PINCH-1 promotes Bcl-2-dependent survival signalling and inhibits JNK-mediated apoptosis in the primitive endoderm

Eloi Montanez[1,2,*,‡], Esra Karaköse[1,*], Denise Tischner[3], Andreas Villunger[3] and Reinhard Fässler[1,‡]

[1]Max Planck Institute of Biochemistry, Department for Molecular Medicine, 82152 Martinsried, Germany
[2]Walter-Brendel-Centre of Experimental Medicine, Ludwig-Maximilians University Munich, 81377 Munich, Germany
[3]Division of Developmental Immunology, Biocentre, Innsbruck Medical University, Innsbruck 6020, Austria

*These authors contributed equally to this work
‡Authors for correspondence (Eloi.Montanez@med.uni-muenchen.de; Faessler@biochem.mpg.de)

Accepted 25 July 2012
Journal of Cell Science 125, 5233–5240

doi: 10.1242/jcs.112029

Summary

The focal adhesion (FA) protein PINCH-1 is required for the survival of primitive endoderm (PrE) cells. How PINCH-1 regulates this fundamental process is not known. Here, we use embryoid bodies (EBs) and isolated EB-derived PrE cells to investigate the mechanisms by which PINCH-1 promotes PrE survival. We report that loss of PINCH-1 in PrE cells leads to a sustained activity of JNK and the pro-apoptotic factor Bax. Mechanistically, the sustained JNK activation was due to diminished levels of the JNK inhibitory factor Ras suppressor protein-1 (RSU-1), whose stability was severely reduced upon loss of PINCH-1. Chemical inhibition of JNK attenuated apoptosis of PrE cells but failed to reduce Bax activity. The increased Bax activity was associated with reduced integrin signalling and diminished Bcl-2 levels, which were shown to inhibit Bax. Altogether our findings show that PINCH-1 is a pro-survival factor that prevents apoptosis of PrE cells by modulating two independent signalling pathways; PINCH-1 inhibits JNK-mediated apoptosis by stabilising the PINCH-1 binding protein RSU-1 and promotes Bcl-2-dependent pro-survival signalling downstream of integrins.

Key words: Integrin, PINCH-1, JNK, RSU-1, Bax, Bcl-2, Apoptosis, Primitive endoderm

Introduction

During the peri-implantation stage of the blastocyst, the inner cell mass (ICM) cells facing the blastocyst cavity differentiate into the primitive endoderm (PrE), which deposits laminin (LN) and assembles the first embryonic basement membrane (BM) between themselves and the remaining undifferentiated ICM cells. The BM induces the differentiation of the PrE into extraembryonic visceral endoderm (ExVE) cells and of the adjacent ICM cells into primitive ectoderm (epiblast), while ICM cells that are not in contact with the BM undergo apoptosis leading to the formation of the proammniotic cavity (Li et al., 2003). The ExVE, which contributes to the yolk sac, is progressively displaced by the definitive endoderm that arises from the epiblast and is required for organogenesis (Lewis and Tam, 2006). The mechanisms that regulate differentiation and survival of the PrE are not fully understood.

Attachment of cells to extracellular matrix (ECM) proteins of BMs is mediated by integrins, which represent a superfamily of adhesion proteins composed of α and β subunits (Hynes, 2002). The β1 integrins are the largest subfamily. They are highly expressed in the early mouse embryo and are essential for their development. Deletion of the gene encoding β1 integrin in mice leads to peri-implantation lethality characterised by impaired adhesion and aberrant differentiation of the PrE and epiblast (Fässler and Meyer, 1995; Stephens et al., 1995; Li et al., 2003). It has been shown that β1 integrins promote differentiation of PrE by activating Ras/mitogen-activated protein kinases (MAPKs) (Liu et al., 2009).

Integrins have short cytoplasmic domains that lack catalytic activities. Therefore, their signal transduction relies on the recruitment of signalling and adaptor proteins such as the integrin-linked kinase (ILK), PINCH and parvin (IPP) complex. ILK is a pseudokinase, which binds the LIM-only domain containing proteins PINCH-1 or PINCH-2 with its N-terminal ankyrin repeats and the calponin homology domains containing proteins α-, β- or γ-parvin with the ILK kinase-like domain (Legate et al., 2006; Lange et al., 2009; Legate et al., 2009; Fukuda et al., 2009; Wickström et al., 2010). PINCH-1 is ubiquitously expressed throughout mammalian development and adult life, while PINCH-2 expression becomes detectable at mid-gestation, and high levels are restricted to muscle, heart and brain (Wu, 1999; Tu et al., 1999; Braun et al., 2003). PINCH-1 can bind several actin modulating proteins (Legate et al., 2006; Wickström et al., 2010), the Ras suppressor protein 1 (RSU-1) and the protein phosphatase 1α (PP1α). The latter two interactions were shown to modulate MAPK and phosphoinositol 3-kinase (PI3K)/AKT signalling, respectively (Masuelli and Cutler, 1996; Dougherty et al., 2008; Eke et al., 2010).

Deletion of the PINCH-1-encoding gene (known as *Lims1*) in mice leads to embryonic lethality also shortly after implantation. PINCH-1-deficient embryos display an abnormal epiblast polarity, impaired cavitation, detachment of PrE and epiblast cells from the BM, and severe apoptosis of the PrE (Liang et al., 2005; Li et al., 2005). Similarly, embryonic stem (ES)-cell-

derived embryoid bodies (EBs), which mimic peri-implantation development (Coucouvanis and Martin, 1995; Montanez et al., 2007) identified PINCH-1 as an important survival factor for PrE cells (Li et al., 2005). PINCH-1 was also shown to protect tumour cells from apoptosis by enhancing the activities of the pro-survival proteins ERK1/2 and AKT (Chen et al., 2008; Eke et al., 2010; Sandfort et al., 2010). How PINCH-1 regulates survival of the PrE is not known.

Cell survival and apoptosis are essential for development and postnatal life. Apoptosis is characterised by a number of morphological changes including cell contraction, membrane blebbing, chromatin condensation and DNA fragmentation (Meier et al., 2000). Two main signalling pathways initiate apoptosis in mammalian cells; the intrinsic pathway, which is triggered by intracellular stress signals such as DNA damage or ECM detachment and leads to the release of cytochrome c and other pro-apoptotic factors from the mitochondria, and the extrinsic pathway, which is induced by the activation of cell death receptors leading to the recruitment and activation of the caspase-8, which initiates apoptosis (Tait and Green, 2010). In certain cells activation of caspase-8 is not sufficient to trigger apoptosis and therefore they also activate the mitochondrial pathway to amplify the apoptotic signal. The anti-apoptotic factors of the Bcl-2 family members (Bcl-2, Mcl-1) establish a finely tuned activity balance of pro-apoptotic Bcl-2 family members that fall into two classes; the BH3-only proteins such as Bim, Bid, Puma or Bmf, and the Bax-like proteins that control the formation of the mitochondrial outer permeabilization pore and the release of pro-apoptotic factors (Tait and Green, 2010). Both apoptosis pathways converge on caspase-3, which executes apoptosis (Degterev et al., 2003). Integrins have a dual role in cell survival. While integrin-mediated adhesion protects cells from apoptosis by upregulating Bcl-2 expression (Zhang et al., 1995), unligated integrins can trigger apoptosis by recruiting and activating caspase-8 (Stupack et al., 2001). Jun N-terminal kinases (JNKs) also play a critical role in the regulation of apoptosis during embryo development, either by inducing the expression of pro-apoptotic genes or by controlling the activities of mitochondrial pro- and anti-apoptotic proteins (Davis, 2000; Dhanasekaran and Reddy, 2008).

In the present article we investigated the role of PINCH-1 in differentiation and survival of the PrE. We found that PINCH-1 is dispensable for PrE differentiation but promotes PrE survival through tuning Bcl-2 and Bax activity, and through inhibiting JNK-mediated apoptosis.

Results

PINCH-1 is dispensable for differentiation of the PrE

Integrin-mediated signalling is critical for the differentiation of PrE (Liu et al., 2009). To elucidate whether PINCH-1 is required for this integrin-mediated task, we generated EBs from wild-type (WT) and PINCH-1$^{-/-}$ ES cells (Montanez et al., 2007). After 7 days in suspension, WT ES cells developed into cystic EBs consisting of an outer layer of cubical-shaped PrE cells, an inner layer of columnar pseudostratified epiblast cells, a thin and continuous BM between epiblast and PrE cells, and a central cavity (Fig. 1A). Immunostaining with antibodies against endoderm markers revealed that PrE cells expressed α-fetoprotein (AFP) and disabled homologue 2 (Dab2) in their cytoplasm and the endoderm-specific transcription factor GATA4 in their nuclei (Fig. 1A; data not shown). PINCH-1$^{-/-}$ EBs displayed abnormal epiblast polarity, a discontinuous BM, detached PrE and impaired cavitation (Fig. 1A). In addition, DAPI staining showed condensed and fragmented nuclei in PrE cells of all PINCH-1$^{-/-}$ EBs analysed (Fig. 1A). However, we found no apparent defects in the expression and localisation of endoderm markers in PINCH-1$^{-/-}$ PrE cells (Fig. 1A; data not shown).

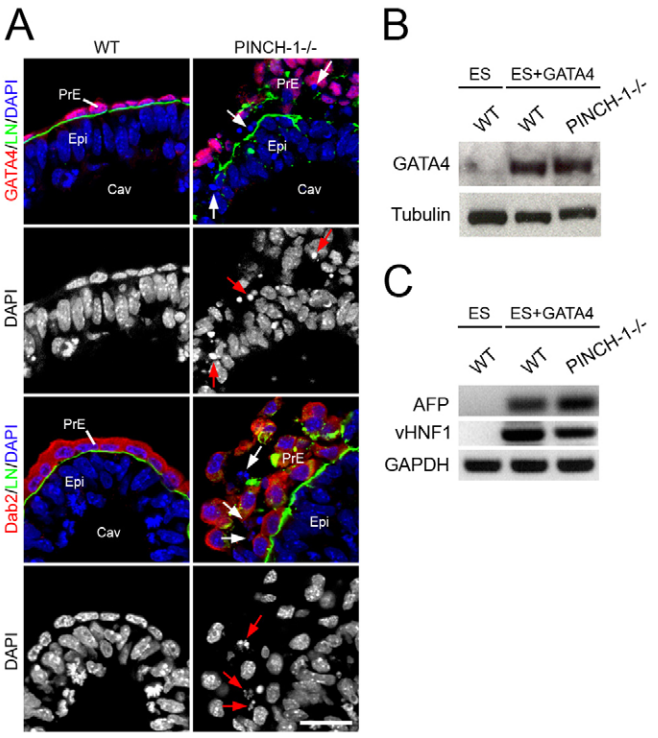

Fig. 1. PINCH-1 is dispensable for PrE differentiation but required for PrE survival. (**A**) Immunostaining of cross-sections derived from 7-day-old WT and PINCH-1$^{-/-}$ EBs for the endoderm markers GATA4 and disabled homologue 2 (Dab2). BMs were visualised with a specific antibody against LN111 and nuclei were visualised with with DAPI. PINCH-1$^{-/-}$ PrE cells have condensed and fragmented nuclei (arrows). Scale bar: 30 μm. PrE, primitive endoderm; Epi, epiblast; Cav, cavity. (**B**) Western blot of GATA4 in protein lysates of untreated WT ES cells and GATA4-transfected WT and PINCH-1$^{-/-}$ ES cells. Tubulin served as a loading control. (**C**) RT-PCR for genes encoding the endoderm markers α-fetoprotein (AFP) and variant hepatocyte nuclear factor 1 (vHNF1) in untreated WT ES cells and GATA4-transfected WT and PINCH-1$^{-/-}$ ES cells. GAPDH served as a loading control.

Forced expression of GATA4 in ES cells is sufficient to induce PrE differentiation (Fujikura et al., 2002). To confirm our results with the EBs, we expressed GATA4 in WT and PINCH-1$^{-/-}$ ES cells (Fig. 1B) and monitored the expression of the visceral endoderm markers AFP and variant Hepatocyte Nuclear Factor 1 (vHNF1) by RT-PCR. We induced similar levels of AFP and vHNF1 in WT and PINCH-1$^{-/-}$ cells (Fig. 1C) indicating that PINCH-1 is dispensable for PrE differentiation.

Loss of PINCH-1 in PrE cells triggers activation of the intrinsic apoptotic pathway

The condensed and fragmented nuclei confirm a role for PINCH-1 in PrE cell survival. To further corroborate this finding we performed TUNEL assays and an immunostaining with anti-

active caspase-3. In WT cystic EBs, apoptotic cells were mainly observed at the centre of EBs where cells are eliminated to create the pro-amniotic-like cavity and occasionally within the epiblast and very rarely in PrE cells (Fig. 2A). In PINCH-1$^{-/-}$ EBs, the rate of apoptotic PrE cells was high (Fig. 2A). Quantitative analysis of TUNEL-positive PrE cells showed a 3-fold increase of apoptotic cell numbers in PINCH-1$^{-/-}$ EBs when compared to WT EBs (Fig. 2B). Almost all apoptotic PrE cells in PINCH-1$^{-/-}$ EBs were detected adjacent to the BM suggesting that apoptosis might occur already before cell detachment (Fig. 2A). To further confirm this observation, we isolated PrE cells from WT and PINCH-1$^{-/-}$ EBs (Fig. 2C), cultured them on the BM component fibronectin (FN) (Liu et al., 2009) and analysed apoptosis 48 hours later. Similarly like the PrE cells in PINCH-1$^{-/-}$ EBs, PINCH-1$^{-/-}$ PrE cells plated on FN underwent apoptosis and displayed caspase-3 activation (Fig. 2D). Quantitation of TUNEL-positive PrE cells showed a 3-fold increase of apoptosis in PINCH-1$^{-/-}$ PrE cells when compared to WT cells (Fig. 2E). Together, these results indicate that loss of PINCH-1 in PrE cells triggers caspase-3-dependent apoptosis.

Next we determined the mechanism leading to apoptosis in PrE cells lacking PINCH-1 expression. Unligated integrins recruit caspase-8 to the cell membrane where it is activated and initiates apoptosis (Stupack et al., 2001). To determine whether caspase-8 is activated in PINCH-1$^{-/-}$ PrE cells, we performed immunostaining of WT and PINCH-1$^{-/-}$ EBs using an antibody against active caspase-8. In PINCH-1$^{-/-}$ and in WT EBs active caspase-8-positive cells were mainly observed in the centre of the

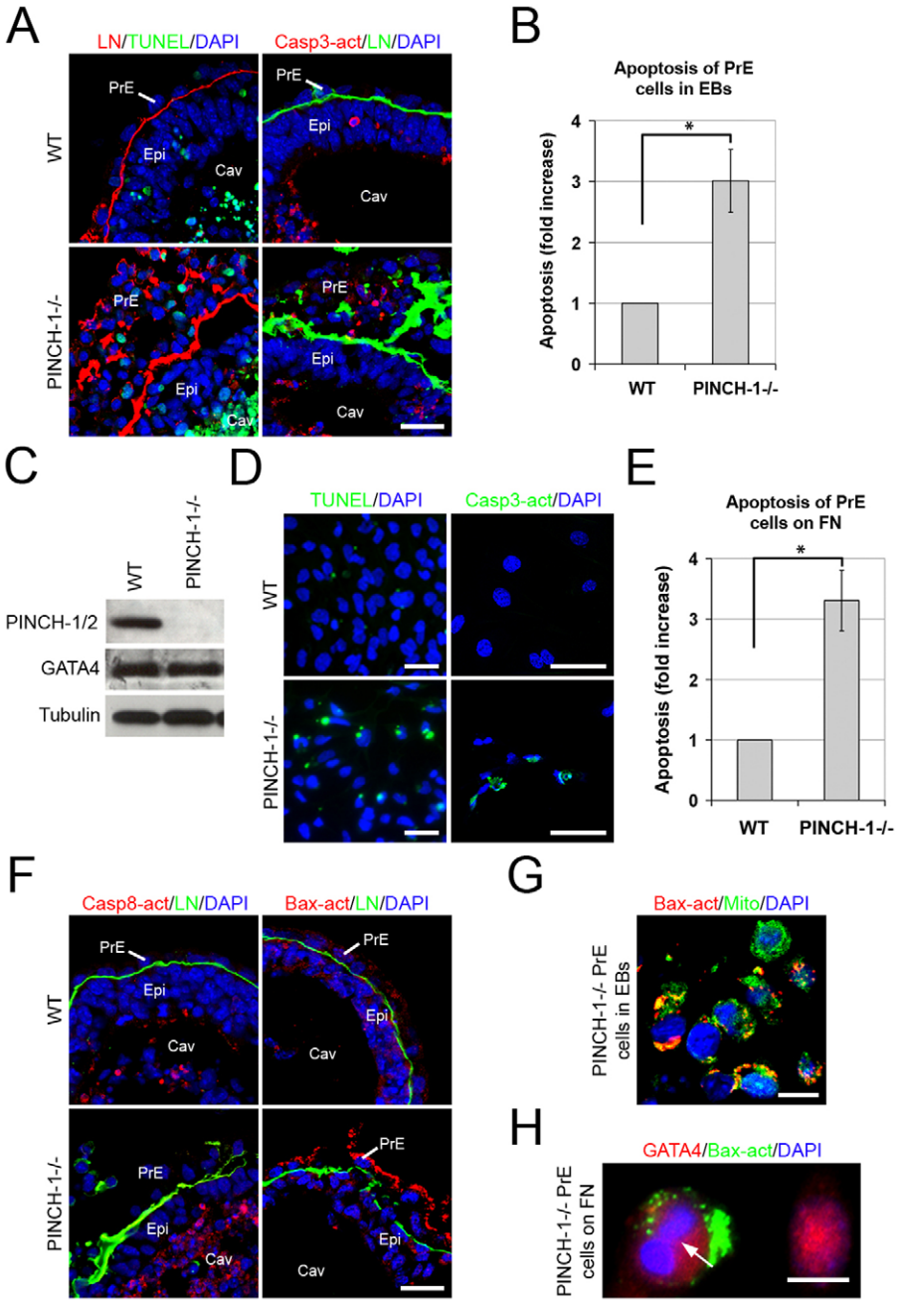

Fig. 2. Deletion of PINCH-1 triggers PrE cell apoptosis. (**A**) Apoptosis was assessed in sections derived from WT and PINCH-1$^{-/-}$ EBs by using a TUNEL assay and immunostaining for cleaved caspase 3. BMs were visualised with an anti-LN111 antibody and nuclei were visualised with DAPI. Scale bar: 30 μm. PrE, primitive endoderm; Epi, epiblast; Cav, cavity. (**B**) Quantification of TUNEL-positive PrE cells in EB sections. The ratios of TUNEL-positive PrE cells to total PrE cells were determined, and the mean ratio for WT PrE cells was set at 1 to indicate the relative fold change of apoptosis in PINCH-1$^{-/-}$ PrE cells. A minimum of 30 EBs from three independent preparations were analysed. (**C**) Protein levels of PINCH-1 and GATA4 in lysates of PrE cells from WT and PINCH-1$^{-/-}$ EBs. Tubulin served as a loading control. (**D**) Apoptosis was assessed in WT and PINCH-1$^{-/-}$ PrE cells cultured for 48 hours on FN by TUNEL assay and cleaved-caspase 3 immunostaining. Nuclei were stained with DAPI. Scale bar: 100 μm. (**E**) Quantification of TUNEL-positive PrE cells cultured on FN for 48 hours. The ratios of TUNEL-positive PrE cells to total PrE cells were determined, and the mean ratio for WT PrE cells was set at 1 to indicate the relative fold change of apoptosis in PINCH-1$^{-/-}$ PrE cells. A minimum of 150 cells from three independent WT and PINCH-1$^{-/-}$ preparations were analysed. (**F**) Immunostaining of sections from WT and PINCH-1$^{-/-}$ EBs for cleaved caspase 8 and active Bax. BMs were visualised with antibodies against LN and nuclei were visualised with DAPI. Scale bar: 30 μm. PrE, primitive endoderm; Epi, epiblast; Cav, cavity. (**G**) Immunosignals of active Bax are overlapping with the mitochondrial marker (MitoTracker®) in PINCH-1$^{-/-}$ PrE cells. Scale bar: 10 μm. (**H**) Immunostaining of GATA4 and active Bax in PINCH-1$^{-/-}$ PrE cells cultured on FN for 48 hours. Nuclei were stained with DAPI. Active Bax is found in cells with condensed and fragmented DNA (arrow). Scale bar: 40 μm. *P<0.05.

EBs and very rarely in the epiblast and in PrE cells (Fig. 2F). To test whether a mitochondrial dysfunction is involved in the apoptosis of PINCH-1$^{-/-}$ PrE cells we performed immunostaining of WT and PINCH-1$^{-/-}$ EBs using the 6A7 Bax antibody, which recognises activated Bax. In contrast to WT PrE cells, which lacked active Bax in the cytoplasm, PINCH-1$^{-/-}$ PrE cells displayed a strong and punctate staining for active Bax (Fig. 2F) that co-localised with the mitochondrial marker MitoTracker® (Fig. 2G). In line with these results, adherent apoptotic PINCH-1$^{-/-}$ PrE cell also contained active Bax (Fig. 2H). Together, these results indicate that loss of PINCH-1 in PrE cells triggers the intrinsic mitochondrial apoptotic pathway.

Loss of PINCH-1 leads to increased JNK activation

We next sought to elucidate the molecular mechanism(s) by which PINCH-1 regulates Bax activity in PrE cells. It has been reported that PINCH-1 protects tumour cells from Bax-dependent apoptosis through phosphorylation and activation of ERK1/2 and AKT or by downregulating expression and phosphorylation of the pro-apoptotic protein Bim (Chen et al., 2008; Eke et al., 2010). To test whether this is also occurring in PrE cells, we determined the phosphorylation levels of ERK1/2 and AKT in WT and PINCH-1$^{-/-}$ PrE cells cultured for 24 or 48 hours on FN. PINCH-1$^{-/-}$ PrE cells showed similar steady state phosphorylation levels of ERK1/2 and AKT-Ser473 as WT cells excluding a role of both signalling molecules in Bax activation (Fig. 3A). To test whether Bim is involved in apoptosis of PINCH-1$^{-/-}$ PrE cells, we determined the levels of total and the levels of phosphorylated Bim. WB analysis with a specific antibody that recognises total Bim and a phosphorylation-induced mobility shift of Bim upon phosphorylation revealed no differences between WT and PINCH-1$^{-/-}$ PrE cells (Fig. 3B). Together, these results indicate that (i) ERK, AKT or Bim activity are not involved in regulating survival of PINCH-1$^{-/-}$ PrE and that (ii) PINCH-1 is employing different mechanism(s) to support cell survival in PrE than tumour cells.

Sustained activation of JNK also triggers the mitochondrial apoptotic pathway leading to cell death (Ip and Davis, 1998). To test whether loss of PINCH-1 affects JNK activity, we measured phosphorylation levels of JNK in WT and PINCH-1$^{-/-}$ PrE cells cultured for 48 hours on FN and found increased JNK phosphorylation levels in PINCH-1$^{-/-}$ PrE cells compared to WT cells (Fig. 3A). To determine whether the increased JNK activity was responsible for the augmented Bax activity in PINCH-1$^{-/-}$ PrE cells, we treated the cells with the specific JNK inhibitor SP600125 or DMSO (vehicle) and assayed Bax activation 48 hours later by WB. As expected, SP600125 treatment reduced JNK phosphorylation in PINCH-1$^{-/-}$ PrE cells (Fig. 3C). However, the treatment did not alter the activation rate of Bax, indicating that blockade of JNK signalling is not ablating Bax activity in PINCH-1$^{-/-}$ PrE cells (Fig. 3C).

Sustained JNK activation in PINCH-1$^{-/-}$ PrE cells triggers apoptosis

To test whether sustained JNK activity is involved in the apoptosis of PINCH-1$^{-/-}$ PrE cells we treated WT and PINCH-1$^{-/-}$ PrE cells with SP600125 or DMSO (vehicle) (Fig. 3C) and assayed apoptosis 48 hours later. The blockade of JNK reduced the apoptosis rate of PINCH-1$^{-/-}$ PrE cells by around 40–50%

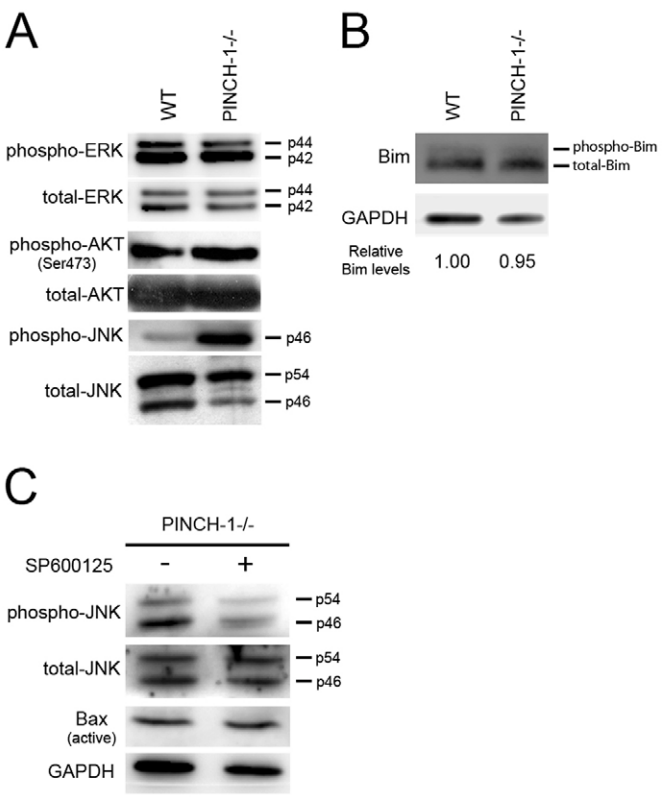

Fig. 3. Increased JNK activity in PINCH-1$^{-/-}$ PrE cells. (**A**) Representative western blots (n=3) for total and phospho-specific forms of ERK, AKT and JNK in lysates of WT and PINCH-1$^{-/-}$ PrE cells cultured for 48 hours on FN. (**B**) Representative western blot (n=3) for Bim (phospho-induced band shift) in protein lysates from WT and PINCH-1$^{-/-}$ PrE cells cultured on FN. GAPDH levels were determined to adjust protein loading. Bim levels in WT and PINCH-1$^{-/-}$ PrE cells were determined by densitometry and levels in WT PrE cells were normalised to 1. (**C**) Representative western blots (n=3) for total and phospho-specific forms of JNK and Bax in lysates from PINCH-1$^{-/-}$ PrE cells cultured on FN and treated with or without JNK inhibitor (SP600125; 10 μM). GAPDH levels were determined to adjust protein loading.

(Fig. 4A) indicating that JNK plays a prominent role in PINCH-1-dependent survival of PrE cells.

We next sought to elucidate the mechanism by which PINCH-1 regulates JNK activity in PrE cells. PINCH-1 interacts with RSU-1, which was shown to negatively regulate the activity of JNK (Masuelli and Cutler, 1996; Kadrmas et al., 2004; Dougherty et al., 2008). To determine the level as well as spatial expression pattern of RSU-1 during peri-implantation, we performed immunostaining of E6.5 embryos and of 7-day-old cystic EBs using a specific antibody against RSU-1. The immunostaining revealed high RSU-1 expression in PrE, where it partially localises adjacent to LN, and practically no RSU-1 in the epiblast (Fig. 4B,C). To determine whether RSU-1 co-localises with PINCH-1 in PrE cells, we expressed PINCH-1–GFP-encoding cDNA in freshly isolated PrE cells from WT EBs and stained them with RSU-1 antibody. We found that PINCH-1 and RSU-1 co-localise in focal adhesions (FAs) of WT PrE cells (Fig. 4D; supplementary material Fig. S1). The RSU-1 staining was weaker in PrE cell derived from PINCH-1$^{-/-}$ EBs when compared to PrE cells of WT EBs (Fig. 4C). WB assays also revealed significantly reduced levels of RSU-1 in PrE cells

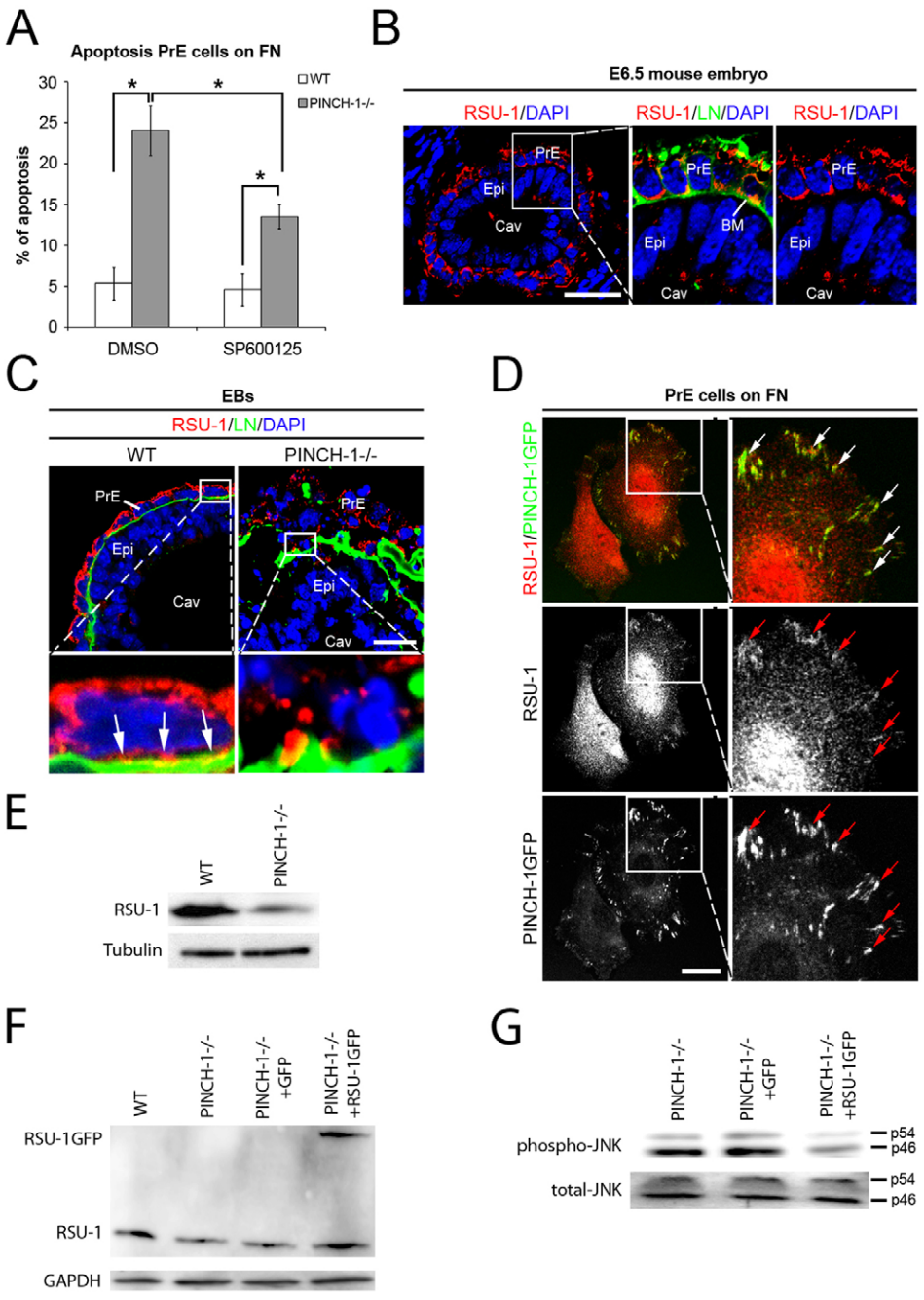

Fig. 4. Sustained JNK activation triggers apoptosis of PINCH-1$^{-/-}$ PrE cells. (**A**) Quantification of the percentage of TUNEL-positive WT and PINCH-1$^{-/-}$ PrE cells cultured on FN and treated with the JNK inhibitor SP600125 (10 µM) or DMSO (vehicle). 150 cells were analysed in each group of three independent experiments. (**B**) Immunostaining of RSU-1 in E6.5 cross sections derived from WT embryos. BMs were visualised with anti-LN111 antibodies and were visualised nuclei with DAPI. Scale bar: 30 µm. PrE, primitive endoderm; Epi, epiblast; Cav, cavity; BM, basement membrane. (**C**) Immunostaining of RSU-1 in WT and PINCH-1$^{-/-}$ EBs. BMs were visualised with anti-LN111 antibodies and nuclei with DAPI. Arrows point to RSU-1 signals that are adjacent to the LN111-positive BM. Scale bar: 30 µm. PrE, primitive endoderm; Epi, epiblast; Cav, cavity. (**D**) Immunostaining of RSU-1 in freshly isolated PrE cells transfected with PINCH-1–GFP-encoding cDNA. Scale bar: 25 µm. Arrows point to FAs. (**E**) Western blots for RSU-1 in lysates from WT and PINCH-1$^{-/-}$ PrE cells cultured for 48 hours on FN. Tubulin levels were determined to adjust protein loading. (**F**) Western blots for RSU-1 in lysates of WT and PINCH-1$^{-/-}$ PrE cells, and PINCH-1$^{-/-}$ PrE cells transfected with either GFP or RSU-1–GFP. GAPDH levels were determined to adjust protein loading. (**G**) Western blots for total JNK and phospho-JNK in lysates of PINCH-1$^{-/-}$ PrE cells transfected with GFP or RSU-1–GFP. *P<0.05.

derived from PINCH-1$^{-/-}$ EBs (Fig. 4E). Overexpression of a GFP-tagged RSU-1 cDNA in PINCH-1$^{-/-}$ PrE cells (Fig. 4F) reduced the levels of phosphorylated JNK (Fig. 4G). Together these results indicate that the PINCH-1 stabilises RSU-1, which in turn abates JNK activity in PrE cells.

PINCH-1$^{-/-}$ PrE cells display reduced Bcl-2 protein levels

We observed elevated Bax activity in PINCH-1$^{-/-}$ PrE cells that was not controlled by JNK (Fig. 3C). Bax activity can be inhibited by Bcl-2, whose expression in turn can be regulated by FN-induced α5β1 integrin signalling (Zhang et al., 1995; Tait and Green, 2010). To test whether α5β1 integrin signalling is impaired in PINCH-1$^{-/-}$ PrE cells, we isolated PrE cells and analysed their spreading on FN. Within 40 minutes after plating WT PrE cells spread on FN and formed FAs and actin stress fibres, as revealed by paxillin and phalloidin staining, respectively (Fig. 5A; supplementary material Fig. S2A). In contrast, PINCH-1$^{-/-}$ PrE cells failed to spread on FN and developed very few FAs and stress fibres (Fig. 5A; supplementary material Fig. S2A). The PINCH-1$^{-/-}$ PrE cells remained round even 16 hours after plating and developed multiple membrane blebs (Fig. 5B). Similar defects were observed when GATA4-transduced PINCH-1$^{-/-}$ ES cells were cultured on FN (supplementary material Fig. S2B).

As previously reported for other cells we also found that the diminished α5β1–FN function in PINCH-1$^{-/-}$ PrE cells is associated with reduced Bcl-2 levels (Fig. 5C). This finding was

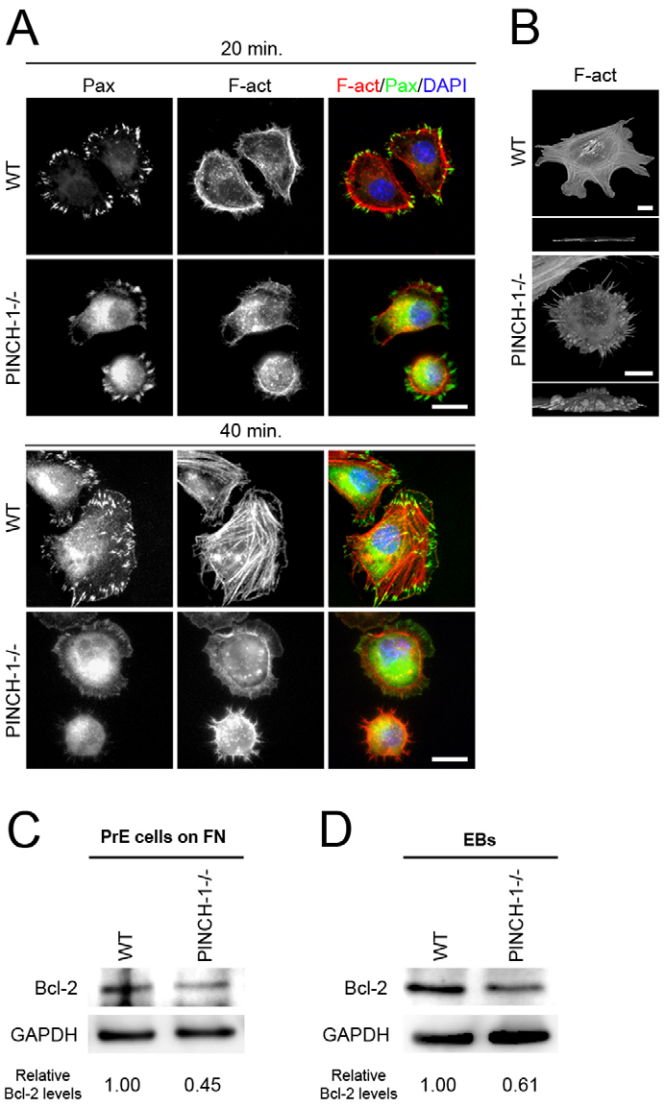

Fig. 5. Reduced Bcl-2 protein levels in PINCH-1$^{-/-}$ PrE cells. (**A**) Immunostaining for paxillin in WT and PINCH-1$^{-/-}$ PrE cells cultured for 20 and 40 minutes on FN. F-actin was stained with phalloidin–TRITC and nuclei were stained with DAPI. Scale bars: 25 µm. (**B**) The *Z*-projections of WT and PINCH-1$^{-/-}$ PrE cells cultured for 16 hours on FN. Scale bar: 25 µm. (**C**) Western blots for Bcl-2 in lysates from WT and PINCH-1$^{-/-}$ PrE cells cultured for 48 hours on FN. GAPDH levels were determined to control protein loading. Bcl-2 levels were determined by densitometry and levels in WT PrE cells were normalised to 1. (**D**) Western blots for analysis Bcl-2 in lysates of WT and PINCH-1$^{-/-}$ EBs. GAPDH levels were determined as a control for protein loading. Bcl-2 levels were determined by densitometry and levels in WT PrE cells were normalised to 1.

further confirmed by WB analysis of protein lysates from WT and PINCH-1$^{-/-}$ EBs (Fig. 5D). To exclude reduced integrin expression as cause for the impaired integrin signalling of PINCH-1$^{-/-}$ PrE cells, we measured integrin surface levels by flow cytometry. The levels of α5 and β1 integrins were similar in WT and PINCH-1$^{-/-}$ PrE cells (supplementary material Fig. S3), and αv, α6 and β4 integrins were below the detection level in both genotypes (supplementary material Fig. S3). Together, these results indicate that PINCH-1 supports α5β1 integrin binding to FN and Bcl-2 expression in PrE cells.

Discussion

In the present study we show that PINCH-1 is essential for PrE cell survival but is not transducing β1 integrin-mediated signals required for the differentiation of the PrE. Mechanistically, loss of PINCH-1 triggers death of PrE cells by impairing two, independent signalling pathways; PINCH-1 loss leads to a diminished RSU-1 stability followed by sustained JNK activity, and to reduced Bcl-2 expression elicited by an impaired α5β1 integrin downstream signalling.

During peri-implantation, the PrE deposits the first embryonic BM, which is essential for further development (Li et al., 2003). β1 integrins are highly expressed in the early mouse embryo and are essential for PrE differentiation as well as BM deposition and assembly (Fässler and Meyer, 1995; Liu et al., 2009). β1 integrins mediate differentiation of PrE by inducing the nuclear translocation of the endoderm-specific transcription factor GATA4, which in turn induces the expression of BM proteins and orchestrates endoderm maturation (Liu et al., 2009). β1 integrins are also essential for the stable adhesion, spreading and polarity of PrE cells on the FN-rich BM (Liu et al., 2009). Consistent with these fundamental functions, deletion of the β1-integrin-encoding gene arrests mouse development at the peri-implantation stage (Fässler and Meyer, 1995; Stephens et al., 1995). Deletion of the PINCH-1-encoding gene also results in embryonic lethality at peri-implantation associated with impaired cell adhesion (Liang et al., 2005; Li et al., 2005). The findings of our study show that PINCH-1 is not required for the differentiation of ES cells into PrE but for the adhesion and spreading of PrE. This indicates that β1 integrins mediate PrE adhesion and spreading through PINCH-1, but not their differentiation.

We also observed a pronounced apoptosis in the PrE of PINCH-1$^{-/-}$ EBs. Normal development depends on a delicate equilibrium of controlled cell death and cell survival. Developing organisms often produce excess of cells during organogenesis, which are removed at later stages by apoptosis. These waves of apoptosis are involved in morphogenetic processes such as folding and fusion of epithelial sheets or cavitation and are therefore important for organogenesis (Weil et al., 1997). In mammalian embryos, the first wave of apoptosis occurs at peri-implantation when the embryo forms the pro-amniotic cavity (Meier et al., 2000). In a previous study we reported that the apoptosis leading to the formation of the pro-amniotic cavity is abrogated in PINCH-1$^{-/-}$ embryos, while the survival of the developing PrE is impaired (Li et al., 2005).

A prime candidate for regulating the apoptosis rate in PrE is JNK. The activity of JNK was sustained in developing flies lacking PINCH expression leading to pronounced migration defects (Kadrmas et al., 2004). JNK signalling can also regulate apoptosis and cell survival during development (Davis, 2000). A transient activation of JNK is associated with cell survival, whereas a sustained activation is associated with apoptosis (Ip and Davis, 1998). Similarly like in flies, we found that loss of PINCH-1 results in sustained JNK activity in PrE cells. Interestingly, we could also reduce but not abolish apoptosis of PINCH-1$^{-/-}$ PrE cells by inhibiting JNK. This indicates that PINCH-1 promotes survival of the PrE by inhibiting JNK-mediated apoptosis, and in addition through JNK-independent pathway(s). The JNK inhibitory function of PINCH in flies is believed to occur through RSU-1, whose levels are diminished in flies lacking PINCH (Kadrmas et al., 2004). In line with the fly data, loss of PINCH-1 expression in PrE also reduces RSU-1 protein levels.

Furthermore, we found that normalisation of the levels of phosphorylated JNK can be accomplished by overexpressing RSU-1 in PINCH-1$^{-/-}$ PrE cells, which indicates that the reduction of RSU-1 and not the loss of PINCH expression leads to increased JNK activity. The high levels of RSU-1 in PrE cells co-localise with PINCH-1 in FAs where the activity of RSU-1 towards JNK is then most likely regulated through the interaction with PINCH-1. A similar co-dependence of RSU-1 and PINCH-1 protein levels has also been reported for human embryonic kidney cells (Dougherty et al., 2005), which further supports the notion that the interaction with PINCH-1 maintains RSU-1 stability in several, maybe even all cell types.

Since the chemical inhibition of JNK is not sufficient to fully rescue the survival of PINCH-1$^{-/-}$ PrE cells we were interested to find the JNK-independent pathway(s) regulated by PINCH-1. Integrins are capable of regulating both the extrinsic as well as the intrinsic apoptotic pathways. We could exclude a major defect in the extrinsic apoptotic pathway by showing that caspase-8 activity was similar in WT and PINCH-1$^{-/-}$ PrE cells. The intrinsic pathway depends on the activities of anti- as well as pro-apoptotic members of the Bcl-2 family (Tait and Green, 2010). The pro-apoptotic Bcl-2 family member Bim (Ley et al., 2005) was shown to be regulated by PINCH-1 in cancer cells (Chen et al., 2008). In these cells, PINCH-1 induces the activity of ERK, which triggers Bim phosphorylation and degradation, and thus prevents Bim-mediated activation of the pro-apoptotic effector Bax (Chen et al., 2008). Interestingly, PINCH-1$^{-/-}$ PrE cell have normal levels of phosphorylated ERK and Bim, while the activity of Bax was increased. There are two possibilities from published reports that could explain increased Bax activity in PINCH-1$^{-/-}$ PrE cells. On one hand the increased activation of Bax could originate from an impaired activation of the pro-survival kinase AKT, whose activity is controlled by the PP1α phosphatase in a PINCH-1-dependent manner in tumour cells (Eke et al., 2010; Sandfort et al., 2010). The normal phosphorylation levels of AKT in PINCH-1$^{-/-}$ PrE cells, however, exclude an impaired function of AKT or of upstream regulators of AKT. On the other hand, Bax can also be regulated by the anti-apoptotic protein Bcl-2, either by directly binding and blocking Bax activity or by sequestering BH3-only proteins from Bax (Tait and Green, 2010). Moreover, the expression of Bcl-2 can be regulated by α5β1 integrins (Zhang et al., 1995), which are prominently expressed on PrE cells (Liu et al., 2009). The α5β1 integrin ligand FN is also enriched in the BM between PrE and epiblast (Liu et al., 2009). In support for an integrin/PINCH-1/Bcl-2 pathway we found that the function of α5β1 integrin is impaired and the levels of Bcl-2 were reduced in PINCH-1$^{-/-}$ PrE cells. How PINCH-1 regulates Bcl-2 expression is not clear and needs to be addressed in future studies.

In summary, our data show that PINCH-1 regulates PrE survival differently than in cancer cells. Furthermore, we provide evidence that PINCH-1 acts downstream of integrins to regulate the activity of JNK and to maintain the levels of Bcl-2. Both signalling pathways contribute to the survival of the PrE.

Materials and Methods

ES cells and EBs formation

The ES cell lines used for this study were WT and PINCH-1$^{-/-}$ ES cells (Li et al., 2005). EB differentiation was initiated from ES-cell aggregates in suspension culture as described before (Montanez et al., 2007). To force ES cell differentiation into the PrE lineage they were electroporated with the pCAG-IP-GATA4 vector as described by Fujikura et al. (Fujikura et al., 2002).

Antibodies and reagents

The following antibodies and reagents were used for the analyses: rat antibody against LN-α1 chain (Chemicon); rabbit antibody against GAPDH (Calbiochem); rat antibody against tubulin (Chemicon); Dab2 (clone 52) monoclonal antibody (BD Biosciences); GATA4 (Santa Cruz Biotechnologies); rabbit antibody against cleaved (activated) caspase-3 (Cell Signaling Technology); rabbit antibody against cleaved (activated) caspase-8 (Cell Signaling Technology); rabbit antibodies against PKB/Akt and phospho-PKB/Akt (Thr308, Ser473) (Cell Signaling Technology); rabbit antibodies against JNK and phospho-JNK (Cell Signaling Technology); rabbit antibodies against ERK and phospho-ERK (Cell Signaling Technology); rabbit antibody against Bax (6A7) (Sigma), hamster antibody against Bcl-2 (BD Pharmingen), rabbit antibody against Bim (Assay Desings), Paxillin (Transduction laboratories), mouse antibody against PINCH-1/2 (BD Biosciences) and rabbit antibodies against RSU-1 (Dougherty et al., 2008). Secondary antibodies were purchased from the Jackson ImmunoResearch Laboratories Inc., Molecular Probes and BioRad. TRITC-conjugated phalloidin was used to detect F-actin (Molecular Probes). MitoTracker® Green FM was used to detect mitochondria (Invitrogen). Apoptotic cells were detected using the *In Situ* Cell Death Detection kit (Roche Diagnostics).

EB lysis and immunoblotting

WT and PINCH-1$^{-/-}$ EBs were grown for 7 days in suspension, washed once in phosphate-buffered saline (PBS) and lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics).

Histology and immunostaining

Histology and immunofluorescence analyses of EBs and embryos were performed as described previously (Montanez et al., 2007). All animal experiments were performed according to approved guidelines.

Isolation and transfection of PrE cells

PrE cells were dissociated from 7-day-old WT and PINCH-1$^{-/-}$ EBs by repeated pipetting and were then separated from the remaining EBs by differential settling through gravity. The PrE cells were grown on FN (10 μg/ml)-coated glass coverslips or 60-mm dishes for 48 hours. The cells were then either fixed with 3% paraformaldehyde for immunostaining or harvested for western blot analysis. PrE cells were transfected with the following vectors pEGFP-N1 (BD Biosciences), pEGFP-N1-RSU-1 (Dougherty et al., 2005) and pEGFP-N1-PINCH-1 (Braun et al., 2003) using Lipofectamine (Invitrogen).

RT-PCR

RT-PCR assays were performed as described previously (Fujikura et al., 2002).

Flow cytometry

Flow cytometry assays were performed as described previously (Montanez et al., 2008).

JNK inhibition treatment

PrE cells were freshly isolated from PINCH-1$^{-/-}$ EBs, cultured on FN (10 μg/ml)-coated glass coverslips for 48 hours, starved with 1% FCS containing medium for 3 hours and then treated with SP600125 (10 μM) (Sigma) or DMSO (vehicle) for 1 hour. The extent of apoptosis was assayed 48 hours later as described above.

Statistical analysis

Statistical analysis was performed using unpaired Student's *t*-test, and $P<0.05$ was considered statistically significant. Calculations were performed using GraphPad Prism (GraphPad Software). Results are presented as means ± standard deviation.

Funding

The work was supported by the Sonderforschungsbereich [grant number SFB021 to A.V. and R.F.]; the Tiroler Zukunftsstiftung (to A.V. and R.F.); and the Max Planck Society (to R.F.).

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.112029/-/DC1

References

Braun, A., Bordoy, R., Stanchi, F., Moser, M., Kostka G, G., Ehler, E., Brandau, O. and Fässler, R. (2003). PINCH2 is a new five LIM domain protein, homologous to PINCHand localized to focal adhesions. *Exp. Cell Res.* **284**, 237-248.

Chen, K., Tu, Y., Zhang, Y., Blair, H. C., Zhang, L. and Wu, C. (2008). PINCH-1 regulates the ERK-Bim pathway and contributes to apoptosis resistance in cancer cells. *J. Biol. Chem.* **283**, 2508-2517.

Coucouvanis, E. and Martin, G. R. (1995). Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. *Cell* **83**, 279-287.
Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239-252.
Degterev, A., Boyce, M. and Yuan, J. (2003). A decade of caspases. *Oncogene* **22**, 8543-8567.
Dhanasekaran, D. N. and Reddy, E. P. (2008). JNK signaling in apoptosis. *Oncogene* **27**, 6245-6251.
Dougherty, G. W., Chopp, T., Qi, S. M. and Cutler, M. L. (2005). The Ras suppressor Rsu-1 binds to the LIM 5 domain of the adaptor protein PINCH1 and participates in adhesion-related functions. *Exp. Cell Res.* **306**, 168-179.
Dougherty, G. W., Jose, C., Gimona, M. and Cutler, M. L. (2008). The Rsu-1-PINCH1-ILK complex is regulated by Ras activation in tumor cells. *Eur. J. Cell Biol.* **87**, 721-734.
Eke, I., Koch, U., Hehlgans, S., Sandfort, V., Stanchi, F., Zips, D., Baumann, M., Shevchenko, A., Pilarsky, C., Haase, M. et al. (2010). PINCH1 regulates Akt1 activation and enhances radioresistance by inhibiting PP1α. *J. Clin. Invest.* **120**, 2516-2527.
Fässler, R. and Meyer, M. (1995). Consequences of lack of β1 integrin gene expression in mice. *Genes Dev.* **15**, 1896-1908.
Fujikura, J., Yamato, E., Yonemura, S., Hosoda, K., Masui, S., Nakao, K., Miyazaki Ji, J. and Niwa, H. (2002). Differentiation of embryonic stem cells is induced by GATA factors. *Genes Dev.* **16**, 784-789.
Fukuda, K., Gupta, S., Chen, K., Wu, C. and Qin, J. (2009). The pseudoactive site of ILK is essential for its binding to α-Parvin and localization to focal adhesions. *Mol. Cell* **36**, 819-830.
Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687.
Ip, Y. T. and Davis, R. J. (1998). Signal transduction by the c-Jun N-terminal kinase (JNK)—from inflammation to development. *Curr. Opin. Cell Biol.* **10**, 205-219.
Kadrmas, J. L., Smith, M. A., Clark, K. A., Pronovost, S. M., Muster, N., Yates, J. R., 3rd and Beckerle, M. C. (2004). The integrin effector PINCH regulates JNK activity and epithelial migration in concert with Ras suppressor 1. *J. Cell Biol.* **167**, 1019-1024.
Lange, A., Wickström, S. A., Jakobson, M., Zent, R., Sainio, K. and Fässler, R. (2009). Integrin-linked kinase is an adaptor with essential functions during mouse development. *Nature* **461**, 1002-1006.
Legate, K. R., Montañez, E., Kudlacek, O. and Fässler, R. (2006). ILK, PINCH and parvin: the tIPP of integrin signalling. *Nat. Rev. Mol. Cell Biol.* **7**, 20-31.
Legate, K. R., Wickström, S. A. and Fässler, R. (2009). Genetic and cell biological analysis of integrin outside-in signaling. *Genes Dev.* **23**, 397-418.
Lewis, S. L. and Tam, P. P. (2006). Definitive endoderm of the mouse embryo: formation, cell fates, and morphogenetic function. *Dev. Dyn.* **235**, 2315-2329.
Ley, R., Ewings, K. E., Hadfield, K. and Cook, S. J. (2005). Regulatory phosphorylation of Bim: sorting out the ERK from the JNK. *Cell Death Differ.* **12**, 1008-1014.
Li, S., Edgar, D., Fässler, R., Wadsworth, W. and Yurchenco, P. D. (2003). The role of laminin in embryonic cell polarization and tissue organization. *Dev. Cell* **4**, 613-624.
Li, S., Bordoy, R., Stanchi, F., Moser, M., Braun, A., Kudlacek, O., Wewer, U. M., Yurchenco, P. D. and Fässler, R. (2005). PINCH1 regulates cell-matrix and cell-cell adhesions, cell polarity and cell survival during the peri-implantation stage. *J. Cell Sci.* **118**, 2913-2921.
Liang, X., Zhou, Q., Li, X., Sun, Y., Lu, M., Dalton, N., Ross, J., Jr and Chen, J. (2005). PINCH1 plays an essential role in early murine embryonic development but is dispensable in ventricular cardiomyocytes. *Mol. Cell. Biol.* **25**, 3056-3062.
Liu, J., He, X., Corbett, S. A., Lowry, S. F., Graham, A. M., Fässler, R. and Li, S. (2009). Integrins are required for the differentiation of visceral endoderm. *J. Cell Sci.* **122**, 233-242.
Masuelli, L. and Cutler, M. L. (1996). Increased expression of the Ras suppressor Rsu-1 enhances Erk-2 activation and inhibits Jun kinase activation. *Mol. Cell. Biol.* **16**, 5466-5476.
Meier, P., Finch, A. and Evan, G. (2000). Apoptosis in development. *Nature* **407**, 796-801.
Montanez, E., Piwko-Czuchra, A., Bauer, M., Li, S., Yurchenco, P. and Fässler, R. (2007). Analysis of integrin functions in peri-implantation embryos, hematopoietic system, and skin. *Methods Enzymol.* **426**, 239-289.
Montanez, E., Ussar, S., Schifferer, M., Bösl, M., Zent, R., Moser, M. and Fässler, R. (2008). Kindlin-2 controls bidirectional signaling of integrins. *Genes Dev.* **22**, 1325-1330.
Sandfort, V., Eke, I. and Cordes, N. (2010). The role of the focal adhesion protein PINCH1 for the radiosensitivity of adhesion and suspension cell cultures. *PLoS ONE* **5**, e13056.
Stephens, L. E., Sutherland, A. E., Klimanskaya, I. V., Andrieux, A., Meneses, J., Pedersen, R. A. and Damsky, C. H. (1995). Deletion of beta 1 integrins in mice results in inner cell mass failure and peri-implantation lethality. *Genes Dev.* **9**, 1883-1895.
Stupack, D. G., Puente, X. S., Boutsaboualoy, S., Storgard, C. M. and Cheresh, D. A. (2001). Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J. Cell Biol.* **155**, 459-470.
Tait, S. W. and Green, D. R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat. Rev. Mol. Cell Biol.* **11**, 621-632.
Tu, Y., Li, F., Goicoechea, S. and Wu, C. (1999). The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. *Mol. Cell. Biol.* **19**, 2425-2434.
Weil, M., Jacobson, M. D. and Raff, M. C. (1997). Is programmed cell death required for neural tube closure? *Curr. Biol.* **7**, 281-284.
Wickström, S. A., Lange, A., Montanez, E. and Fässler, R. (2010). The ILK/PINCH/parvin complex: the kinase is dead, long live the pseudokinase! *EMBO J.* **29**, 281-291.
Wu, C. (1999). Integrin-linked kinase and PINCH: partners in regulation of cell-extracellular matrix interaction and signal transduction. *J. Cell Sci.* **112**, 4485-4489.
Zhang, Z., Vuori, K., Reed, J. C. and Ruoslahti, E. (1995). The alpha 5 beta 1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc. Natl. Acad. Sci. USA* **92**, 6161-6165.