johnson, 1980), begging the question of whether LKB1 is involved in the earliest polarization steps in the embryo. *Lkb1*-null embryos live until around embryonic day (E)9.0 with defects in turning, somitogenesis and vasculogenesis (Jishage et al., 2002). However, because *Lkb1* is expressed in the oocytes of these zygotic-null animals (Szczepańska and Maleszewski, 2005), this question remains unaddressed.

Aside from roles in polarity formation, LKB1 is a tumor suppressor that is mutated in >30% of non-small-cell lung carcinomas (Sanchez-Cespedes et al., 2002; Wingo et al., 2009) and ~20% of cervical cancers (Wingo et al., 2009). Human mutations in *LKB1* cause Peutz-Jeghers syndrome, characterized by benign intestinal hamartomatous polyps and an increased cancer risk, reiterated in heterozygous *Lkb1*-null mice (Katajisto et al., 2007; Miyoshi et al., 2002). LKB1 can phosphorylate 13 other kinases, including AMPK, MARKs (PAR-1), NUAks and MELK (PIG-1) (Lizcano et al., 2004). LKB1-dependent phosphorylation of AMPK is important for energy regulation and cell growth involving mTOR signaling, and has been implicated in LKB1 tumor suppressor activity (reviewed in Hardie and Alessi, 2013). Alternatively, LKB1 activation of MARKs regulates pancreatic acinar cell polarity and possibly the actin cytoskeleton (Granot et al., 2009; Yamahashi et al., 2011), whereas NUAks control cortical axon branching (Courchet et al., 2013). Thus, LKB1 can control a wide variety of cell processes by controlling target phosphorylation in a context-dependent manner.

In *C. elegans*, PAR-4 activation of PIG-1 is required for the shedding and/or extrusion of certain cell lineages in the absence

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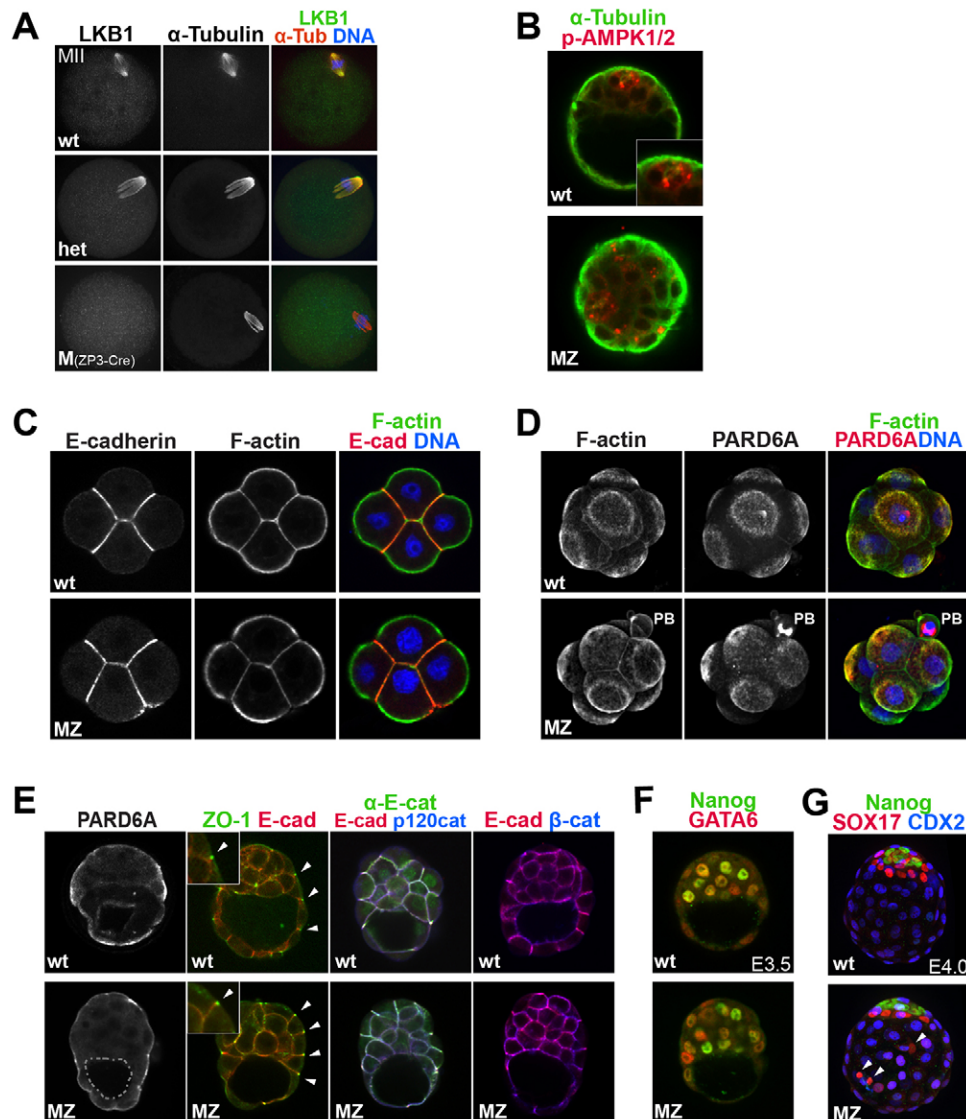
of normal programmed cell death (Denning et al., 2012). Cell extrusion is an active epithelial process observed in vertebrates and invertebrates, where cells are ejected basally or apically (but not both) from an epithelium through actomyosin-based contractions by neighboring cells (reviewed in Eisenhoffer and Rosenblatt, 2013). They can be dying prior to ejection (Gibson and Perrimon, 2005; Rosenblatt et al., 2001) or alive in the case of transformed cells (Hogan et al., 2009; Kajita et al., 2010; Leung and Brugge, 2012; Vidal et al., 2006) or during tissue homeostasis (Eisenhoffer et al., 2012; Marinari et al., 2012).

Taken together, current data show that LKB1 has many diverse roles across species, but the relationship between LKB1 control of tumorigenesis, metabolism, epithelial integrity and polarity is unclear. Here, we completely remove *Lkb1* from the early mouse embryo in order to ask whether it is involved in the first steps of polarity formation, as seen in invertebrates. We find that LKB1 has no role in establishing or maintaining polarity through to gastrulation stages. However, we find that loss of LKB1 leads to impaired epithelial integrity and cell extrusion from the epithelia, a process involving E-cadherin.

## RESULTS

### LKB1 is not required for establishment of polarity or lineage specification in the preimplantation embryo

Although LKB1/PAR-4 is essential for polarity formation in *C. elegans* and *Drosophila*, mouse zygotic *Lkb1*<sup>-/-</sup> embryos survive until neurulation (Jishage et al., 2002). Given that cell polarization and epithelia formation play important roles in early embryogenesis, we hypothesized that maternal *Lkb1* expression in the oocyte rescues preimplantation development in zygotic mutants. To test this, we generated a maternal deletion of *Lkb1* in the conditional *Lkb1*<sup>lox/lox</sup> mouse line using the oocyte-driven Zp3-Cre (De Vries et al., 2004). LKB1 localized to the mitotic spindle of metaphase II (MII) oocytes from wild-type and *Lkb1*<sup>+/-</sup> females as described previously (Fig. 1A) (Szczepańska and Maleszewski, 2005), but was completely absent from MII oocytes derived from *Lkb1*<sup>lox/lox</sup>; Zp3-cre females (Fig. 1A, *n*=7), confirming efficient excision of *Lkb1* maternally. It was also reported that phosphorylated (p-)AMPK1/2, a target of LKB1, is associated with the mitotic apparatus, where it is specifically localized to the spindle poles,



**Fig. 1. LKB1 is dispensable for polarity establishment and lineage specification.**

(A) LKB1 colocalizes with  $\alpha$ -tubulin to the mitotic spindle of MII oocytes in wild-type (wt) and *Lkb1*<sup>+/-</sup> (Het) oocytes, but is absent from an oocyte expressing ZP3-Cre (maternal *Lkb1* loss, M). (B) p-AMPK1/2 localizes to cytoplasmic puncta within dividing cells of both wild-type and maternal-zygotic *Lkb1* mutant (MZ) blastocysts.  $\alpha$ -tubulin labels the mitotic spindle. The inset shows topmost dividing cell in the wild type. (C) Confocal Z-slice shows normal localization of E-cadherin and F-actin (using phalloidin) at cell–cell contacts in an eight-cell maternal-zygotic embryo. (D) Confocal Z-stack shows normal apical enrichment of F-actin and PARD6A in a polarized eight-cell maternal-zygotic embryo. PB, polar body. (E) Confocal Z-slices of polarity markers in  $\sim$ E3.5 blastocysts. PARD6A is normally localized to the apical surface of maternal-zygotic mutants (dotted line shows blastocoel).  $\alpha$ -E-catenin, E-cadherin,  $\beta$ -catenin and p120-catenin colocalize at all points of cell–cell contact in wild-type and maternal-zygotic mutants. Co-labeling of E-cadherin and ZO-1 (arrowheads) shows wild-type tight junction localization (green spot) above basolateral adherens junctions (red cell–cell boundary) in maternal-zygotic mutants. The inset shows a higher-magnification view of a junction of each embryo. Note that  $\alpha$ -E-catenin variably shows nuclear staining in both wild-type and maternal-zygotic embryos. (F) ‘Salt-and-pepper’ localization of Nanog and GATA6 is intact in E3.5 maternal-zygotic blastocysts. (G) Segregation of EPI (Nanog), primitive endoderm (SOX17) and trophoblast (CDX2) is largely normal in expanded E4.0 maternal-zygotic blastocysts. Arrowheads show misplaced primitive endoderm cells in this embryo.

kinetochores and cell cortex of dividing intestinal cells (Wei et al., 2012). Similarly, we observed p-AMPK1/2 in cytoplasmic puncta and spindle poles (not shown) in dividing cells of wild-type blastocysts (Fig. 1B). This staining disappeared after incubation of the antibody with phosphorylated peptide or pretreatment of the embryos with  $\lambda$  protein phosphatase (not shown). Notably, p-AMPK1/2 localization did not change in maternal-zygotic *Lkb1*-null embryos using two different antibodies, suggesting that LKB1 is not required for AMPK phosphorylation at early stages (Fig. 1B).

To determine the role of maternal *Lkb1* in polarity establishment, we immunostained eight-cell and blastocyst-stage embryos for apical and basolateral markers. We observed no difference in basolateral localization of E-cadherin (Fig. 1C, Z-sections) and  $\beta$ -catenin (not shown) between wild-type and maternal-zygotic eight-cell embryos. Furthermore, apical poles were properly established at the eight-cell stage as visualized by F-actin enrichment (Fig. 1C,D) and apical localization of the markers PARD6A (Fig. 1D,E) and aPKCz (not shown) throughout preimplantation stages. In blastocysts, the basolateral and adherens junction markers E-cadherin,  $\alpha$ -E-catenin (also known as catenin  $\alpha$ -1),  $\beta$ -catenin and p120-catenin (also known as catenin  $\delta$ -1) colocalized at all points of cell–cell contact in maternal-zygotic mutants, as confirmed by z-stack analysis (Fig. 1E). Establishment of tight junctions also appeared to be normal in maternal-zygotic mutants, as determined by proper localization of ZO-1 (Fig. 1E, arrowheads) and occludin (not shown) just apical to E-cadherin-labeled adherens junctions between every trophectoderm cell (Fig. 1E, insets).

Polarity establishment precedes and drives lineage specification in the preimplantation embryo (Anani et al., 2014; Yamanaka and Ralston, 2010). At early blastocyst stages in maternal-zygotic mutants, Nanog and GATA6 were expressed in the classic ‘salt and pepper’ pattern throughout the ICM as in the wild type (Fig. 1F) (Chazaud et al., 2006). Later, when EPI and primitive endoderm have segregated into separate layers, wild-type and maternal-zygotic mutant ICMs were indistinguishable in size and organization (Fig. 1G). Notably, we occasionally observed primitive endoderm and EPI cells incorrectly located away from the ICM, as described below (Fig. 1G, arrowheads). Taken together, these results show that a complete lack of LKB1 does not affect polarity establishment and lineage specification during preimplantation stages.

### LKB1 is required for blastocyst outgrowth formation

As it is challenging to observe embryonic development during E3.5–4.5 implantation stages, we asked whether these properly established lineages were capable of further development by challenging them in *in vitro* outgrowth assays. Within 24 h of plating, E3.5 blastocysts of all genotypes adhered to the gelatin-coated surface (Fig. 2A, 18–21 h; supplementary material Movie 1), and within 3 days the blastocysts formed a small outgrowth of epiblast and endoderm cells supported by a minimal bed of trophoblast cells (Fig. 2A, 60–63 h). After 5–6 days of culture, wild-type and maternal-only *Lkb1* mutant outgrowths were very large, and extraembryonic parietal endoderm started to emerge to populate the dish (Fig. 2A, 111 h–144 h, arrow). By contrast, zygotic mutant outgrowths unexpectedly stopped growing after 3 days of culture and collapsed or were dead by 6 days of culture (Fig. 2A, 90–126 h; Fig. 2E,  $n=7/7$ ; supplementary material Movie 1). Interestingly, by 3 days of culture, maternal-zygotic mutant outgrowths were clearly distinct from other genotypes, as

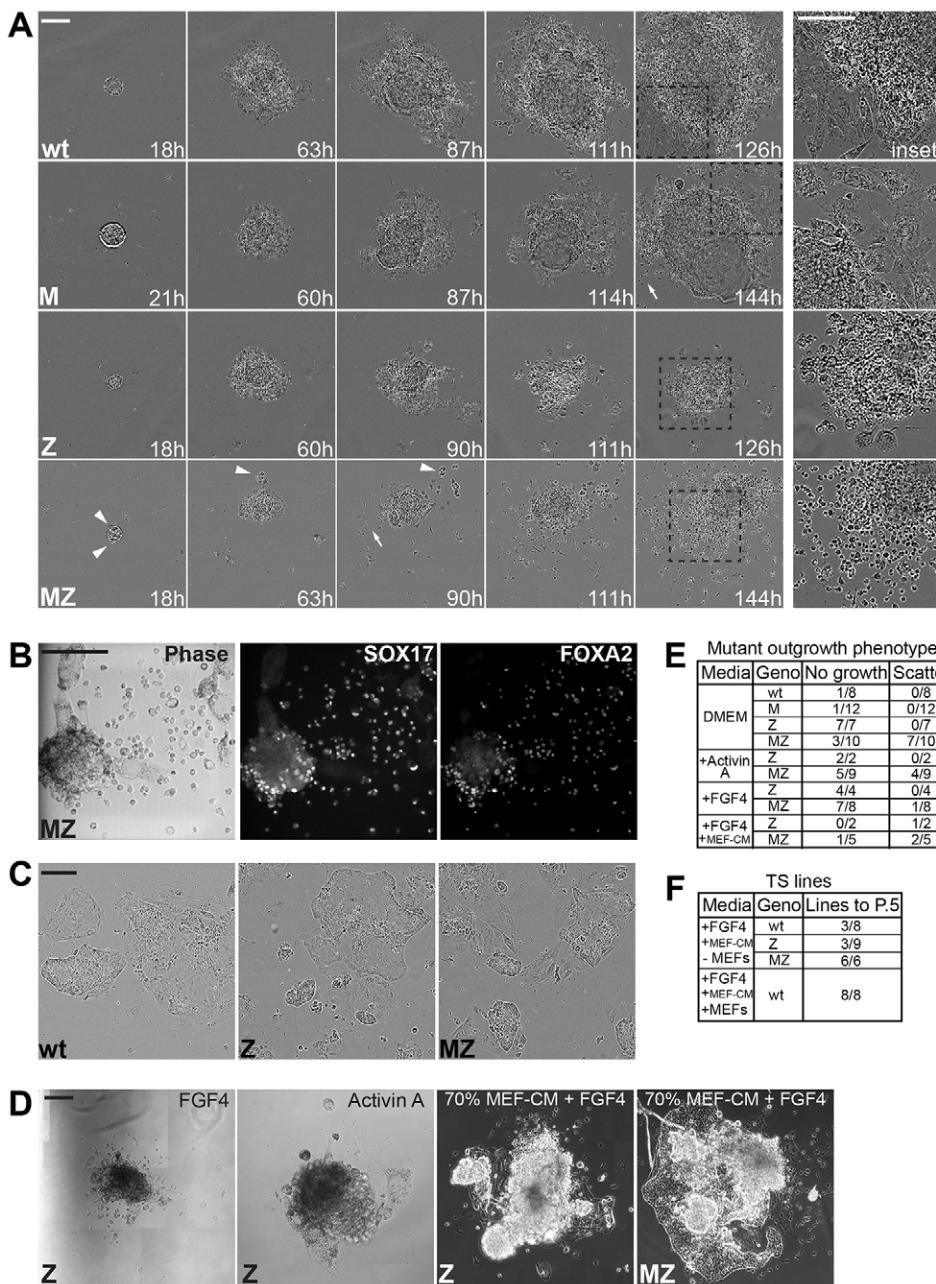
cells were often seen outside of the blastocyst that detached from the embryo and adhered separately (Fig. 2A, arrowheads; supplementary material Movie 1). Unlike zygotic mutant outgrowths, maternal-zygotic outgrowths continued to grow; however, significant numbers of cells resembling parietal endoderm emerged from the outgrowth early (Fig. 2A, 90–111 h, arrow; supplementary material Movie 1). By 6 days of culture, maternal-zygotic outgrowths were made up almost entirely of scattered cells, with a minimal ICM cluster and very few trophoblast giant cells (Fig. 2A, 144 h; supplementary material Movie 1). In both zygotic and maternal-zygotic mutant outgrowths, the few giant cells [which expressed the giant cell marker TROMA-3 (also known as KRT19), not shown] were often found at a significant distance from the outgrowth and did not touch each other (Fig. 2B). To determine the identity of the early-scattering cells in maternal-zygotic outgrowths, we immunostained fixed cultures and found that they robustly expressed the primitive endoderm markers SOX17 and FOXA2 (Fig. 2B), suggesting that these cells are still alive and that parietal endoderm exits precociously from maternal-zygotic outgrowths.

The reduced number of trophoblast giant cells suggested that there might be defects in trophoblast lineage development. We therefore attempted to isolate trophoblast stem cell (TSC) lines by culturing blastocysts in 70% mouse embryonic fibroblast-conditioned medium (MEF-CM) and FGF4, without MEFs (to allow genotyping). Under these non-MEF conditions, only 3/8 wild-type blastocysts gave rise to TSC lines that survived to at least the fifth passage (Fig. 2C,F, compare to 8/8 with MEFs). However, despite the lack of MEF support, maternal-zygotic blastocysts easily gave rise to TSC lines (Fig. 2C,F,  $n=6/6$ ). For both zygotic and maternal-zygotic *Lkb1* mutant lines, FGF4 was required to maintain TSCs, as its removal resulted in trophoblast differentiation within 2 weeks, as reported previously for wild-type cultures (Tanaka et al., 1998). These results suggest that the lack of trophoblast in outgrowth assays is not due to a trophoblast development defect but rather to something that either maternal-zygotic or zygotic *Lkb1* mutants require *in vitro* that is unnecessary in wild-type outgrowths.

We next asked whether the conditions used for TSC lines could rescue trophoblast development in outgrowth assays. FGF4 alone did not significantly change the numbers of trophoblast giant cells or the size of the epiblast in zygotic mutant outgrowths, nor did activin A, which is required for TSC maintenance (Fig. 2D,E) (Erlebacher et al., 2004). However, in both zygotic and maternal-zygotic *Lkb1* mutant outgrowths, the addition of FGF4 and 70% MEF-CM resulted in larger epiblast outgrowths supported by a TSC-like bed of cells (Fig. 2D,E). No giant cells were observed in either culture, consistent with TSC conditions suppressing trophoblast differentiation. Of note, maternal-zygotic outgrowths still exhibited early endoderm scattering. Thus, both maternal-zygotic and zygotic mutants are able to form TSC lines but cannot form proper trophoblast giant cells in normal outgrowth assays. This suggests that trophoblast development requires an additional pathway of support *in vitro* without LKB1 function.

### Maternal-zygotic embryos are developmentally delayed and arrest before zygotic *Lkb1* mutant embryos

To determine whether LKB1 is required for further development *in utero*, we collected E4.5–9.5 *Lkb1* litters and performed immunostaining or *in situ* hybridization for developmental markers. Notably, pups with maternal deletion of *Lkb1* were born and were fertile as adults, demonstrating there is no strict



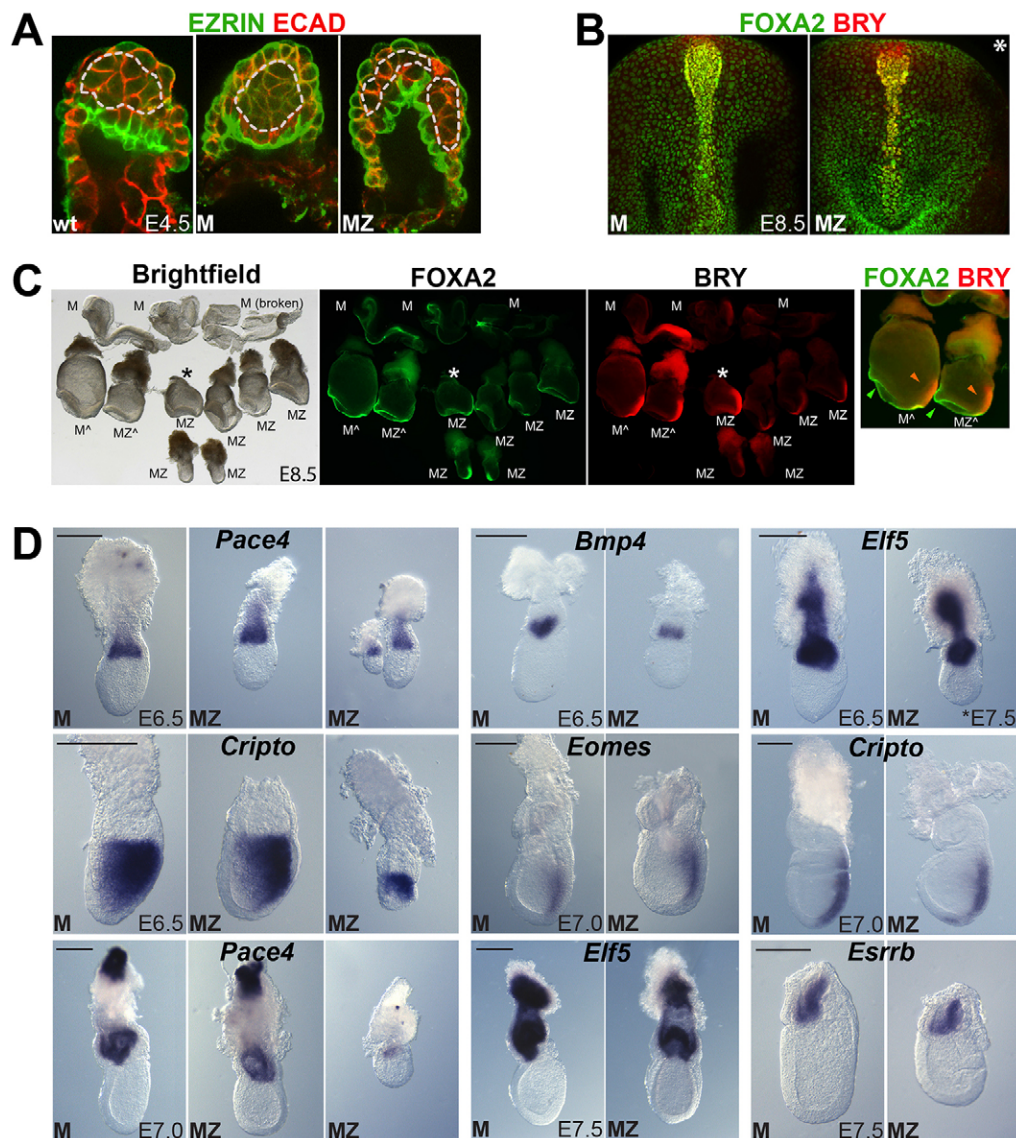
**Fig. 2. LKB1 is required for normal blastocyst outgrowths, but not TSC derivation.** (A) Time-lapse phase-contrast imaging of blastocyst outgrowths over 6 days. Insets show a higher-magnification view of the area in the dashed squares. Arrows indicate the start of parietal endoderm exit. Arrowheads show early detaching cells. (B) The maternal-zygotic mutant (MZ) outgrowth stain shows scattered cells expressing nuclear endodermal markers SOX17 and FOXA2. (C) The morphology of TSC colonies is similar between wild-type (wt), zygotic (Z) and maternal-zygotic embryos. (D) FGF4 or activin A do not rescue zygotic mutant blastocyst outgrowth survival after 6 days, but TSC conditions (70% MEF-CM+FGF4) do. Note that scattered cells remain in maternal-zygotic outgrowths under TSC conditions. The images shown are composites tiled manually from multiple phase-contrast images. Scale bars: 200  $\mu$ m. (E) A table of outgrowth phenotypes under different conditions. M, maternal *Lkb1* loss. (F) A table of trophoblast stem (TS) cell lines that reached passage 5 (P.5) under different conditions.

maternal requirement for *Lkb1*. At E4.5, no gross differences were observed in the size or shape of embryos with maternal-zygotic deletion of *Lkb1*. However, staining for the polarity markers ezrin (apical) and E-cadherin (basolateral) highlighted inconsistencies in the shape of the developing epiblast, where a normally compact ball of cells was often split or separated (Fig. 3A, dashed lines).

At E6.5 and E7.5, Mendelian ratio litters (50% maternal and 50% maternal-zygotic) were obtained, although maternal-zygotic embryos were almost always smaller or delayed. At E8.5, when zygotic *Lkb1* mutant embryos are still grossly similar to wild-type embryos in shape and size (Jishage et al., 2002), all maternal-zygotic embryos were arrested at an E7.5 developmental stage or earlier, and never progressed further (Fig. 3C). Morphologically, the most advanced embryos had head folds (Fig. 3C, overlay, green arrowheads) but were shortened and had significantly

smaller amniotic cavities (Fig. 3C). Immunostaining for FOXA2 and brachyury (BRY) showed correct posterior expression in the notochord, node and endoderm, and notochord, node and primitive streak, respectively (Fig. 3B,C, orange arrowheads in overlay). *In situ* hybridization for *Bry* and other posterior markers *Cripto* (also known as TDGF1) and *Eomes* confirmed this result at earlier stages (Fig. 3D, *Bry* not shown). Thus, LKB1 is not required for anterior-posterior specification, but might be required for extension to a normal embryo length.

We wondered whether delayed development in maternal-zygotic embryos was due to poor development of the trophoblast, as shown in outgrowth assays, and thus we probed for markers of extraembryonic tissues. *Pace4* (also known as *Pesk6*), *Bmp4*, *Esrrb* and *Elf5* were all correctly expressed in the chorion, extraplacental cone or other extraembryonic ectoderm compartments, regardless of embryo size (Fig. 3D, E6.5 versus



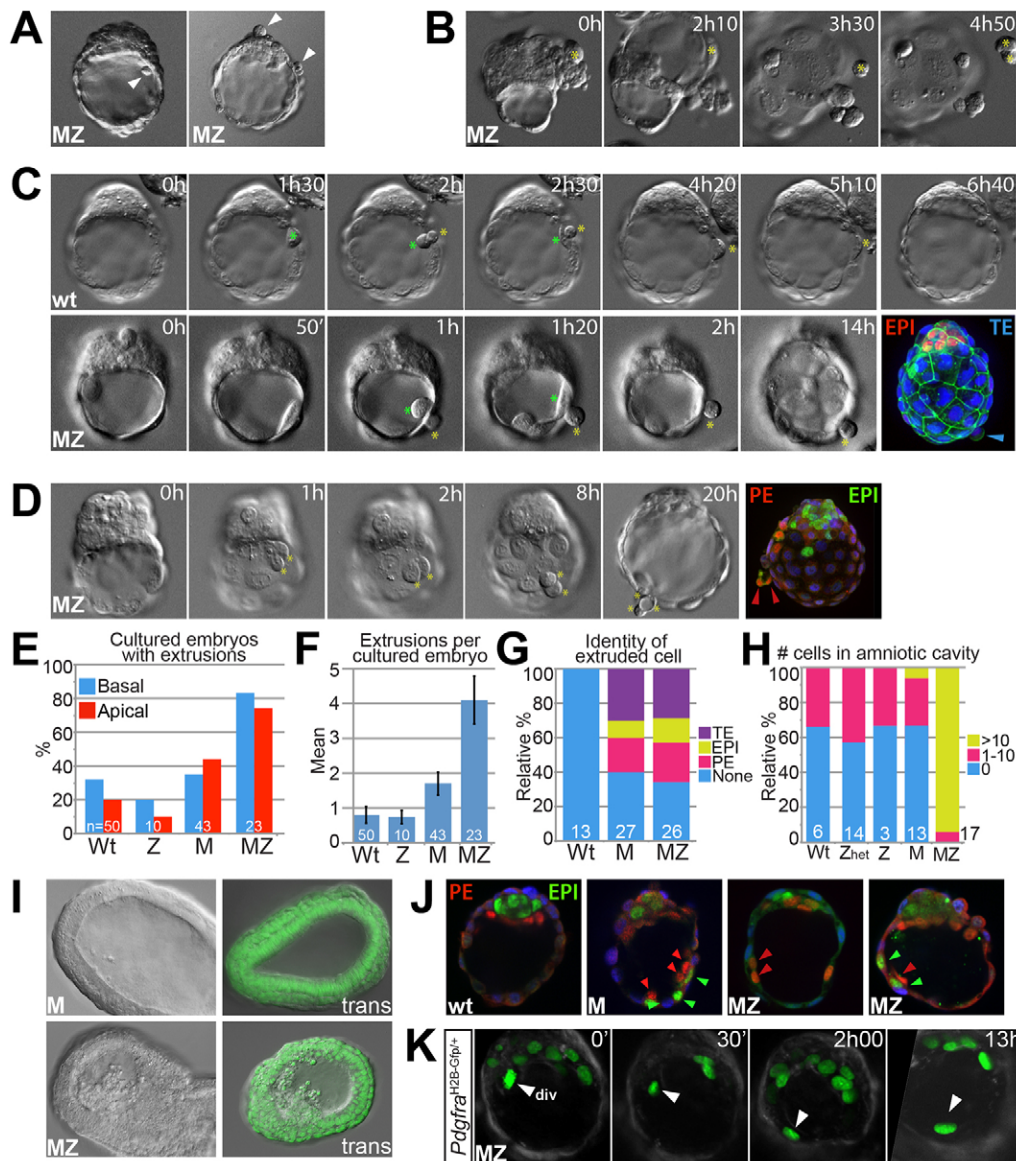
**Fig. 3. Maternal-zygotic embryos arrest at gastrulation stages but have normal expression of anterior-posterior axis and extra-embryonic markers.** (A) E4.5 immunostaining of maternal-zygotic (MZ) post-implantation embryos shows disorganized or split ICMs (dashed lines). Ezrin labels apical and E-cadherin labels basolateral portions of the epithelia. wt, wild type. (B) View of the notochord, node (spoon shape) and primitive streak (orange co-staining above node) of E8.5 embryos. Posterior is up, anterior is down. FOXA2 and brachyury (BRY) are expressed equally in maternal (M) and maternal-zygotic embryos, although maternal-zygotic embryos are smaller. (C) One complete E8.5 litter shows maternal-zygotic delay to morphological stages E6.5–E7.5, with amniotic cavity shortening at E7.5. Compared to normal maternal deletion siblings, three are E8.5 (top) and one is morphologically E7.5 but is normally sized (mid left,  $\wedge$ ). FOXA2 shows strong notochord staining in all embryos, BRY shows strong primitive streak (posterior) staining in all embryos.  $\wedge$ -marked embryos are shown in the higher-magnification overlay: anterior head folds (green arrowhead, FOXA2) and posterior primitive streak (orange arrowhead, FOXA2+BRY) are formed in both maternal and maternal-zygotic mutants. The asterisk indicates the same embryo in multiple panels. (D) *In situ* hybridization for posterior embryonic markers *Cripto* and *Eomes*; extra-embryonic markers *Pace4*, *Bmp4*, *Elf5* and *Esrrb* at E6.5 and/or E7.5. *Pace4* E6.5 shows an example of recovered separable twins. In maternal-zygotic mutants, all markers are expressed at the correct levels and in the correct location as those of stage-matched maternal mutant siblings. Scale bars: 200  $\mu$ m.

E7.5). This was especially evident in twin embryos that were recovered (Fig. 3D, *Pace4* E6.5). Taken together, we conclude that gross specification of embryonic and extraembryonic tissues occurs in both zygotic and maternal-zygotic deletion embryos, but that maternal *Lkb1* is required for embryos with zygotic deletion of *Lkb1* to advance to the 10-somite E8.5 stage (Jishage et al., 2002).

#### Loss of maternal *Lkb1* induces cell extrusion from epithelia in blastocysts

While investigating polarity and lineage commitment in blastocysts and outgrowths, we observed unexpected cell

phenomena (Fig. 1G; Fig. 2A, arrowheads), and began time-lapse imaging of E2.5–4.5 cultured blastocysts. Interestingly, we observed frequent cell extrusions oriented basally (inward towards the blastocoel) or apically (outward) from embryos with maternal-zygotic loss of *Lkb1* (Fig. 4A,E, 83%  $\geq 1$  basal, 74%  $\geq 1$  apical), with an average of four extrusions per embryo (Fig. 4F). Extrusions did occur in wild-type blastocysts but at lower frequencies, especially apically (Fig. 4E, 32%  $\geq 1$  basal, 20%  $\geq 1$  apical; supplementary material Movie 2). Interestingly, blastocysts with zygotic loss of *Lkb1* were similar to wild-type blastocysts (Fig. 4E), whereas blastocysts with maternal deletion



**Fig. 4. LKB1 loss results in extrusions from the blastocyst.** (A) Extrusion in maternal-zygotic (MZ) blastocysts can occur basally (towards the blastocoel) or apically. Arrowheads, extruded cells. (B) Time-lapse DIC imaging of a cultured maternal-zygotic blastocyst shows extrusions dividing and migrating away from the embryo. Asterisks label an extruded cell and its daughter. Zona pellucidae are removed for all time-lapse cultures. (C) Extrusions can occur after cell division. Here, the divisional plane of trophectoderm (TE) cells is parallel to the blastocyst surface, leaving daughters that are perpendicular. After division, an inner daughter (green asterisk) and outer daughter (yellow asterisk) are visible. For the wild-type (wt) blastocyst, supplementary material Movie 2 shows a delay in reintegration of the outside daughter (images rotated and reflected from film). For the maternal-zygotic blastocyst, supplementary material Movie 3 shows the outside daughter extruding and continuing to express the trophectoderm marker CDX2 (arrowhead). EPI is shown by Nanog staining; the green channel shows E-cadherin (cell–cell contacts). (D) Extrusions can happen without division. Asterisks, two cells emerging from the primitive endoderm (PE) layer and traveling along the blastocyst wall until finally extruding through the mural trophectoderm and continuing to express the primitive endoderm marker SOX17 (arrowheads). Times are rounded to the nearest hour. Because of embryo drift, some frames are rotated for comparison. (E) A graph showing the number of cultured embryos displaying any extrusions, and the direction of extrusions. Z, zygotic mutant; M, maternal mutant. (F) The mean number of extrusions per cultured embryo. Data show the mean  $\pm$  s.e.m. (G) Identity of extruded cells from a subset of time-lapse cultured embryos, as assessed by immunostaining for trophectoderm (CDX2), EPI (Nanog) and primitive endoderm (SOX17). 'None' indicates that neither CDX2, Nanog nor SOX17 was present in the DNA-stained nucleus. (H) A graph showing the number of cells detected in the amniotic cavity of E7.5 embryos. In most cases, only one or two cells were found in embryos in the 1–10 category. Het, heterozygous zygotic mutant. (I) Left, DIC images of whole lateral views of amniotic cavities from maternal and maternal-zygotic mutant E7.5 embryos. Right, overlay of DIC and DAPI nuclear stains (falsely colored green) in transverse (trans) slices of the same embryos. Cell masses have pyknotic nuclei. (J) EPI cells can be found inserted into the mural trophectoderm in maternal and maternal-zygotic embryos (green arrowheads). Primitive endoderm cells are often found adjacent to the mural trophectoderm (red arrowheads). (K) Time-lapse confocal images of a MZ; *Pdgfra*<sup>H2B-Gfp/+</sup> embryo showing an already-specified primitive endoderm cell dividing, leaving a daughter to migrate along the blastocyst wall to settle adjacent to the mural trophectoderm opposite the ICM (arrowhead). Div, division.

of *Lkb1* fell between wild-type or zygotic mutants and maternal-zygotic mutants, with double the number of apical extrusions and a similar number of basal extrusions to that observed in wild-type blastocysts (Fig. 4E, 35%  $\geq 1$  basal, 44%  $\geq 1$  apical). Many extruded cells were obviously alive when extruded, as they could continue to divide, remained at the surface for hours and could even delaminate from the embryo entirely to divide on the surface of the dish (Fig. 4B; supplementary material Movie 3). Extrusions could occur immediately after divisions (Fig. 4C) or after non-division-associated cell movements (Fig. 4D; supplementary material Movie 4). With division-related extrusions, one or both daughters could extrude. We wondered whether division-related extrusions could be due to abnormal mitotic spindle placement and division plane (Wei et al., 2012), but saw examples of both daughters re-integrating into the trophectoderm epithelia from similar divisions in wild-type embryos (Fig. 4C, compare upper and lower rows, yellow asterisks). This suggests that LKB1 could be required for divided epithelial cells to reintegrate into an epithelium after cell division.

We asked whether certain lineages were susceptible to extrusion, but we found examples from all three lineages by immunostaining embryos after culture (Fig. 4C, trophectoderm; Fig. 4D, primitive endoderm, EPI not shown; Fig. 4G). Primitive endoderm extrusions often swelled to make large cysts (Fig. 4D). A comparison of extrusion lineage identity demonstrated that only maternal or maternal-zygotic mutant extrusions were clearly expressing one of three lineage markers in a non-fragmented nucleus (Fig. 4G). Taken together, these data show that the loss of *Lkb1* greatly increases the rate of apical and basal cell extrusion, and these cells can be alive and derived from any lineage.

We investigated whether cell extrusions might continue to occur later during development. Differential interference contrast (DIC) images of either whole or transverse slices of E7.5 maternal-zygotic embryos showed that nearly 95% had cellular masses inside the amniotic cavity, some of which were very large (Fig. 4H,I). Nuclear staining indicated that most of these cells were pyknotic (Fig. 4I). In wild-type and maternal mutant embryos, and those with heterozygous or homozygous zygotic loss of *Lkb1* (Fig. 4H,I), only small isolated groups of pyknotic cells could be found in a subset of embryos. This suggests that significant numbers of cells are lost in maternal-zygotic embryos, possibly through a process of epithelial extrusion found only occasionally in wild-type embryos.

In addition to extrusions in maternal and maternal-zygotic mutant blastocysts, cells expressing Nanog (EPI) or SOX17 (primitive endoderm) were often found away from the ICM (Fig. 1G; Fig. 4J). Primitive endoderm cells nestled basally to the trophectoderm layer (Fig. 4J, red arrowheads) but, interestingly, EPI cells could be found inserted into the trophectoderm layer (green arrowheads). We visualized primitive endoderm cells in cultured maternal-zygotic embryos using the *Pdgfra*<sup>H2B-GFP/+</sup> reporter line, and found that primitive endoderm cells moving away from the ICM were specified before mislocalization (Fig. 4K). Thus, without LKB1, the embryo failed to maintain blastocyst epithelial integrity as well as ICM cell organization.

#### Extruded cells express appropriate polarity markers and are only sometimes apoptotic

To investigate what contributes to extrusions in maternally deleted *Lkb1* embryos, we performed immunostaining for polarity and junction markers. We observed expanded apical domains

visualized by staining of P-ERM (phosphorylated ezrin/radixin/moesin) and PARD6B in apical extrusions (Fig. 5A, white arrowheads). In some extrusions, E-cadherin was clearly expressed at the lateral junction where the extrusion touched the underlying blastocyst (Fig. 5A, asterisk) but, in others, the apical surface encompassed the entire extrusion and had little E-cadherin (Fig. 5A, lower left, upper right). In extrusions with both apical and basolateral compartments, ZO-1 was strongly expressed at tight junctions (Fig. 5A, green arrowheads). These results suggest that extruded cells are not inherently unable to polarize or maintain their polarity.

At peri-implantation stages, the primitive endoderm layer secretes laminins to form the basal lamina of the embryo (Fig. 5B,C). We immunostained flushed E4.5 embryos for laminin but found no gross discontinuities in the basal lamina (Fig. 5B). Furthermore, SOX17-labeled primitive endoderm cells were able to produce laminin even if extruded or mislocalized outside the primitive endoderm layer in the blastocyst (Fig. 5C), suggesting that there was no change in the functional identity of extruded cells.

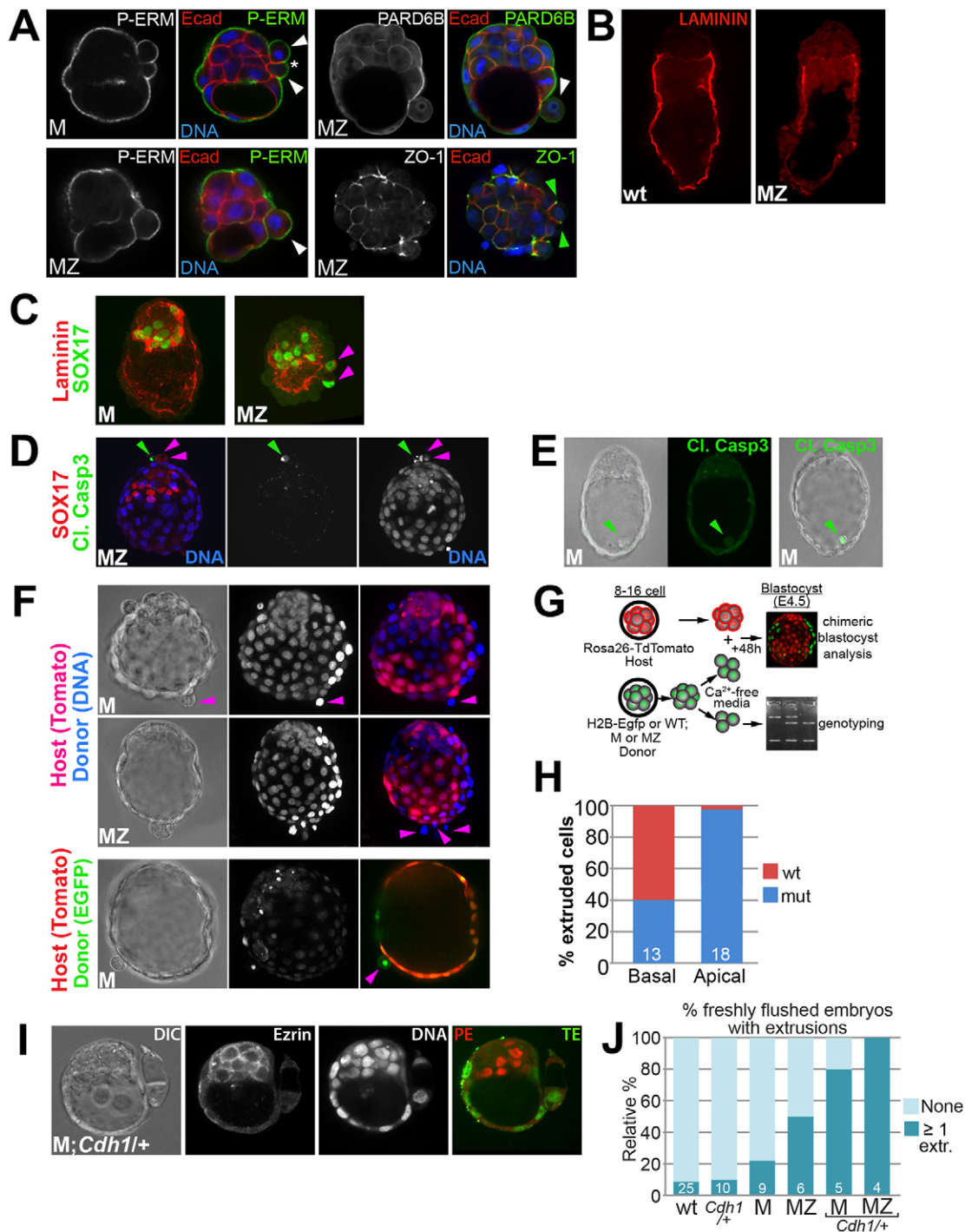
Cell extrusion is one of the important mechanisms eliminating sick, dying or dead cells from epithelia for epithelial homeostasis (reviewed in Eisenhoffer and Rosenblatt, 2013). However, we regularly observed extruded cells dividing at the surface of the blastocyst (Fig. 4B), although we also observed spontaneous lysis of extrusions (supplementary material Movie 3) or extrusions with fragmented nuclear staining and no lineage marker expression (Fig. 4G, blue). To determine whether extrusions were undergoing apoptosis after extruding, we immunostained blastocysts for cleaved caspase-3, an indicator of caspase pathway activation. We found no relationship between extrusions and caspase activation, as some extruded cells had staining and some did not, even within the same embryo and regardless of whether extrusion occurred apically or basally (Fig. 5D,E).

#### Apical cell extrusions occur cell autonomously

We asked whether extrusions in *Lkb1* mutant embryos are due to a cell-autonomous defect by making chimeras of wild-type and mutant cells. Unmarked or H2B-EGFP-expressing maternal or maternal-zygotic mutant donor embryos were dissociated using Ca<sup>2+</sup>-free medium, and some cells were reserved for genotyping (Fig. 5G). Mutant donor cells were aggregated with wild-type *TdTomato* 8–16-cell hosts, and allowed to develop in culture until E4.5 (Fig. 5G). We observed that basal extrusions came from either wild-type or mutant cells (Fig. 5H, 60% wild type, 40% maternal or maternal-zygotic), but that apical extrusions were almost exclusively mutant for *Lkb1* (Fig. 5F,H, 4% wild type, 96% maternal or maternal-zygotic). These results suggest that apical extrusions are due to a cell-autonomous defect.

#### Reduced expression of E-cadherin increases epithelial extrusion frequency in *Lkb1* maternal mutants

Because we did not observe gross abnormalities in tight or adherens junction protein localization in mutant embryos (Fig. 1C–E), we speculated that epithelial extrusion in maternal *Lkb1* mutants could be due to defects in spontaneous junction dynamics between epithelial cells, especially in the trophectoderm where both basal and apical extrusions occur. To test whether E-cadherin might play a role in epithelial extrusion in maternal or maternal-zygotic mutant embryos, we removed one copy of *Cdh1*, the gene encoding E-cadherin, and scored how



**Fig. 5. Extruded cells express junction markers, are not always apoptotic and occur more frequently with heterozygous loss of *Cdh1*.** (A) Extruded cells have an expanded apical domain marked with P-ERM and PARD6B (white arrowheads). E-cadherin can be expressed at cell–cell contacts of extrusions (asterisk) and ZO-1 can be expressed at tight junctions where touching adjacent cells (green arrowheads). M, maternal mutant; MZ, maternal-zygotic mutant. (B) E4.5 maternal-zygotic embryos do not show any gross abnormalities in the basal lamina. wt, wild type. (C) Extruded (pink arrowheads) or misplaced (yellow arrowhead) primitive endoderm (PE) cells still secrete laminin. (D) Apically extruded cells do (green arrowheads) or do not (pink arrowheads) stain with the antibody against cleaved caspase 3 (Cl. Casp3). (E) Examples of E4.5 embryos with basolateral extrusions (arrowheads) that do or do not stain with the antibody against cleaved caspase 3. (F) Cell autonomy experiments performed as in the schematic shown in G using wild-type–mutant chimeras show that apical extrusions (arrowheads) come from mutant cells. The donor could be unmarked or marked with H2B–EGFP (lower row). (G) Schematic of cell-autonomy experiments. The donor was dissociated into parts in Ca<sup>2+</sup>-free medium to use a small amount of cellular material for genotyping. (H) Almost all apical extrusions are derived from mutant cells (Mut, maternal or maternal-zygotic). (I) Removal of one copy of *Cdh1* in a maternal mutant embryo causes a two-cell extrusion forming a mini-blastocyst (to the right of the blastocyst). TE, trophoblast. (J) *Cdh1* heterozygous (*Cdh1*<sup>+/+</sup>) embryos do not exhibit more extrusions than wild-type embryos. Loss of one copy of *Cdh1* increases the percentage of freshly flushed maternal and maternal-zygotic mutant embryos that have extrusions (extr.).



many freshly flushed E3.5 blastocysts had extrusions. In separate litters, 1/10 *Cdh1* heterozygotes had one apical cell extrusion that was pyknotic, compared to 2/25 freshly flushed wild-type embryos (Fig. 5J). Thus, heterozygous *Cdh1* loss does not cause extrusions over basal levels. In maternal/maternal-zygotic mutant crosses, we found that 22% of maternal mutant and 50% of maternal-zygotic mutant freshly flushed blastocysts had one extrusion or more (Fig. 5J). However, loss of one copy of *Cdh1* doubled the frequency of extrusions so that 80% of maternal;*Cdh1*<sup>+/-</sup> and 100% of maternal-zygotic;*Cdh1*<sup>+/-</sup> had one or more extrusions (Fig. 5I,J). These results demonstrate a genetic interaction between *Lkb1* and *Cdh1*, suggesting that components of adherens junctions might be involved in preventing epithelial extrusions in concert with LKB1.

## DISCUSSION

LKB1 and its invertebrate homolog PAR-4 are required for the establishment and/or maintenance of polarity in *C. elegans* embryos; *Drosophila* oocytes, follicle cells and retinal cells; and mammalian mammary epithelia, pancreatic B cells, testes and neurons (reviewed in Partanen et al., 2013). Furthermore, overexpression of LKB1 and its binding partner STRAD is sufficient to polarize isolated intestinal epithelial cells *in vitro*, a phenomenon reminiscent of mouse blastomeres remaining polarized in the absence of cell–cell contacts (Baas et al., 2004; Ziomek and Johnson, 1980). However, here, we conclusively find that complete removal of murine *Lkb1* is dispensable for the first steps of polarity establishment at the eight-cell stage or even for continued polarity maintenance through to implantation.

Despite maternal-zygotic and zygotic mutant embryos surviving through gastrulation, we observed a marked inability of both genotypes to form normal outgrowths *in vitro*. Zygotic mutant outgrowths collapsed within a few days of culture. Conversely, maternal-zygotic outgrowths exhibited precocious formation and/or migration of parietal endoderm, a cell type that is only detected after 5 or 6 days of culture, when the outgrowth is very large. The endoderm behavior is not a result of over-proliferation of primitive endoderm in the blastocyst, as primitive endoderm formation in maternal-zygotic embryos was comparable to that of controls. Clearly, *Lkb1* loss makes cells refractory to signals in the medium that would normally support blastocyst outgrowths.

It is proposed that signals from the ICM, like FGF4, are required for trophoblast growth and expansion (Tanaka et al., 1998) (reviewed in Pfeffer and Pearton, 2012). Despite very poor development and differentiation of trophoblast in outgrowths, maternal-zygotic and zygotic mutant embryos formed robustly growing TSC lines in 70% MEF-CM and FGF4, even in the absence of MEFs. A MEF-CM supplement could be either restoring a crucial signaling pathway that is lacking in *Lkb1*-null cells or providing a new signal that compensates for the loss of LKB1 activity. Because TSC conditions rescued epiblast growth in zygotic and maternal-zygotic mutant outgrowths as well, this trophoblast-maintaining activity must, in turn, be involved in signaling from the trophoblast to the epiblast.

Although LKB1 is dispensable for polarity establishment in preimplantation embryos, we find that LKB1 is required for the maintenance of epithelial integrity through the prevention of epithelial extrusions and irregular cell movements. In wild-type embryos, extrusion of one cell (out of ~100 in the blastocyst) can occur in ~40% of cultured embryos. Interestingly, extrusion can occur basally or apically, which is unusual compared to other

systems that extrude cells in only one direction (reviewed in Eisenhoffer and Rosenblatt, 2013). Wild-type extruded cells never express lineage markers but exhibit pyknotic nuclei or are dead, suggesting that preimplantation extrusion normally occurs to remove dead cells from expanding epithelia. Conversely, in maternal-zygotic mutants, up to 80% of embryos exhibit extrusions, and embryos can undergo several extrusion events. Aberrant mitotic spindle orientation has been reported in *Lkb1*<sup>+/-</sup> gastrointestinal polyps (Wei et al., 2012), and LKB1/PAR-4 is able to phosphorylate MARK/PAR-1 kinases to effect microtubule skeleton organization (Kojima et al., 2007). We wondered whether aberrant mitotic spindle placement might explain extrusion events in mutants, but found that although the divisional plane of most trophectoderm cell divisions was orthogonal to the epithelial surface, a fair number of divisions occurred in parallel to the epithelial surface in both mutants and wild-type controls. Interestingly, we observed an instance of a delay in the re-integration of an apically placed daughter in a parallel division in wild-type controls. In a similar division in an *Lkb1* mutant, the apically placed daughter did not re-integrate and remained detached (Fig. 4C). These results suggest that LKB1 is required for cells to recover their position in epithelia when local environmental stresses, such as cell division, occur during epithelial morphogenesis. Thus, mitotic spindle orientation might play a partial role in *Lkb1*-null epithelial extrusion, but it cannot be the only driver, as cells can extrude outside of a division event (Fig. 4D).

Interestingly, transformed cells or live cells extruding from naturally overcrowded tissues do not require division to extrude and, furthermore, can extrude in the absence of apoptosis (Eisenhoffer et al., 2012; Marinari et al., 2012), similar to extrusion-like events in *Lkb1*-null embryos. Additionally, in both *Lkb1*-mutant blastocysts and outgrowths, cells could surprisingly detach from the embryo, migrate away and continue to divide. This is striking behavior reminiscent of the extrusion of Ras<sup>V12</sup>-, Src- and Erb2-transformed cells extruding detrimentally from epithelia in mammalian or invertebrate model systems (Hogan et al., 2009; Kajita et al., 2010). As *Lkb1* is frequently deleted in lung and cervical cancers and mutated in tumor-prone Peutz-Jaeger patients, extrusions caused by *Lkb1* loss in blastocysts might shed light on its possible roles as a tumor suppressor. Indeed, intestinal adenoma polyps have been found to lack normal cell extrusion (Eisenhoffer et al., 2012), begging the question of whether extrusion plays any part in the formation of hamartoma polyps caused by *Lkb1* haploinsufficiency.

LKB1 phosphorylates at least 14 downstream targets, including AMPK, MARK (PAR-1) and MELK, doing so in conjunction with its binding partners STRAD and MO25 (also known as CAB39) (Katajisto et al., 2007; Partanen et al., 2013; Partanen et al., 2012). *Pig-1*, the *C. elegans* MELK homolog, was recently found to be required for the shedding and/or extrusion of certain lineages of cells in the genetic absence of programmed cell death (Denning et al., 2012). This activity is exclusively regulated upstream by PAR-4 with STRAD or MO25 homologs. Furthermore, cell extrusion is accompanied by loss of expression of cell adhesion molecules at cell surfaces, making another compelling connection between LKB1 activity, adhesion regulation and epithelial maintenance. However, there are key differences between this study and ours. First, *Pig1* and *Par-4* are required for the extrusion of very specific cell types; whereas, in mouse blastocysts, LKB1 is required to repress loss of any cell type. Second, all extruded cells exhibit apoptotic morphology

(despite not being able to activate caspase signaling), whereas we clearly see the extrusion of living cells in *Lkb1*<sup>-/-</sup> blastocysts. Still, there are likely to be common points between how LKB1 and partners regulate epithelial integrity and extrusion across species.

One of the concerted events required for cell extrusion is a loss of cell–cell contacts with neighboring cells (reviewed in Eisenhoffer and Rosenblatt, 2013). In the blastocyst, all apical extrusions and most basal extrusions exit the trophectoderm layer, a classic epithelium in which cells are linked by adherens and tight junctions. *Lkb1*-null extruded cells were still able to form junctions where they touched neighboring cells, suggesting that the extruding cell itself is not deficient in junction formation. We found that loss of one copy of *Cdh1* exacerbates the extrusion phenotype in maternal or maternal-zygotic mutant embryos, implicating a relationship between adherens junctions and LKB1 in limiting these events. *Cdh1* is also a tumor suppressor, and it will be interesting to focus on how these two pathways might interact in a cancer-related context. Further studies to identify downstream targets of LKB1 will be important to allow us to understand the mechanisms by which these cells physically extrude and how this might relate to LKB1 tumor suppressor activity.

## MATERIALS AND METHODS

### Mice

Conditional *Lkb1*<sup>lox/lox</sup> mice (Bardeesy et al., 2002), obtained from the National Cancer Institute (Frederick, MD), were maintained on a mixed C57BL/6;CD1 background. Tg(Zp3-Cre)82Kw/KnwJ mice (De Vries et al., 2004) were obtained from The Jackson Laboratory (Bar Harbor, ME). The *Lkb1* maternal deletion allele was generated from *Lkb1*<sup>lox/lox</sup>;Zp3-Cre females crossed with *Lkb1*<sup>+/-</sup> males (maternal or maternal-zygotic) and the zygotic allele from *Lkb1*<sup>+/-</sup> crosses (zygotic). *Pdgfra*<sup>tm11(EGFP)Sor</sup> (Hamilton et al., 2003), Tg(HIST1H2BB/EGFP)1Pa/J (Hadjantonakis and Papaioannou, 2004), *Gt(ROSA)26Sor*<sup>tm14(CAG-tdTomato)Hze/J</sup> (Madisen et al., 2010) and *Cdh1*<sup>tm2Kem</sup> lines (Boussadia et al., 2002) were obtained from The Jackson Laboratory (Bar Harbor, ME) and crossed with *Lkb1*<sup>+/-</sup> males. Cre-deleted alleles of *tdTomato* and *Cdh1* were generated by crossing with the Zp3-Cre line.

### Embryo collection and culture

Oocytes and embryos were collected from oviducts/uteri by flushing with FHM medium (EMD Millipore, MA) or from decidua by dissection in DPBS. Embryos were fixed immediately or cultured in microdrops (15 µl/drop) of KSOM+AA medium (EMD Millipore) covered with mineral oil in a 37°C, 5% CO<sub>2</sub> incubator or cultured similarly in 35-mm glass-bottomed dishes (MatTek Corp., MA) for imaging. Imaged E3.5 embryos were treated with acid Tyrode's solution to remove the zona pellucidae. Freshly flushed embryos for immunostaining often had intact zona pellucidae.

### Whole-mount immunostaining

Pre- or peri-implantation embryos (with the zona pellucida intact where indicated) were fixed in 4% formaldehyde (Polyscience Inc., PA) in 1× PBS for 45 min at room temperature, and post-implantation embryos in 2% formaldehyde for 20 min. All embryos were permeabilized in 0.3% Triton X-100-PBS, washed in 0.1% Tween 20-PBS (PBT) and incubated in blocking solution (PBT with 10% FBS) for 1 h. For λ protein phosphatase treatment (New England Biolabs, MA), embryos were incubated after Triton X-100 treatment in 1× λ protein phosphatase buffer and 1× MnCl<sub>2</sub> for 30 min at 30°C before blocking. Embryos were then incubated with primary antibodies overnight in block at 4°C, washed in PBT and incubated with secondary antibodies at room temperature for 1 h in PBT. For P-AMPK1/2 staining, primary antibody was alternatively incubated with 6× volume of P-AMPKα1/2 Thr172 peptide (Santa Cruz

Biotechnology, TX) in block for 30 min at room temperature prior to staining. Embryos were mounted with POPO-1 (Molecular Probes-Life Technologies, ON, Canada), 1:1000 with 100 µg/ml RNase A in PBT. F-actin was visualized with phalloidin (Molecular Probes-Life Technologies). Primary antibodies used were against: LKB1 (Cell Signaling Technology, MA), α-tubulin (Developmental Studies Hybridoma Bank, IA), p-AMPK1/2 (Cell Signaling Technology, MA; and Epitomics, CA), α-E-catenin (Cell Signaling Technology), p120-catenin (BD Transduction Laboratories, CA), uvomorulin (E-cadherin, Sigma, MO), β-catenin (Cell Signaling Technology), PARD6A (Abcam, Cambridge, UK), PARD6B (Santa Cruz Biotechnology), aPKCz (Santa Cruz Biotechnology), ZO-1 (Invitrogen-Life Technologies, ON, Canada), Nanog (Cosmobio, Tokyo, Japan), GATA6 (R&D Systems, MN), SOX17 (R&D Systems), CDX2 (Biogenex, Fremont, CA), FoxA2 (Cell Signaling Technology), TROMA1 (DSHB), ezrin (Cell Signaling Technology), P-ERM (Cell Signaling Technology), laminin (DSHB) and cleaved caspase-3 (Cell Signaling Technology). Secondary antibodies were from Molecular Probes-Life Technologies (Alexa Fluor) or Jackson ImmunoResearch (West Grove, PA) (Dylight).

### Whole-mount *in situ* hybridization

Embryos were fixed overnight in 4% formaldehyde (Polyscience Inc.) in 1× PBS, and *in situ* hybridization was performed as per the Janet Rossant laboratory protocol: [http://www.sickkids.ca/research/rossant/protocols/conlon/wm1\\_conlon.pdf](http://www.sickkids.ca/research/rossant/protocols/conlon/wm1_conlon.pdf). The following probes were a kind gift from Pantelis Georgiadis (University of Cyprus): *Pace4*, *Bmp4*, *Elf5*, *Cripto*, *Eomes*, *Esrrb*, *Bmp4*.

### Time-lapse microscopy and image analysis

A Zeiss Axio observer, 10× (Zeiss Fluor NA 0.5) or 20× (Zeiss Plan Apo NA 0.8) objective, Zeiss AxioCam MRm CCD camera and an environmental chamber were used for DIC and GFP epifluorescence time-lapse imaging or phase-contrast imaging of fixed outgrowths. A Quorum spinning-disc confocal microscope, Leica 20× PlanAPO objective (McGill CIAN imaging facility) was used for time-lapse imaging or imaging fixed samples. An IncucyteFLR (Essen BioScience) in a cell-culture incubator was used for phase-contrast time-lapse images of outgrowths and TSC lines. For embryo time-lapse, images were taken every 10 min in 10–20-µm slices, using Axiovision or Velocity software. For outgrowths, a single image was taken every 3–6 h. Whole-mount post-implantation embryos were imaged on a Zeiss Lumar.v12 with ApoLumar S1.2X objective and epifluorescence.

### Outgrowths and TSC lines

Blastocysts without zona pellucidae were plated on gelatin-coated 12-well dishes and cultured for 7 days in DMEM (Wisent) or RPMI (Wisent, for TSC line conditions) +20% ES-FBS (Wisent) and occasionally imaged using an Incucyte. Where described, 25 ng/ml FGF4 (R&D, 235-F4-025) in PBS/0.1% BSA and 1 µg/ml heparin (Sigma, H3149), 5 ng/ml activin A (R&D, 338-AC-010) and/or 70% MEF-conditioned RPMI medium was added. MEF-conditioned medium was derived from mitomycin-C-treated MEFs harvested as described previously (Behringer, 2014). A subset of outgrowths were phase-contrast imaged every 3 h using an IncuCyte FLR (Essen Bioscience). For TSC lines, blastocysts from pure CD1 backgrounds without zona pellucidae were plated on gelatin-coated four-well dishes in RPMI+70% MEF-conditioned medium+20% ES-FBS+25 ng/ml FGF4 and 1 µg/ml heparin. Outgrowths were disaggregated by trypsinization and pipetting at +4 days, and the lines were passaged at 50–70% confluency. Medium was replaced every third day or the day before passage. Genotyping was done from supernatants or harvested cells after trypsinization. For MEF-supported CD1 wild-type TSC lines, MEFs were seeded as feeders prior to TSC passaging (Behringer, 2014).

### Aggregate chimeras

Zona pellucidae were removed from donors and hosts using acid Tyrode's solution. H2B-EGFP-expressing or wild-type maternal or maternal-zygotic mutant 8–16 cell embryos were cultured for 15 min in

Ca<sup>2+</sup>-free KSOM (Millipore) and dissociated into small groups with microneedles and pipetting. The isolated groups were transferred to indented microdrop 35-mm plates with Rosa26-TdTomato 8–16 cell hosts and cultured to the blastocyst stage or used for genotyping.

### Embryo genotyping

Immunostained/scanned embryos were recovered individually and genotyped by PCR. Fluorescent-line embryos were genotyped by fluorescence. Using Sigma Extract-N-Amp PCR kit, embryos was transferred to 5 µl of E+TP mixture solution by mouth pipette, incubated for 10 min at room temperature and treated at 56°C for 30 min, 24°C for 5 min and 95°C for 5 min, then neutralized with 5 µl of N solution for PCR. The *Lkb1* primers used were (Bardeesy et al., 2002): *Lkb1\_36*, 5'-GGGCTTCCACCTGGTGCCAGCCTGT-3'; *Lkb1\_39*, 5'-GAGATGGGTACCAGGAGTTGGGGCT-3'; *Lkb1\_37*, 5'-GATGGAG-GACCTTTGGCCGGTCA-3'. *Cdh1* primers were as described previously (Boussadia et al., 2002).

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

D.K. and Y.Y. conceived of and designed experiments. D.K. performed most of the experiments and analyzed the data. S.A. performed the oocyte staining. N.H.-Y. maintained mouse colonies and performed some culture experiments and genotyping. S.P. performed part of the outgrowth assay. M.S. performed the initial preliminary study. D.K. and Y.Y. wrote and edited the manuscript.

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### Supplementary material

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