PINCH1 regulates cell-matrix and cell-cell adhesions, cell polarity and cell survival during the peri-implantation stage

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Summary

PINCH1 is composed of 5 LIM domains, binds integrin-linked kinase (ILK) and locates to integrin-mediated adhesion sites. In order to investigate PINCH1 function we generated mice and embryonic stem (ES) cell-derived embryoid bodies (EBs) lacking the *PINCH1* gene. Similar to mice lacking β1 integrin or *Ilk*, loss of *PINCH1* arrested development at the peri-implantation stage. In contrast to β1 integrin or *Ilk* mutants, however, disruption of the *PINCH1* gene produced implantation chambers with visible cell clumps even at embryonic day 9.5. In order to define the phenotype leading to the peri-implantation lethality we made *PINCH1*-null EBs and found similar but also additional defects not observed in β1 integrin or *Ilk* mutant EBs. The similarities included abnormal epiblast polarity, impaired cavitation and detachment of endoderm and epiblast from basement membranes. Additional defects, which were not observed in β1 integrin- or ILK-deficient mice or EBs, included abnormal cell-cell adhesion of endoderm and epiblast as well as the presence of apoptotic cells in the endodermal cell layer. Although ILK and PINCH1 were shown to be involved in the phosphorylation of serine-473 of PKB/Akt, immunostaining with specific antibodies revealed no apparent alteration of PKB/Akt phosphorylation in PINCH1-deficient EBs. Altogether these data demonstrate an important role of PINCH1 for integrin function, actin organization, cell-cell adhesion and endodermal cell survival during the implanting of mouse embryos.

Supplementary material available online at
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Key words: PINCH (LIMS1), ILK, Integrin, Adhesion, Implantation, PKB/Akt

Introduction

Adhesion between cells and adhesion of cells to the extracellular matrix (ECM) are fundamental for the development of multicellular organisms. Cell adhesion receptor proteins that mediate such interactions include cadherins and integrins. Integrins are essential for cell migration but are also involved in the induction of cell polarity and differentiation, and in the regulation of cell proliferation and programmed cell death. Integrins are heterodimeric transmembrane glycoproteins consisting of a non-covalently bound α and β subunit. The interaction of integrins with their ligands at the extracellular side leads to the connection of the integrin cytoplasmic tails with the actin cytoskeleton, to the clustering of integrins into focal adhesion sites (FAs), and finally to the activation of various intracellular signalling pathways (Brakebusch and Fässler, 2003). Since the integrin cytoplasmic domains lack both an actin binding domain as well as enzymatic activity, all of these effects are mediated by integrin-associated molecules.

PINCH1 and 2 (Zhang et al., 2002a; Braun et al., 2003) are proteins that localise to FAs where they interact with the integrin-linked kinase (ILK), which in turn binds the cytoplasmic tails of β1 and β3 integrins (reviewed by Wu, 1999; Grashoff et al., 2004). The PINCH proteins are highly similar, encoded by different genes and composed of five tandemly arranged LIM domains followed by putative nuclear localisation/export signals (Braun et al., 2003; Zhang et al., 2002a). LIM domains are double zinc-binding motifs that facilitate interactions with other proteins or nucleic acid (Kadrmas and Beckerle, 2004). Consistent with such binding properties, the first LIM domain of PINCH has been shown to bind ILK and the fourth LIM domain the SH2/SH3 adaptor protein Nck2 (or Grb4), which in turn can associate with several receptor tyrosine kinases and thereby links integrins to growth factor signalling (Tu et al., 1998). Furthermore, mammalian PINCH1 and 2 and *C. elegans* PINCH have been shown to be present in the nucleus of a variety of cell types (Hobert et al., 1999; Zhang et al., 2002a; Campana et al., 2003). Whether nuclear PINCH1 and 2 binds nuclear proteins and/or DNA, however, is not known.

Loss-of-function studies in *C. elegans* and *D. melanogaster* and cell biological studies with mammalian cells, in which

either the interaction of PINCH1 with ILK was disrupted by overexpressing dominant-negative forms of PINCH1 or PINCH1 levels were depleted by RNA interference provided several compelling lines of evidence that PINCH1 is essential for integrin functions and actin organization (Zhang et al., 2002b; Fukuda et al., 2003). Loss of PINCH expression in flies and worms revealed a crucial role of PINCH in integrin-mediated cell adhesion and linkage of actin to integrin adhesion sites (Clark et al., 2003; Hobert et al., 1999). Cell biological studies showed that PINCH1 promotes cell adhesion, spreading, motility, survival and ECM assembly (Wu, 1999). These studies also demonstrated that the interaction of PINCH with ILK is necessary to target both proteins to FAs and to prevent degradation of the PINCH-ILK-parvin complex (Zhang et al., 2002c; Fukuda et al., 2003).

Several biochemical and cell biological studies uncovered a prominent role for ILK in promoting cell survival. The proposed molecular mechanism involves ILK recruitment into FAs and phosphorylation of Ser473 of PKB/Akt (Delcommenne et al., 1998). Phosphorylation of Ser473 by ILK and of Thr308 by 3-phosphoinositide-dependent kinase 1 (PDK1) leads to full activation of PKB/Akt and inhibition of programmed cell death. In agreement with the ability of PINCH1 to stabilise and to target ILK to FAs, depletion of PINCH1 in HeLa cells leads to diminished phosphorylation of Ser473 of PKB/Akt (Fukuda et al., 2003). Interestingly, however, depletion of PINCH1 also reduced Thr308 phosphorylation and induced apoptosis even in cells overexpressing a constitutive active form of PKB/Akt (Fukuda et al., 2003). This indicates that PINCH1 is able to modulate PKB/Akt activity in an ILK-dependent and an ILK-independent manner and furthermore, that PINCH1 functions in the survival pathway both upstream of PKB/Akt as well as downstream or alternatively, independent of PKB/Akt.

In order to directly test the physiological function of PINCH1 in vivo we generated mice and embryoid bodies (EBs) carrying a disrupted *PINCH1* gene. We report here that mice lacking PINCH1 expression arrest their development at the peri-implantation stage. Absence of PINCH1 in EBs led to abnormal epiblast polarity, impaired cavitation and detachment of primitive endoderm from basement membranes (BMs). These defects were also observed in β1 integin- and ILK-deficient EBs, although they were more pronounced. Additional PINCH1-specific defects included diminished survival of primitive endoderm and abnormal cell-cell interactions of endoderm and epiblast.

Materials and Methods

Generation of PINCH1-deficient mice and ES cells

A 650 bp fragment from a *PINCH1* EST clone (GenBank accession number AI 195455) was used to screen a PAC library. Several clones were identified and used to generate the PINCH1 constructs. To delete the *PINCH1* gene in mice we generated a conventional targeting construct by replacing part of exon 3, the entire exon 4 and a large part of intron 5 with a neomycin cassette (neo knockout allele; termed $PINCH1^{+/neo}$) and electroporated it into ES cells. Out of 360 ES clones isolated, 13 underwent homologous recombination and five of them were injected into host blastocysts to generate germline chimeric mice. Second, we also constructed a conditional *PINCH1* null allele (floxed allele), in which exon 4 was flanked by *loxP* sites and a neo-tk cassette. Upon electroporation and G418 selection 360 surviving ES cell clones were isolated and eight of them identified as targeted clones. Three of them were used to remove the neo-tk cassette by transient expression of the Cre recombinase, which gave rise to ES cell clones either lacking the neo-tk cassette (termed $PINCH1^{+/fl}$) or ES cell clones lacking the neo-tk cassette as well as exon 4 (termed $PINCH1^{+/-}$). Three $PINCH1^{+/-}$ ES cell clones were used to generate mice. Insertion of the neo cassette into exon 3 disrupts the *PINCH1* gene and Cre-mediated deletion of exon 4 gives rise to splicing of exon 3 into exon 5, which leads to a reading frame shift and if at all, to a short, non-functional N-terminal PINCH1 peptide. The phenotype of $PINCH1^{neo/neo}$ mice and $PINCH1^{-/-}$ mice was identical and therefore, both mutations were designated as *PINCH1*-null or *PINCH1*-deficient.

To obtain $PINCH1^{-/-}$ ES cells, two PINCH1 ES cell clones lacking exon 4 and the neo-tk cassette ($PINCH1^{+/-}$) were re-electroporated again with the floxed PINCH1 construct. ES cell clones with a recombination event on the wild-type allele were again Cre-treated to obtain ES cells with a homozygous deletion of $PINCH1^{-/-}$. They were identified by Southern blot assay and by PCR genotyping. The following primers were used for the PCR genotyping: 5′-CTA-GGCTGGTAATGCAGGCC-3′ and 5′-CCTGCCAATGATGAATT-CAC-3′ to determine the wild-type or floxed allele; 5′-CTAG-GCTGGTAATGCAGGCC-3′ and 5′-CGCAGTTGGCACAGTTGA-AG-3′ to determine the constitutive null allele.

Antibodies

The following antibodies were used for the analyses: mouse monoclonal antibodies against ILK (clone 3, Transduction lab; clone 65.1.9, Upstate Biotech); rat mAb against E-cadherin (Zymed); rat mAb against ZO-1 (Chemicon); mouse mAb against GM130 (BD Biosciences Pharmingen); mouse mAb against Ki67 (BD Biosciences Pharmingen); rabbit antibody against cleaved (activated) caspase-3 (Cell Signalling Technology); rabbit antibody against integrin β1 (Fässler and Meyer, 1995); rabbit antibodies against PKB/Akt and phospho-PKB/Akt (Thr308, Ser473) (Cell Signalling Technology); rat mAb against α-tubulin. Secondary Abs were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA), Molecular Probes (Eugene, OR, USA) and BioRad (Hercules, CA, USA).

To generate PINCH1- and PINCH2-specific antibodies, peptides derived from the C-termini of PINCH1 (CLKKLSETLGRK) or PINCH2 (CAQPKSVDVNSL), respectively, were synthesized and conjugated to keyhole limpet haemocyanin (KLH) using a commercial kit (Imject Maleimide Activated mcKLH Kit, Pierce Biotechnology Inc., Rockford, IL, USA). Rabbits were immunized with 400 μg of conjugate four times at 2-week intervals and bled 1 week after the last injection. The antiserum was affinity-purified using a commercial kit (SulfoLink Kit, Pierce Biotechnology Inc.); high affinity antibodies were eluted from the column using 100 mM glycine buffer, pH 2.7 and then dialyzed against phosphate-buffered saline (PBS).

To test the two affinity-purified polyclonal antibody preparations, lysates were prepared from mouse bladder and western blots were carried out. The PINCH1 antibodies recognized a single band of around 35 kDa and PINCH2 antibodies a single band around 36 kDa (see Fig. S1A in supplementary material). To exclude cross-reaction GST-tagged PINCH1 and 2 were expressed in bacteria, blotted onto membranes and cross-hybridized with both antibodies. PINCH1 antibodies recognized PINCH1 but not PINCH2 and vice versa (see Fig. S1B in supplementary material).

Apoptotic cells in implantation chambers or EBs were detected using either the In Situ Cell Death Detection kit (Roche Diagnostics) or the cleaved caspase-3 immunostaining.

Generation of embryoid bodies

Wild-type R1 cells and *PINCH1*-null ES cells were cultured as described previously (Li et al., 2002). To evaluate epiblast

differentiation, ES cell aggregates were grown in suspension culture for 9 days as previously described (Li et al., 2002).

EB lysis and immunoblotting

Normal, *PINCH1*-null and *Ilk*-null EBs were grown for 7 days in suspension, harvested, lysed and immunoblotted as described previously (Li et al., 2002). The experiments were carried out with three different samples, the bands were measured densitometrically and the protein loading was controlled by immunostaining for tubulin.

Northern blot assay

Total RNA isolation, gel separation, blotting and hybridization were performed as previously described (Fässler et al., 1995). A PINCH1 cDNA probe (Braun et al., 2003) was random primed using standard protocols.

Histological analysis, immunostaining, electron microscopy

Immunohistochemistry of embryos or tissues and immunofluorescence studies of EBs were performed as described previously (Sakai et al., 2003). For further analysis of EB morphology, EBs were fixed in glutaraldehyde, embedded in Epon, and prepared as thick and thin sections, the former used for light microscopy and the latter for electron microscopy (described by Li et al., 2002).

Results

Deletion of the *PINCH1* gene leads to peri-implantation lethality

We used two independent strategies to disrupt the PINCH1 gene in mice. We made a conventional targeting construct by replacing part of exon 3, the entire exon 4 and a large part of intron 5 with a neomycin cassette (+/neo) and a conditional *PINCH1* null allele, in which exon 4 was flanked by *loxP* sites and a neo-tk cassette (Fig. 1A). The neo-tk cassette was removed by transiently expressing Cre recombinase, giving rise to ES cell clones either lacking the neo-tk cassette (+/fl) or lacking the neo-tk cassette as well as exon 4 (+/–) (Fig. 1A). The *PINCH1*$^{+/neo}$ and *PINCH*$^{+/-}$ ES cells were used to generate mice. Both null mutations produced the same phenotype and are therefore, not further distinguished.

Mice with a heterozygous *PINCH1*-null mutation appeared normal. Among 391 viable, 3-week-old offspring from heterozygous intercrosses, 144 were of wild-type and 247 of heterozygous genotypes. No homozygous mutant mice were among the progeny (Fig. 1B) indicating that loss of *PINCH1* expression results in embryonic lethality.

To determine when loss of *PINCH1* expression arrests development, embryos derived from heterozygous intercrosses were dissected at different developmental stages, genotyped and analysed. During midgestation (E10.5-13.5) we analysed 45 implantation chambers; 15 of them were empty and the remaining 30 contained either wild-type or heterozygous embryos. At E7.5 we analysed 78 embryo chambers, of which seven contained cell remnants that were PCR genotyped homozygous for the *PINCH1*-null allele (Fig. 1C,D) and 15 contained so few cells that it was not possible to genotype them. At E6.5 we sectioned 30 embryo chambers and found 21 normally developed egg cylinders (putative wild-type or heterozygous) and nine embryos with various degrees of

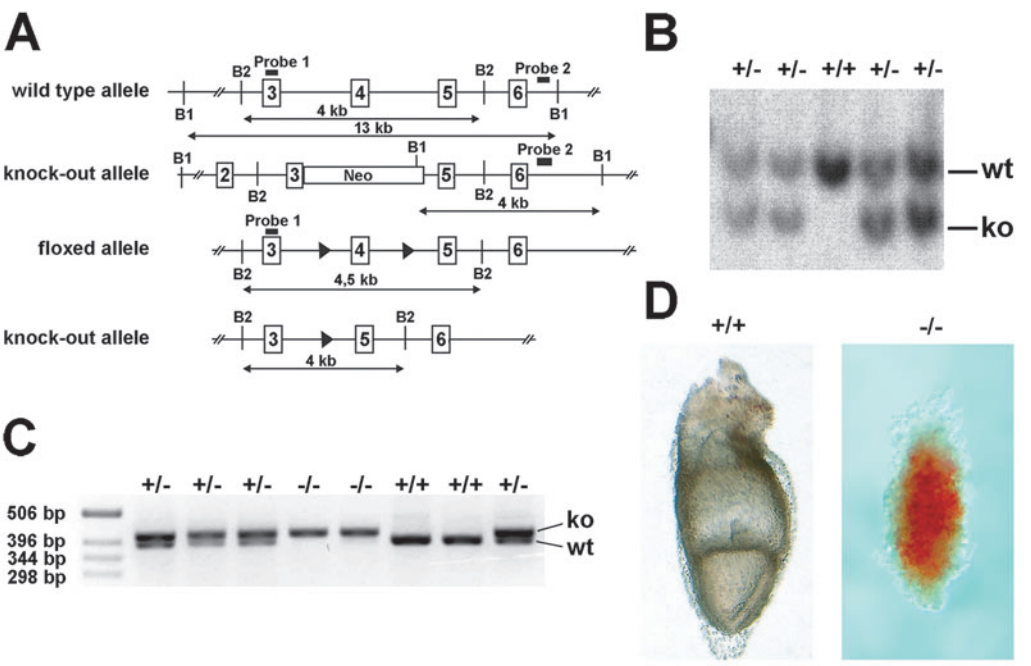

Fig. 1. Strategy of *PINCH1* gene disruption and embryos from *PINCH1* heterozygous intercrosses. (A) Partial map of the wild-type *PINCH1* allele, *PINCH*1neo, *PINCH1*-floxed allele (*PINCH1*fl), and the *PINCH1*-null allele after Cre-mediated recombination. Exons and *loxP* sequences are indicated as rectangles and triangles, respectively. The DNA fragment length obtained after Southern blotting and the internal (probe 1) and external (probe 2) probes are indicated. Restriction sites are: B1, *Bam*HI; B2, *Bgl*II. (B) Southern blot of *PINCH1*neo tail DNA digestion with *Bam*HI and hybridization with probe 2. The sizes of the wild-type (13 kb) and targeted (4 kb) bands are indicated. (C) PCR genotyping of E7.5 embryos derived from heterozygous intercrosses. (D) Whole-mount morphology of E7.5 wild-type and remnants of *PINCH1*-null embryos.

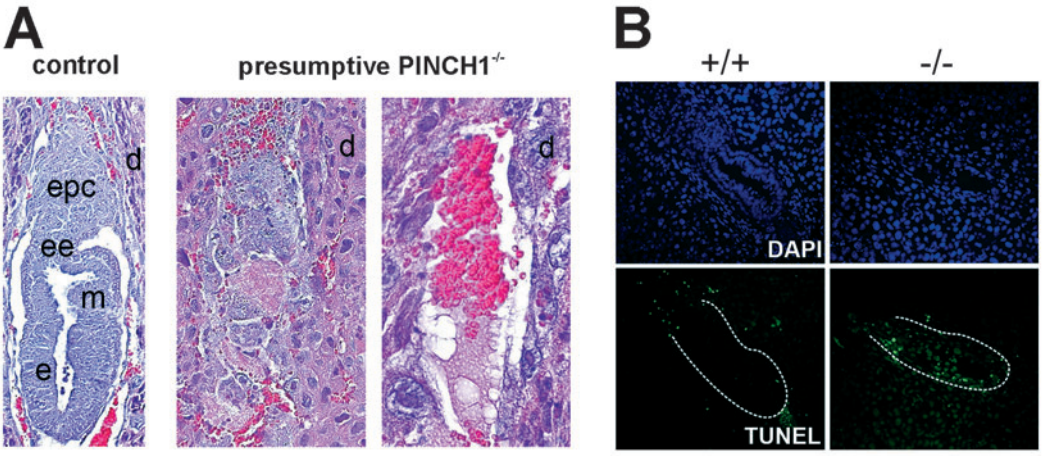

Fig. 2. Peri-implantation lethality in the absence of PINCH1 expression. (A) Haematoxylin and Eosin staining of sections from E6.5 implantation chambers from heterozygous intercrosses shows that the presumptive mutant chambers contained either degenerated embryos (middle image) or blood cells (right image). d, decidua; epc, ectoplacental cone; ee, extraembryonic ectoderm; e, ectoderm; m, mesoderm. (B) TUNEL staining on sections from E6.5 decidual chambers shows multiple apoptotic cells in the remnants of the *PINCH1*-null embryo proper (marked region). Control embryos were TUNEL negative. The embryos were identified on consecutive sections stained with DAPI.

degeneration (putative *PINCH1*-null) ranging from complete absence of embryos to egg cylinders infiltrated by maternal blood cells (Fig. 2A). These data together with the expected number of normal appearing *PINCH1*-null E3.5 blastocysts (data not shown) indicate that loss of *PINCH1* expression allows normal pre-implantation development, subsequent implantation into the uterus and initiation of the decidua reaction leading to the formation of a normal number of embryo chambers. *PINCH1*-null embryos, however, deteriorate rapidly after implantation. In order to test whether mutant embryos underwent apoptosis we performed TUNEL assays on sections derived from E5.5 and E6.5 embryos. While normal embryos showed no TUNEL-positive signals, sections of putative *PINCH1*-null embryos exhibited many TUNEL-positive signals (Fig. 2B).

Endoderm detachment and partial inversion of F-actin distribution in *PINCH1*-null EBs

To obtain *PINCH1*$^{-/-}$ ES cells we re-electroporated two *PINCH1*$^{+/-}$ ES cell clones with the floxed *PINCH1* construct. ES cell clones with a recombination event on the wild-type allele were treated with Cre to delete the *PINCH1* gene (Fig. 1A). Homozygous inactivation of the *PINCH1* gene was confirmed by Southern and western blotting (data not shown).

To investigate the mechanism underlying the PINCH1 phenotype, we generated EBs and monitored formation of the endoderm, the embryonic BM, the epiblast and a proamnionic-like cavity by light microscopy of live and sectioned EBs for up to 12 days. In wild-type EBs, the endoderm started to form in 2 days as a single layer of tightly associated squamous cells (primitive endoderm), which gradually became cuboidal in morphology (visceral endoderm). The epiblast developed as a second layer of elongated, pseudostratified epithelial cells in 5-7 days and the remaining ICM cells underwent apoptosis and created a sharply defined central cavity (Fig. 3A and Fig. 7A). By contrast, the endoderm formed 1 day later in *PINCH1*-null EBs and often developed into a loosely associated, multilayer of cells that sometimes detached from the BM (Fig. 3B,F, Fig. 4B and Fig. 7C). The *PINCH1*-null epiblast was not polarized and consisted of multiple layers of polygonal cells that failed to elongate. In addition, the cavitation was some times incomplete (Fig. 4B). After 9 days in culture, most *PINCH1*-null EBs began to degenerate by an apoptotic mechanism, while no significant deterioration occurred in the normal EBs cultured for 12 days (data not shown).

To elucidate the spatial relationship between PINCH1, integrins and BM, EBs were immunostained with different antibodies. In wild-type EBs, PINCH1 and β1 integrin were expressed in endoderm and epiblast and concentrated at the BM zone co-localizing with laminin γ1 (Fig. 3C,E). PINCH2 was not expressed in either normal or *PINCH1*-null EBs (data not shown). Immunostaining of laminin γ1 and other BM components revealed a linear deposition beneath the *PINCH1*-null endoderm indicating that PINCH1 is not required for BM expression and assembly (Fig. 3D and data not shown). Expression and localization of β1 integrin was visible at the BM zone of normal endoderm and epiblast (Fig. 3E). By sharp contrast, β1 integrin localized to the subcortical area of *PINCH1*-null endoderm and epiblast (Fig. 3F) indicating that PINCH1 is required for β1 integrin recruitment to cell-matrix adhesion sites.

Rhodamine-phalloidin staining of wild-type EBs showed the actin cytoskeleton in the apical belt and along the lateral plasma membranes (Fig. 3G). In *PINCH1*-null EBs, the apical actin belt assembled, although it was less prominent. Interestingly, actin aggregates were visible adjacent to the basal plasma membranes (arrowheads in Fig. 3H) and parallel actin fibres were absent along the apicobasal axis.

Abnormal polarity of epiblast and abnormal localization of endodermal tight junctions

To further characterize the epiblast polarity defects, EBs were immunostained with different antibodies. GM130 staining showed that the Golgi apparatus of normal epiblast

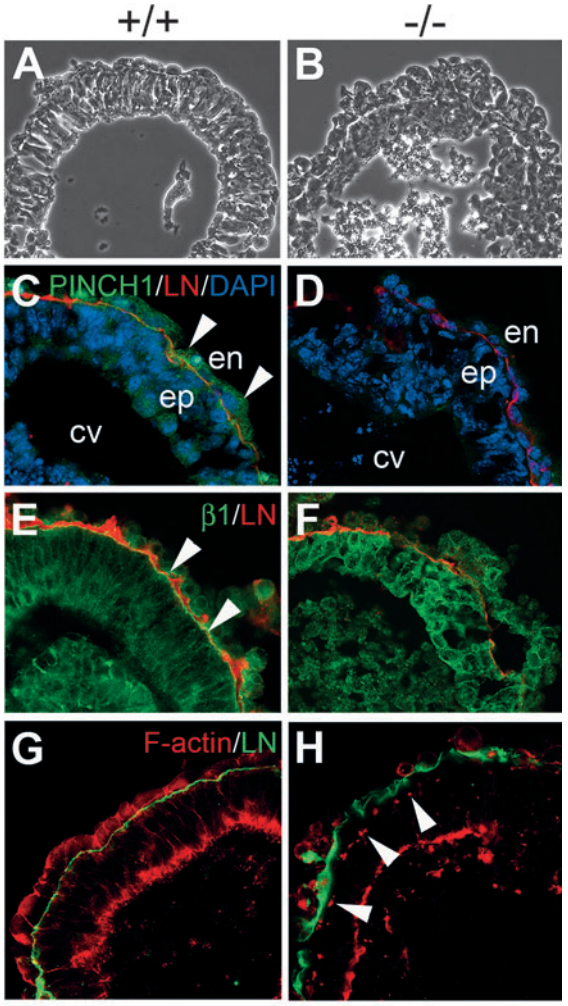

Fig. 3. Endodermal detachment and disorganized F-actin in *PINCH1*-null EBs. (A,B) Phase-contrast images of wild-type EB (A) showing the epiblast layer consisting of pseudostratified columnar epithelial cells, and *PINCH1*-null EB (B) showing a multi-layered and polygonal epiblast cell layer sometimes associated with incomplete cavitation. (C,D) Immunofluorescence of PINCH1 (green) and laminin γ1 (red) revealed PINCH1 expression at the basement membrane zone of normal endoderm and epiblast and in the nucleus of some endodermal cells (C). *PINCH1*-null EBs lack PINCH1 signals but show a linear laminin γ1 staining (D). Nuclei are counterstained with DAPI (blue). (E,F) β1 integrin (green) is condensed in the basal plasma membranes of normal endoderm and epiblast where it colocalizes with laminin γ1 (red; arrowheads; E). Signals for β1 integrin are similarly strong in *PINCH1*-null EBs (F). (G,H) F-actin (red) forms an apical belt and bundles parallel to lateral plasma membranes in the normal, polarized epiblast (G). *PINCH1*-null epiblast contains actin aggregates at the basal plasma membranes and lacks the parallel actin bundles (H).

repositioned from the perinuclear region to the apical junctional region with radial orientation (Fig. 4A). While most *PINCH1*-null epiblast cells retained their Golgi apparatus in the perinuclear region, some showed apical distribution in a nonlinear fashion (Fig. 4B). E-cadherin was enriched at the apices of normal epiblast (Fig. 4C). Although the apical accumulation of E-cadherin was less evident in *PINCH*-null

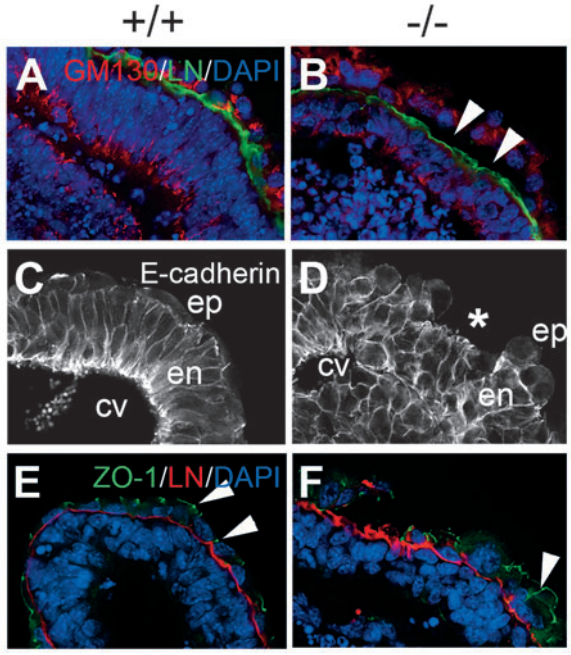

Fig. 4. Abnormal epiblast polarity and disruption of endodermal tight junctions in *PINCH1*-null EBs. (A,B) In normal EBs the cis-Golgi matrix protein GM130 (red) is localized to the apical region of the epiblast and to the basolateral region of the endoderm. GM130 is also observed in the apical side of the remaining ICM cells facing the cavity. In *PINCH1*-null EBs, GM130 is peri-nuclear in the endoderm, and sometimes apical in the epiblast although in a non-linear fashion (B). Arrowheads showing a large segment of endoderm detached from the basement membrane. (C,D) E-cadherin is localized to the lateral plasma membrane and concentrated at the apex of the epiblast (C). *PINCH1*-null EBs show reduced apical E-cadherin signals in the epiblast and often miss segments of endodermal cells (asterisk; D). (E,F) ZO-1 is found in the apex of the cell-cell junctions of normal endoderm (E, arrowheads). In *PINCH1*-null endoderm, ZO-1 is found at the basal or entire apical and lateral plasma membranes (F, arrowhead).

EBs (Fig. 4D), electron microscopy revealed that adherens junctions formed between the cells facing the incomplete cavities (data not shown). The tight junction (TJ) marker ZO-1 was expressed exclusively by endodermal cells and redistributed from the lateral plasma membranes to the apices to form a punctate pattern that correlated to the ultrastructural TJs (Fig. 4E and Fig. 7G). In *PINCH1*-null endoderm, ZO-1 often remained in the lateral and sometimes even in the basal plasma membranes (Fig. 4F and Fig. 7H), suggesting a defect in TJ maturation and/or localization.

Apoptosis in the endodermal layer of *PINCH1*-null EBs

Since PINCH1 can modulate proliferation and survival of cells in vitro we investigated these processes during EB development. Ki67 staining revealed similar numbers of proliferating cells in wild-type and *PINCH1*-null EBs (Fig. 5A,B). Apoptotic cells were detected in the centre of normal EBs where the cavity formed (Fig. 5C), and occasionally in the epiblast but never in the endoderm. By contrast, apoptosis was prominent in the *PINCH1*-null endodermal cells (Fig. 5D).

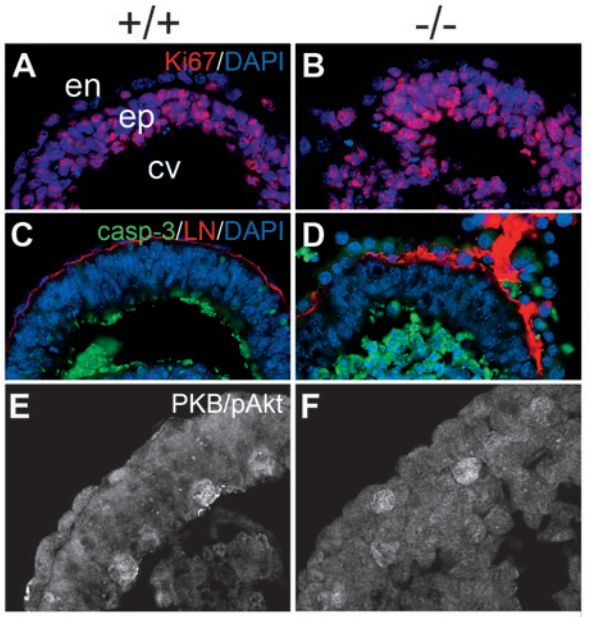

Fig. 5. Apoptosis in *PINCH1*-null EBs. (A,B) Cell proliferation assessed by Ki67 staining (red) is seen in most epiblast and some endodermal cells from both wild-type and *PINCH1*-null EBs. (C,D) Immunostaining for cleaved caspase-3 (green) reveals apoptotic cells in the central portion of normal EBs leading to cavitation. Immunosignals were occasionally detected in the epiblast but rarely in the endoderm (C). *PINCH1*-null EBs have apoptotic endodermal cells with condensed nuclei (D). (E,F) Immunostaining for Ser473 phosphorylated form of PKB/Akt shows singly positive cells in the epiblast and endoderm layer both in sections derived from normal (E) and *PINCH1*-null EBs (F).

Immunostaining for phosphorylated Ser473 of PKB/Akt was visible throughout the cytoplasm of only a few cells of the endoderm or epiblast both in wild-type and *PINCH1*-null EBs (Fig. 5E,F). Also the number of PKB/Akt-positive cells was similar between normal and mutant EBs.

It has been reported that the stability of ILK and PINCH is interdependent (Fukuda et al., 2003). To test whether this is also the case in EBs, we performed western blot analysis with lysates obtained from *PINCH1*- and *Ilk*-null EBs. We found that ILK is threefold reduced but not absent in *PINCH1*-null EBs and vice versa, PINCH1 is threefold reduced but not absent in *Ilk*-null EBs (Fig. 6A). To test whether the remaining ILK is the result of the expression of an N-terminal PINCH1 peptide (from exon 1 to exon 3), which can be translated after Cre-mediated deletion of exon 4 of the *PINCH1* gene, we isolated total RNA and performed a northern blot assay. Fig. 6B shows that deletion of exon 4 led to a decay of the mutant mRNA indicating that *PINCH1* mutant EBs express little or no N-terminal PINCH1 peptide and that the reduced ILK expression is stabilized by a PINCH1-independent manner. In agreement with the western blot data, immunostaining confirmed reduced expression of ILK in *PINCH1*-null EBs. In wild-type EBs ILK was enriched at the BM zone of endoderm and epiblast (Fig. 6C). In *PINCH1*-null EBs ILK expression levels were reduced but not absent. Furthermore, ILK localized to the subcortical region of endodermal cells and epiblast-like cells (Fig. 6C). The specificity of the anti-ILK antibody was evaluated by staining *Ilk*-null EBs, which revealed no immunosignal (Fig. 6D).

Ultrastructural analyses revealed severe cell-cell and cell-BM adhesion defects

During the development of normal EBs the primitive endoderm appears as a single layer of tightly associated squamous cells, followed by the differentiation of the elongated pseudostratified epiblast cells and the apoptosis of the remaining ICM cells, giving rise to a central cavity (Fig. 7A). In *PINCH1*-null EBs the endoderm formed but often detached from the BM (Fig. 7B,C), the epiblast failed to polarize and consisted of multiple layers of polygonal cells, and the cavitation was sometimes incomplete (Fig. 7C). Transmission electron microscopy of normal EBs showed that endodermal cells tightly adhered to the BM, had a cuboidal shape, numerous microvilli at their apices (Fig. 7D) and TJs symmetrically assembled at the apical contacts of two cells (Fig. 7G). The normal epiblast consisted of a single layer of pseudostratified columnar cells. By contrast, *PINCH1*-null endodermal cells were round, connected to each other by thin plasma membrane extensions, contained fewer microvilli and were often detached from the BMs (Fig. 7C,E,F). TJs were often observed in the middle or basal portion of cell-cell contacts and were occasionally distributed asymmetrically (Fig. 7H). The *PINCH1*-null epiblast had multiple layers of polygonal cells with different staining intensity in Methylene Blue-stained thick sections (Fig. 7B,C,E), suggesting heterogeneity within the cell population.

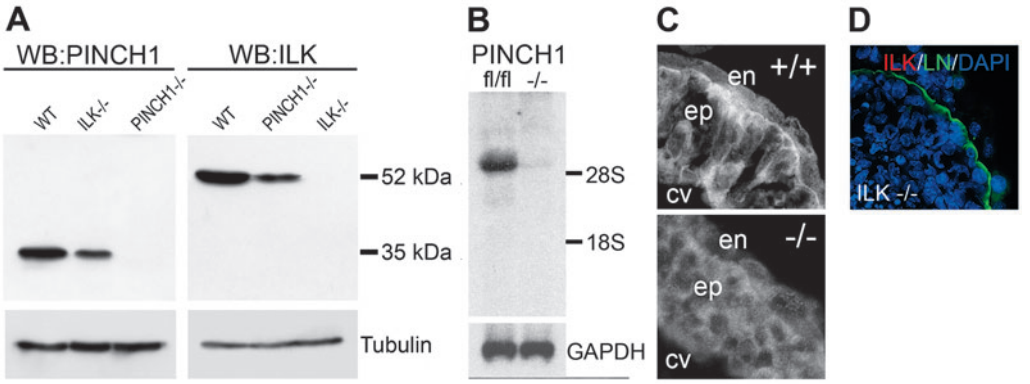

Fig. 6. ILK expression in *PINCH1*-null EBs. (A) Western blot analysis of PINCH1 and ILK in *PINCH1*-null and *Ilk*-null EBs. (B) Northern blot analysis of PINCH1 in control and *PINCH1*-null cells. (C) Immunostaining of ILK in wild-type and *PINCH1*-null EBs. (D) Immunostaining of ILK in *Ilk*-null EBs.

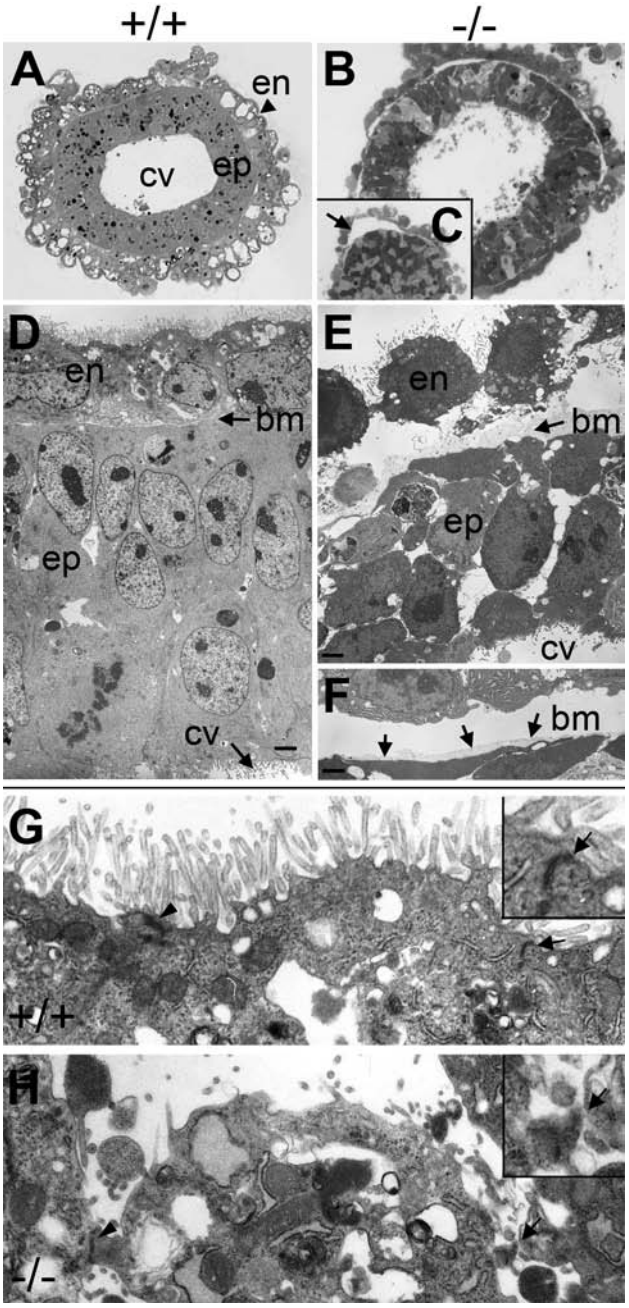

Fig. 7. Ultrastructural changes in *PINCH1*-null EBs as seen by Methylene Blue staining. (A-C) (A)Section of a typical wild-type EB with vacuole-containing endodermal cells (en), pseudo-stratified epiblast (ep) and a sharply defined central cavity (cv). (B,C) In *PINCH1*-null EBs the endoderm is frequently detached (arrow in C) and cells of the ICM and epiblast show different staining intensities (B). (D) Ultrastructural analysis of wild-type EBs revealed cuboidal endodermal cells (en), a thin basement membrane (bm) and an elongated epiblast (ep; D). (E,F) *PINCH1*-null endodermal and epiblast cells are connected through thin plasma membrane extensions. The epiblast cells are non-polarized, irregular (E) and the endoderm is often detached from the basement membrane (F). Normal endoderm has symmetric tight junctions (arrowheads in G; the inset shows 2× magnification of the tight junction marked by an arrow). (H) In *PINCH1*-null endoderm, tight junctions often assemble at the middle or basal portion of cell-cell contacts. Sometimes asymmetric tight junctions form in *PINCH1*-null endoderm with one lateral plasma membrane of a cell not in direct contact with that of another (inset).

Discussion

Cell culture and biochemical studies demonstrated that the interaction of PINCH1 with ILK is a prerequisite to locate both proteins to integrin-mediated adhesion sites and to prevent their proteasome-mediated degradation (Zhang et al., 2002c; Fukuda et al., 2003). These findings led to the hypothesis that PINCH1 and ILK are obligate partners in controlling cell behaviour such as cell shape modulation, motility and survival (Fukuda et al., 2003) and imply that ablation of either gene in vivo would lead to very similar, if not identical, phenotypes. This assumption is consistent with findings of genetic studies in worms and flies, which revealed that loss-of-function alleles of either the *PINCH* or the *Ilk* gene cause indistinguishable defects, characterized by abnormal muscle attachment and embryonic lethality (Hobert et al., 1999; Zervas et al., 2001; Mackinnon et al., 2002; Clark et al., 2003). In order to test whether this is also true for mice we disrupted the *PINCH1* gene in the mouse germline and in ES cells, investigated the consequences in vivo and in ES cell-derived EBs, and compared the phenotypes with those reported for the *Ilk*-null mice (Sakai et al., 2003).

We generated two independent mouse strains carrying a constitutive PINCH1 null mutation and found that, similar to mice lacking the *Ilk* gene, they died shortly after implantation. Interestingly, however, the *PINCH1*-null mice proceeded further in their development than ILK-deficient or β1 integrin-deficient mice. *PINCH1*-null embryos died at around E6.5-7.5 and showed variable degrees of degeneration. In a few cases we were even able to isolate remains of *PINCH1*-deficient embryos at stages as late as E8.5 and E9.5. By contrast, *Ilk*-null or β1 integrin-null embryos are completely absorbed at around E5.5-6.5. Such a temporal difference in lethality suggests that the disruption of the *PINCH1* gene can cause overlapping but must also different defects to the disruption of the *Ilk* or the β1 integrin gene in vivo. These differences are unlikely to be caused through compensation by PINCH2; the expression of PINCH2 is not detectable before E14.5-15.5 (Braun et al., 2003) and was also absent in normal as well as PINCH1-deficient EBs.

EBs have emerged as an elegant experimental system to investigate normal or pathological peri-implantation. As with the surface of the inner cell mass (ICM) of blastocysts the outermost cells of ES cell aggregates differentiate into primitive endoderm and deposit a BM, which is required to polarize the adjacent cells into the columnar epiblast and to induce apoptosis in the remaining cells, resulting in the first cavity called the amniotic cavity. Using EBs derived from mutant ES cells we were able to show in a previous report that mice lacking β1 integrin or ILK die from different causes at peri-implantation. While β1 integrin-deficient mice fail to deposit a BM between primitive endoderm and epiblast, *Ilk*-null mice produce a BM but fail to polarize the epiblast and to cavitate (Aumailley et al., 2000; Li et al., 2002; Sakai et al., 2003).

In agreement with our in vivo analysis, *PINCH1*-null EBs developed primitive endoderm, deposited a BM and formed, in

the majority of cases, fully expanded cavities and hence clearly progressed much further than *Ilk*-null EBs. A more detailed analysis of *PINCH1*-null EBs using immunostaining and electron microscopy disclosed a few similarities with ILK-deficient EBs but strikingly, also additional defects. Similarly, though not as severely as in *Ilk*-null EBs, loss of PINCH1 expression gave rise to an abnormally polarized epiblast with aberrant arrangement of the Golgi stacks, partial inversion of F-actin deposition and diffuse distribution of E-cadherin. One possible explanation for the polarity defect could be that loss of PINCH1 is associated with loss of ILK expression. Such a phenomenon was reported for PINCH1-depleted HeLa cells and served as an explanation for the similar defects that were apparent in ILK- or PINCH1-depleted cells (Fukuda et al., 2003). Immunostaining of our *PINCH1*-null EBs revealed expression of ILK in the endoderm and epiblast. Quantitative measurements of ILK levels in whole EBs by western blotting demonstrated a reduction of ILK in *PINCH1*-null EBs and vice versa. Since PINCH2 is not expressed in EBs these data reveal that there is an interdependence of the two proteins, which is, however, limited and not complete. This notion was supported by a similar observation in *PINCH1*-null fibroblasts, which also displayed reduced, but not absent, ILK expression (R.B. and R.F., unpublished). The reduction of ILK levels in *PINCH1*-null EBs allows the drafting of several hypotheses. First, loss of ILK leads to severe defects of epiblast polarity and cavitation while the reduction of ILK in *PINCH1*-deficient EBs may account for the milder polarity defect, the ability to cavitate and also to develop further in vivo. Second, there may be functionally different pools of ILK and PINCH1 in cells; one pool of PINCH1 and ILK, in which the function and stability of both proteins depend on the interaction between one another, and additional pools of the two proteins, in which their stability and function is not interdependent.

Such ILK-independent, PINCH1-autonomous functions could be responsible for the additional abnormalities observed in PINCH1- but not in ILK-deficient EBs. The most prominent defects were severe detachment of the primitive endoderm from BMs, profound cell-cell adhesion defects affecting cells of the primitive endoderm and epiblast, and apoptosis of the primitive endoderm. A role of PINCH1 in promoting integrin-mediated adhesion was elegantly demonstrated in PINCH1-deficient worms and flies (Hobert et al., 1999; Clark et al., 2003) and therefore could also expected in EBs. However, it was unexpected that PINCH1 is required for cell-BM adhesion specifically by the primitive endoderm but not by the epiblast. Since neither cell type expresses PINCH2, the epiblast must have a different or an additional mechanism by which it anchors to the BM. The mechanism underlying this cell-type-specific defect is not known.

The presence and the severity of the cell-cell adhesion defects point to an important role of PINCH1 in initiating and/or maintaining cell-cell contacts of both epiblast and endoderm. The latter cells form tight junctions, which were often mislocalized or absent. It is not clear how PINCH1 promotes formation and localisation of cell adhesion junctions. One possibility is that PINCH1 is physically present in cell-cell adhesion sites. However, this has not been reported yet and our immunostaining of normal EBs revealed PINCH1 accumulation at the basal plasma membrane, diffuse staining throughout the cytoplasm but no significant enrichment at cell-cell adhesions. Another possibility is that PINCH1 is indirectly involved in the regulation of cell-cell adhesions. Such a role has been proposed for ILK, which can modulate E-cadherin expression through β-catenin-Lef/Tcf-dependent transcription and expression of the transcriptional repressor snail (Novak et al., 1998; Tan et al., 2001). It will be important to further investigate both possibilities using cells from *PINCH1* mutant mice that exhibit abnormal cell-cell adhesion in vivo.

Depletion of PINCH1 in HeLa cells by RNA interference induced strong apoptosis associated with reduced activation of PKB/Akt (Fukuda et al., 2003). We also observed apoptotic cells in the endoderm of *PINCH1*-null EBs as well as in *PINCH1*-null embryos. Similar to the abnormal adhesion to BMs, this defect was cell-type-specific and was not observed in the epiblast. Although cell-ECM detachment can induce anoikis in many cell types including certain epithelial and endothelial cells (Frisch and Ruoslahti, 1997) we noticed cell death in detached and also equally frequently in attached endodermal cells. This indicates that PINCH1 prevents apoptosis in a rather direct manner and not by impairing integrin-mediated adhesion. A remaining puzzle is how PINCH1 prevents death of the primitive endoderm. The strong nuclear PINCH1 staining specifically in endodermal cells suggested a possible regulation through a nuclear function of PINCH1 (Campana et al., 2003). An alternative possibility is that deletion of PINCH1 results in aberrant regulation of PKB/Akt activity. PKB/Akt is a serine/threonine kinase, which transmits extracellular signals to downstream effectors, including caspases (Lawlor and Alessi, 2001). Phosphorylation of Thr308 by PDK1 and Ser473 by DNA protein kinase (Feng et al., 2004) and ILK (Delcommenne et al., 1998) regulates the kinase activity of PKB/Akt. Depletion of ILK in HeLa cells induced apoptosis and gave rise to reduced Ser473 and Thr308 phosphorylation, suggesting that apoptosis was at least in part induced by aberrant PKB/Akt activity (Fukuda et al., 2003). We did not observe a clear reduction of PKB/Akt Ser473 phsophorylation in *PINCH1*-null endoderm. It is possible that differences escaped detection by our immunostaining approach, or alternatively that PINCH1 modulates PKB/Akt-independent survival pathways in primitive endoderm. A PKB/Akt-independent survival pathway in addition to a PKB/Akt-dependent one has been proposed to operate in HeLa cells (Fukuda et al., 2003). The nature of the PKB/Akt-independent survival pathway is not known.

In conclusion we show that the PINCH1 function is similar to ILK function, in that it is rate-limiting for peri-implantation development. Although the time point of lethality supports the prevailing view that the stability and the functions of both polypeptides are interdependent we show that PINCH1 can also operate in an ILK-independent manner to ensure endoderm survival and cell-cell adhesion, and that the stability of both proteins does not solely depend on their interactions. Finally, we found that certain PINCH1 functions such as apoptosis and BM detachment occurred exclusively in mutant endoderm indicating cell-type-specificity of PINCH1 function.

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