Mutations in the Paxillin-binding Site of Integrin-linked Kinase (ILK) Destabilize the Pseudokinase Domain and Cause Embryonic Lethality in Mice*

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Background: Integrin-linked kinase is believed to be recruited to focal adhesions (FAs) by binding paxillin via a conserved motif in the pseudokinase domain.

Results: The paxillin-binding motif is not required for FA localization but for ILK stability, parvin and paxillin binding, and mouse development.

Conclusion: ILK does not require paxillin for FA localization.

Significance: Reducing ILK stability perturbs cell migration and development.

Integrin-linked kinase (ILK) localizes to focal adhesions (FAs) where it regulates cell spreading, migration, and growth factor receptor signaling. Previous reports showed that overexpressed ILK in which Val386 and Thr387 were substituted with glycine residues (ILK-VT/GG) could neither interact with paxillin nor localize to FA in cells expressing endogenous wild-type ILK, implying that paxillin binding to ILK is required for its localization to FAs. Here, we show that introducing this mutation into the germ line of mice (ILK-VT/GG) caused vasculogenesis defects, resulting in a general developmental delay and death at around embryonic day 12.5. Fibroblasts isolated from ILK-VT/GG mice contained mutant ILK in FAs, showed normal adhesion to and spreading on extracellular matrix substrates but displayed impaired migration. Biochemical analysis revealed that VT/GG substitutions decreased ILK protein stability leading to decreased ILK levels and reduced binding to paxillin and α-parvin. Because paxillin depletion did not affect ILK localization to FAs, the embryonic lethality and the in vitro migration defects are likely due to the reduced levels of ILK-VT/GG and diminished binding to parvins.

Integrin-mediated adhesion of cells to extracellular matrix proteins triggers signaling events that govern important cellular processes such as polarity, migration, proliferation, and survival of cells. Because integrin cytoplasmic domains are short and lack catalytic activities, their signaling depends on protein assemblies that form at the adhesion site and are called focal adhesions (FAs).2 The assembly of FAs is triggered by proteins that bind directly to the cytoplasmic domains of integrin subunits, such as talin, kindlin, paxillin, integrin-linked kinase (ILK), and etc. (1).

ILK is a scaffold protein that consists of five N-terminal ankyrin-like repeats and a C-terminal pseudokinase domain (2). It can either be recruited indirectly to integrin tails through binding kindlins or directly by binding the cytoplasmic domains of β1 and β3 integrins (3). Prior to FA recruitment ILK interacts with the LIM proteins PINCH1 or -2 (encoded by Lim1 and Lim2) (4) and the actin-binding α-, β-, or γ-parvin (encoded by Parva, Parvb, and Parvc) (5) to form the ILK/PINCH/Parvin (IPP) complex. IPP complex assembly is required for FA recruitment and the stability of the individual components (6–8). Upon FA recruitment, the IPP complex associates with F-actin and binds regulators of small GTPases. These properties enable the IPP complex to organize the cytoskeleton during cell spreading, polarization, and migration (9, 10). ILK and its interactors are also implicated in the cross-talk between adhesion- and growth factor receptor-dependent signaling (11, 12), which is essential for cell proliferation, survival, and multiple actin-dependent processes. Genetic studies in mice confirmed these functions. Constitutive loss of ILK abrogates polarity and adhesion of the epiblast resulting in lethality at the peri-implantation stage (13). Tissue-specific ablation identified crucial functions of ILK at later stages of development; ILK gene ablation in keratinocytes for example, revealed adhesion and migration defects and in addition, uncovered a role for anchoring microtubules to FAs required for cell polarity (14, 15). Ablation of the ILK gene in cultured endothelial

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2 The abbreviations used are: FA, focal adhesion; IPP, ILK/PINCH/Parvin; E8, embryonic day 8; FN, fibronectin; ILK, integrin-linked-kinase; MEF, mouse embryonic fibroblast; PaxBS, paxillin-binding site; TRITC, tetramethylrhodamine isothiocyanate; TS, Theiler stage.
Mutations in the PaxBS Destabilize ILK

cells resulted in defective cell spreading, whereas ILK loss in endothelial precursor cells leads to defective vasculogenesis and embryonic lethality between E8.5 and E12.5 (16).

The FA protein paxillin binds directly to α4 integrin tails and indirectly to β-tails through other FA proteins and is also capable of recruiting FA proteins to the adhesion site. Paxillin consists of five leucine and aspartate repeat (LD) motifs followed by four LIM domains (17). Paxillin-null mice die shortly after gastrulation due to defective mesoderm development (18), and paxillin-null cells exhibit defective focal adhesion kinase activation, delayed spreading, shape defects, and impaired migration (18, 19). In a search for paxillin binding motifs in FA proteins, a conserved paxillin-binding site (PaxBS) was identified in α-/β-parvin, ILK, and several other proteins. The binding site of ILK and parvin was mapped to the LD motifs of paxillin. Pulldown experiments with recombinant proteins revealed that co-precipitations of both ILK and α-parvin with paxillin were abolished when their respective PaxBS were mutated. Furthermore, overexpressed PaxBS mutant ILK or α-parvin was unable to localize to FA, whereas paxillin localization was unaffected (20, 21). Although these studies were done in cells that still express endogenous ILK and parvin, the results suggested that paxillin binding through the PaxBS in ILK and parvin is necessary for targeting them to FA. However, recent structural analysis confirmed a direct interaction between paxillin LD repeat-containing peptides and α- or β-parvin but disputed that the proposed PaxBS in parvin represents a counter binding site for paxillin (22–25). Based on these studies, it was proposed that a mutant PaxBS in parvin did not impair paxillin binding but rather the protein fold containing the PaxBS in parvin. Although there is no structural information available detailing the interaction between ILK and paxillin, the PaxBS residues of ILK (Val386/Thr387) were resolved in the ILK pseudokinase domain/α-parvin CH2 domain co-crystal structure (Protein Data Bank code 3KMW) (5). According to this structure, Val386/Thr387 are part of the αF helix (residues 370–387) in the C-terminal lobe of the ILK pseudokinase domain and located on the protein surface and therefore principally allow a direct interaction with paxillin (Fig. 1). The side chains of Val386/Thr387 are in close proximity (3.2–4.0 Å distance) and thus likely interact with Trp383 in the αF helix, Glu389 in the F/G loop, Pro411 and Ile417 in the G/H loop, and Val421 and Met425 in the αH helix. Trp383 in turn seems to stabilize the F/G and G/H loops and thus the αG helix via Pro391 and Pro411. It is therefore possible that the substitutions of Val386/Thr387 with glycines abolish these interactions, leading to increased flexibility of αF to H helices and their connecting loops. Such a destabilization might result in the unfolding and degradation of the mutant ILK, besides perturbing the interactions with parvin or with paxillin.

To better understand the PaxBS mutation in ILK, we generated and analyzed transgenic mice and cells expressing PaxBS mutant ILK (ILK-VT/GG). Mice homozygous for the mutation die between E8.5 and E12.5 and exhibit general growth retardation, likely caused by defective vasculogenesis. In vitro analysis with cells derived from the mutant mice revealed diminished directionality of cell migration. Although PaxBS mutant ILK is normally recruited to FAs, its stability and ability to bind α-parvin and paxillin were decreased. Depletion or overexpression of paxillin neither altered wild-type or PaxBS mutant ILK protein levels nor their subcellular localization, indicating that the defects caused by the PaxBS mutant ILK occur in a paxillin-independent manner.

EXPERIMENTAL PROCEDURES

Mouse Strains—Mice carrying the ILK-VT/GG substitution were generated as described previously for other ILK mutations (2). In brief, a loxP-flanked neomycin cassette was inserted into exon 13 of the murine ILK gene (13). The GTGACA sequence in exon 12 encoding for valine 386 and threonine 387 was mutated to GGCAGC coding for glycines by site-directed mutagenesis (Stratagene) (supplemental Table S1). The construct was electroporated into R1 ES cells (26). Homologous recombinant clones were identified by Southern blot using genomic DNA with an internal and a 3’ external probe, and by PCR using oligonucleotides PBS-ILK-geno_f and PBS-ILK-geno_r (supplemental Table S1). These oligonucleotides were also used for genotyping using standard methods. Mutant ES cells were injected into C57B6 blastocysts to generate germ line chimaeras. Mutant offspring were intercrossed with deleter-Cre transgenic mice (27) to remove the neomycin cassette. Mice were kept and bred according to Bavarian animal welfare laws in local animal facilities, and they were backcrossed at least seven times to the C57BL/6 genetic background prior to analysis.

Cell Culture—Mouse embryonic fibroblasts (MEFs) were isolated at E9.0 by dissolution of whole embryos with trypsin/EDTA and subsequent plating of cells onto collagen I/fibronect-
Mutations in the PaxBS Destabilize ILK

Random migration was performed at 37 °C/5% CO₂ using a live cell imaging setup with one picture taken every 5 min. For random migration assays, sparsely seeded non-dividing polarized cells were tracked for 6 h (20 × 0.4 numerical aperture objective). Cell tracking was performed with ImageJ software and the cell tracking add-on (32), whereas cell velocity and directionality were computed using the chemotaxis and migration tool (version 2.0, Ibidi). Directionality is the quotient of actual and Euclidian distance of a cell track. Haptotactic cell migration was assayed as described previously (33). Transwells with 8-μm pores were coated with fibronectin (0.5 μg/ml), and 1 × 10⁵ cells were added to the upper well in serum-free medium. Cells that migrated through the filter after 4 h were counted and are expressed as migrated cells per high power field (400×).

Antibodies—Primary antibodies used for immunoblotting, immunofluorescence, or flow cytometry are detailed in supplemental Table S2. Appropriate HRP- or fluorophor-conjugated secondary antibodies were obtained from Bio-Rad or Jackson ImmunoResearch Laboratories.

Flow Cytometry—Flow cytometry measurements were performed as described previously (34). Briefly, cells were trypsinized and stained with primary antibodies for 10–30 min on ice, washed, and stained with secondary antibody for 10–30 min on ice. After washing, cells were resuspended in 0.5% (w/v) BSA/PBS. Flow cytometry was performed in triplicates with a FACSCalibur flow cytometer (Becton & Dickinson).

Immunofluorescence—Cryo-sections of 8-μm thickness from embryonic tissues were prepared and embedded according to standard protocols. Cells were grown on glass-coated with 5 mg/liter FN. Samples were either paraformaldehyde-fixed (10 min in 3.7% paraformaldehyde/PBS, permeabilized 3 min with 0.1% Triton X-100/PBS) or methanol/acetone fixed (twice 5 min with ice-cold methanol/acetone 1:1, air drying). Tissue sections were blocked for 1 h in 5% BSA/PBS and then treated with 0.1% Triton X-100/PBS for 20 min, and cells were blocked for 1 h in 1% BSA/0.1% Triton X-100/PBS. Primary antibodies were diluted in blocking solution and applied overnight at 4 °C. After washing with PBS, appropriate secondary antibodies were diluted in blocking solution and applied for 1 h at room temperature. After washing and DAPI staining (1:10,000 in PBS), slides were mounted in Elvanol. Pictures were taken with a TCS SP5 AOBS confocal laser scanning microscope (Leica). All stainings were repeated at least three times.

Western Blotting and Immunoprecipitations—Cells or embryos were homogenized in 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 0.1% w/v SDS, 1.0% (w/v) sodium deoxycholate, 1.0% (v/v) Triton X-100, phosphatase inhibitor cocktails P1 and P2, pH 7.6; all by Sigma) supplemented with Complete protease inhibitors (Roche Applied Science), followed by sonication for 30 s at 4 °C. Equal amounts of total protein per lane were separated on a polyacrylamide gel and transferred to PVDF membranes (Millipore). Membrane blocking and antibody dilution was performed with TBS, pH 7.6, supplemented with 0.1% Tween 20 (Serva) and 2% skim milk (Fluka) or 5% BSA (PAA Laboratories). Subsequently, membranes were incubated for 1 h at room temperature or overnight at 4 °C with primary
Mutations in the PaxBS Destabilize ILK

**RESULTS**

**ILK<sup>ki/ki</sup> Embryos Display a General Growth Retardation and Die Before E12.5**—We generated ILK<sup>+/-</sup>(VT/GG) knock-in mice (abbreviated ILK<sup>ki/ki</sup>) by homologous recombination in ES cells as described previously (2) (supplemental Fig. S1, A–D). ILK<sup>ki/ki</sup> mice were viable, fertile, and indiscernible from their wild-type littermates. Heterozygous intercrosses failed to produce homozygous ILK<sup>ki/ki</sup> mice at birth, indicating that the mutation is embryonic lethal (Table 1). To identify the time of lethality, we performed timed matings and staged isolated embryos by developmental hallmarks and somite pair numbers according to Theiler (35). Between embryonic day (E) 8.0 and 12.5 there were only sporadic embryonic resorptions of controls (ILK<sup>+/-</sup> and ILK<sup>ki/ki</sup>) (Table 2). ILK<sup>ki/ki</sup> embryos developed normally to Theiler stage (TS) 11 corresponding to E8.0. However, after this stage, an increasing percentage was resorbed, and at E12.5, no live ILK<sup>ki/ki</sup> embryos could be isolated (Table 2).

At E8.0, control and ILK<sup>ki/ki</sup> embryos were in the late neural plate/early head-fold stage of development (TS 11a-c). The amnion was not fully closed in ~20% of ILK<sup>ki/ki</sup> embryos, indicating a slight delay in development compared with controls. Although almost all E8.5 control embryos were in the late pre-turning (TS 12b) or turning stage (TS 13), the majority of E8.5 ILK<sup>ki/ki</sup> embryos were still in head-fold (TS 11c/d) or early pre-turning stages (TS 12a) (Table 3). Despite the general retardation, organogenesis, e.g. of the heart was normal (Fig. 2A). At E9.5, wild-type embryos had completed turning and were forming and closing the anterior neuropore (TS 15), whereas 95% of the E9.5 ILK<sup>ki/ki</sup> embryos were either in the midst of or just finished turning (TS 13/14) (Fig. 2A). After E9.5, most ILK<sup>ki/ki</sup> embryos were either in the process of being resorbed or showed a pronounced delay compared with control littermates.

Although the general delay in development of ILK<sup>ki/ki</sup> embryos implies pleiotropic defects, the most striking abnormality was the absence of a vasculature on ILK<sup>ki/ki</sup> yolk sacs at E9.5 and later (Fig. 2B). To define the vascular abnormality in more detail, we investigated yolk sac vasculogenesis at E8.5 and 9.5. In E8.5 controls, precursor cells originating from proximal cavernous blood islands gave rise to a distal primitive vascular plexus close to the embryo proper and demarcated by a continuous FN-containing basement membrane (Fig. 2C). Although FN staining revealed that ILK<sup>ki/ki</sup> yolk sacs also contained proximal blood islands, they lacked discernible distal vascular structures (Fig. 2C). At E9.5, the presence of veins, arteries, and capillaries indicated angiogenic remodeling in control yolk sacs. However, ILK<sup>ki/ki</sup> yolk sacs only displayed a dilated primitive plexus and no discernible arteries, veins, and capillaries (Fig. 2D).

To test whether the ILK-VT/GG mutation allowed integrin-mediated assembly of basement membranes, we performed immunofluorescence stainings for FN and laminin in the embryonic neuroectoderm (Fig. 2E). Control and ILK<sup>ki/ki</sup> embryos showed normal basement membrane assembly without blisters or other cell adhesion defects such as basement membrane splitting. Taken together, these data show that ILK<sup>ki/ki</sup> embryos display a general delay in their development after E8.0 with defective vasculogenesis and death between E8.5 and E12.5. The normal formation of blood islands indicates that early hematopoietic development was normal in ILK<sup>ki/ki</sup> yolk sacs, whereas the subsequent establishment of the vascular plexus was delayed.

ILK<sup>ki/ki</sup> Cells Have a Migration Defect—The formation of the primitive vascular plexus crucially depends on the migration of endothelial precursor cells that originate from yolk sac blood islands and migrate to the distal yolk sac regions (36). We therefore decided to test whether the ILK<sup>ki/ki</sup> mutation affects cell migration. Because we could not obtain enough endothelial cells from yolk sac tissues, we isolated and immortalized embryonic cells (MEFs) from E9.0 control and ILK<sup>ki/ki</sup> embryos. ILK<sup>ki/ki</sup> MEFs showed normal surface expression levels for β1, β3, αV, and α5 integrins as well as normal cell attachment and spreading (Fig. 3, A–C). Consistent with these observations, we found that immediate integrin-dependent signaling such as focal adhesion kinase and AKT phosphorylation were similar in control and ILK<sup>ki/ki</sup> cells following plating on FN (Fig. 3D). However, we found that ILK<sup>ki/ki</sup> MEFs have a significantly reduced capacity to transmigrate through FN-coated porous Transwell membranes (p < 0.001) (Fig. 3E). To determine whether this defect is due to decreased migration velocity or directionality, we performed random migration assays and found that migration velocities of ILK<sup>ki/ki</sup> MEFs were increased, whereas the directionality of migration was decreased (Fig. 3F).

Thus, the developmental vasculogenesis defects likely arise from directional migration defects of endothelial precursor cells.
TABLE 2
Mendelian ratios determined at different stages of embryogenesis

Shown is the number of embryos resulting from timed ILK<sup>+/−</sup> intercrosses harvested at indicated days of embryogenesis. Unknown genotype refers to resorbing embryos where genotyping was impossible. Embryo numbers are given by genotype as normal/delayed/resorbing embryos. Normal refers to embryos comparable with the majority of littermates; delayed denotes embryos at least one stage behind the majority of littermates; resorbing refers to genotypeable embryonic resorbates. p(all, Chi²) gives the result of a Chi square test with ideal Mendelian inheritance of all genotypeable embryos. p(normal, Chi²) excludes delayed and resorbing embryos.

| TS     | ILK<sup>+/−</sup> | ILK<sup>ki/ki</sup> | ILK<sup>ki/ki</sup> | p (all, Chi²) | p (normal, Chi²) |
|--------|------------------|---------------------|---------------------|--------------|-----------------|
| E8.0   | 35 (6)           | 9/0/0               | 4/0/0               | 0.70         | 0.38            |
| E8.5   | 215 (17)         | 41/7/1              | 21/4/6              | 0.18         | 1.1 × 10⁻⁴     |
| E9.5   | 111 (11)         | 25/2/2              | 15/1/2              | 0.06         | 20 × 10⁻⁴      |
| E10.5  | 73 (3)           | 20/0/0              | 1/12/3              | 0.80         | 2.5 × 10⁻⁴     |
| E12.5  | 70 (17)          | 15/0/1              | 0/0/9               | 0.36         | 8.5 × 10⁻⁴     |

The Stability of ILK-VT/GG Protein Is Reduced—To discern how the ILK-VT/GG mutation caused the cellular migration defect, we performed a biochemical characterization of IPP and paxillin expression levels by immunoblotting. IPP protein levels were severely reduced in both ILK<sup>ki/ki</sup> embryos and MEFs compared with controls, whereas paxillin levels were unchanged (Fig. 4, A and B). Because reduced stability of the ILK-VTGG protein could explain the observed reduction in PINCH and α-paxillin levels, we treated control and ILK<sup>ki/ki</sup> MEFs with cycloheximide to inhibit protein synthesis and monitored the decrease of ILK levels over time. Control MEFs displayed a 22 ± 2% reduction of ILK after cycloheximide treatment for 20 h (Fig. 4C). In contrast, ILK<sup>ki/ki</sup> MEFs showed a 58 ± 23% reduction in ILK-VT/GG levels, indicating that the protein turnover of the mutant ILK was 2.6-fold increased in ILK<sup>ki/ki</sup> cells (p = 0.005) (Fig. 4C). By comparison, the reduction of β1 integrin was similar in both cell lines (50 ± 17% versus 50 ± 9%, p = 0.97) (Fig. 4C), indicating that the increased turnover of ILK-VT/GG was specific.

It has been reported that ILK-VT/GG was unable to localize to FAs when ectopically expressed in various cell lines (20, 21). To confirm this finding in ILK<sup>ki/ki</sup> MEFs, we plated MEFs on FN and visualized ILK, parvin, and paxillin by immunostaining. Unexpectedly, all three proteins localized normally to FAs in the absence of endogenous ILK, it failed to localize to FAs in the presence of endogenous ILK (Fig. 4D). This confirms the hypothesis that endogenous ILK interferes with ILK-VT/GG fusion to the fluorophor Venus into ILK-floxed cells expressing endogenous ILK. An aliquot of these cells was used to transiently express the Cre recombine, which deleted the endogenous ILK and yielded ILK-null cells overexpressing either of the two constructs. When examining the localization of Venus-tagged ILK proteins, we observed ILK-WT in FAs as shown by Vinculin co-localization both in cells expressing or lacking endogenous ILK (Fig. 4E). Remarkably, although ILK-VT/GG localized to FA in the absence of endogenous ILK, it failed to localize to FAs in the presence of endogenous ILK (Fig. 4E). This confirms the hypothesis that endogenous ILK interferes with ILK-VT/GG fusion to FAs. Taken together, these data show that the ILK-VT/GG mutation decreases the stability of the IPP complex without affecting paxillin levels or localization.

Overexpression of ILK-VT/GG Fails to Normalize Parvin Levels and Migration—Our data suggest that the mutation of the PaxBS in ILK leads to overall instability of the protein, which in turn decreases levels of PINCH and α-paxillin. To test whether elevated ILK-VT/GG levels would normalize IPP and migration, we stably overexpressed FLAG-tagged WT or ILK-

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FIGURE 2. ILK<sup>ki/ki</sup> embryos display developmental delays and defective yolk sac vasculogenesis. A, representative control or ILK<sup>WT</sup> (ki/ki) E8.5 (TS 12b, 12a) and E9.5 (TS 15, 14) embryos. Arrowheads indicate the heart. B, dark field picture of representative E9.5 control and ILK<sup>ki/ki</sup> yolk sacs. C, FN immunofluorescence staining of proximal or distal E8.5 control or ki/ki yolk sac whole mounts. D, PECAM-1 immunofluorescence staining of E9.5 control or ki/ki yolk sac whole mounts. E, FN or pan-laminin (LM; green) immunofluorescence staining of E8.5 embryonic neuroectoderm. Nuclei were DAPI-stained (blue). Scale bar, 200 μm (A) and 50 μm (B–D).
Mutations in the PaxBS Destabilize ILK

VT/GG in ILK-null fibroblasts. This allowed us to increase ILK-WT levels to 4.2 times and ILK-VT/GG to 2.6 times when compared with the amount of endogenous ILK in parental ILK-floxed cells (Fig. 5A). Although PINCH levels increased concomitantly with ILK-VT/GG, α-parvin levels remained low. The levels of paxillin and of the HSP90, which was shown to stabilize ILK by binding the pseudokinase domain (37), were similar in all cell lines analyzed. Consistent with the inability to normalize α-parvin levels, ILK-VT/GG overexpressing cells showed the same migration defects as ILK<sup>ki/ki</sup> MEFs (Fig. 5B), whereas adhesion was increased (Fig. 5C). Taken together, these results suggest that elevating ILK-VT/GG levels rescued PINCH, but not parvin levels or the migration defect.

ILK-VT/GG Binds Less α-Parvin and Paxillin—ILK protein is stabilized by binding PINCH, parvin, and HSP90 (6–8, 37). The reduced ILK-VT/GG stability raises the possibility that binding to these interactors is impaired. We tested this hypothesis by determining the amounts of bound interactors using anti-FLAG immunoprecipitation followed by immunoblotting (Fig. 5D). PINCH, which binds the ankyrin repeat domains, and HSP90 were co-immunoprecipitated to a similar extent in ILK-WT and ILK-VT/GG-expressing cells. In contrast, co-immunoprecipitation of α-Parvin was severely diminished. Because ILK, PINCH, and parvin protein levels depend on each other, decreased parvin binding by ILK-VT/GG could cause the reduction of IPP protein levels in ILK<sup>ki/ki</sup> cells. Interestingly, the ILK-VT/GG mutation reduced but did not abolish paxillin binding. The reduced amounts of paxillin in ILK-VT/GG precipitates point to either an impaired direct binding of paxillin to ILK-VT/GG or alternatively reduced indirect binding to ILK-VT/GG due to less co-precipitated parvin.

Depletion or Overexpression of Paxillin Does Not Affect Levels or Localization of ILK—If reduced paxillin binding to ILK-VT/GG was responsible for the instability of the mutant ILK protein, depletion of paxillin levels should destabilize ILK-WT. To test this hypothesis, we depleted paxillin in ILK-floxed cells by RNAi with a paxillin-specific shRNA (shPxn) and also rescued the shPxn cells by re-expressing a shPxn-resistant human PAXILLIN cDNA (shPxn+Pxn). Control cells expressed scrambled control shRNA (shCtrl). The shPxn depleted endogenous paxillin by 97% and re-expression of human PAXILLIN resulted in a 13-fold increase in protein levels (Fig. 6A). Using an anti-paxillin antibody with cross-reactivity against Hic-5, we failed to detect a specific signal in immunostainings of shPxn...
Mutations in the PaxBS Destabilize ILK

In the present study, we analyzed the consequences of disrupting the potential PaxBS in the pseudokinase domain of ILK in vivo and in vitro. The substitutions of Val396 and Thr397 with glycines (ILK-VT/GG) were reported to disrupt paxillin-binding and to prevent ILK-VT/GG recruitment to FAs. We show in the present paper that ILK-VT/GG mice developed vasculogenesis defects, resulting in general growth retardation and onset of lethality at midgestation. Interestingly, the VT/GG mutation severely impaired the protein stability and reduced the binding to parvin but did not affect the recruitment of ILK-VT/GG to the FAs of MEFs derived from mutant mice.

cells, suggesting that both paxillin and Hic-5 were absent (Fig. 6B). Depletion of paxillin phenocopied paxillin-deficient cells (19) and showed diminished focal adhesion kinase activity, abnormal cell shape, and impaired spreading. As expected, overexpression of PXN increased focal adhesion kinase activity and cell spreading, indicating that the defects due to paxillin knockdown were specific (Fig. 6, A and B). Despite the pronounced cellular phenotypes following paxillin knockdown, ILK levels and ILK recruitment to FAs were unaffected (Fig. 6, A and B).

To test whether increasing paxillin levels stabilizes ILK-VT/GG, we stably transduced paxillin tagged with mCherry (Cherry-paxillin) into cells expressing ILK-WT or -VT/GG. Increasing paxillin levels did not change ILK-WT or ILK-VT/GG levels (Fig. 6C). Because changes in paxillin expression had no discernible impact on levels or subcellular localization of wild-type or mutant ILK, we conclude that the reduced paxillin binding to ILK-VT/GG plays little or no role in the defects observed in ILK<sup>−/−</sup> mice.

**DISCUSSION**

In the present study, we analyzed the consequences of disrupting the potential PaxBS in the pseudokinase domain of ILK in vivo and in vitro. The substitutions of Val<sup>396</sup> and Thr<sup>397</sup> with glycines (ILK-VT/GG) were reported to disrupt paxillin-binding and to prevent ILK-VT/GG recruitment to FAs. We show in the present paper that ILK-VT/GG mice developed vasculogenesis defects, resulting in general growth retardation and onset of lethality at midgestation. Interestingly, the VT/GG mutation severely impaired the protein stability and reduced the binding to parvin but did not affect the recruitment of ILK-VT/GG to the FAs of MEFs derived from mutant mice.
Mutations in the PaxBS Destabilize ILK

Our results differ from previous in vitro data, where ILK-VT/GG overexpressed in various cell lines was unable to interact with parvin and paxillin and to localize to FAs (20, 21). We could resolve this discrepancy by showing that endogenous wild-type ILK prevents FA localization of co-expressed ILK-VT/GG, whereas in absence of endogenous ILK, FA localization of ILK-VT/GG was normal. Because endogenous wild-type ILK and co-expressed mutant ILK-VT/GG apparently compete for interactors necessary for FA targeting, we conclude that ILK-VT/GG has most likely a reduced affinity to these protein(s) compared with wild-type ILK. This decrease is independent of paxillin because neither overexpression nor near-complete RNAi-mediated depletion of paxillin affected the localization of ILK. A related study has recently shown that deletion of the paxillin gene also did not prevent localization of the obligate ILK binding partner parvin to FAs (22). Although the related family member Hic-5 may have taken over the “recruitment role” of paxillin, the absence of detectable Hic-5 after RNAi-mediated paxillin depletion in our cell system argues against this possibility. Taken together, these findings suggest there is no role for paxillin in FA recruitment of ILK.

Despite the localization of ILK-VT/GG to FAs, we noted a >75% decrease of ILK-VT/GG and concomitantly of PINCH and parvin protein levels in mutant embryos and cells, which was due to an accelerated protein turnover. The decreased protein stability of ILK-VT/GG supports the notion that the amino acid substitutions destabilized folding (4). The destabilization might directly result in unfolding and degradation of ILK and/or affect ILK binding to known direct interactors such as parvin, which would indirectly promote degradation of both ILK and parvin, as both proteins expose a large hydrophobic interface in the non-bound state. Although the mutual dependence of ILK/PINCH/parvin protein stability on their interaction is well documented (6–8), there are currently no data available pointing to an involvement of paxillin in IPP complex stability. Our data revealed that ILK protein stability was unaffected upon paxillin depletion or overexpression. However, parvin protein levels were more severely decreased in mutant cells and embryos compared with ILK or PINCH. Moreover, overexpression of ILK-VT/GG failed to rescue parvin levels, whereas PINCH levels did increase. The explanation for this difference in protein stability could be the ability of ILK, but not of parvin, to directly bind the chaperone HSP90 (37, 41). Because paxillin can associate with the IPP complex through either direct binding to ILK or indirect binding to parvin, it is possible that the reduced paxillin in ILK-VT/GG immunoprecipitates is a consequence of the reduced parvin binding to ILK rather than a reduced direct binding to the PaxBS-deficient ILK.

We have previously reported that cells with a complete loss of ILK expression show impaired cell spreading and directional cell migration (13, 14). Interestingly, the >75% reduction in IPP proteins in ILK-VT/GG cells enabled adhesion and spreading but reduced the directionality of migration. This interesting observation points to a hierarchical requirement of IPP for cellular functions, with efficient directionality of cell migration requiring >25% of normal IPP levels, and cell adhesion and spreading depending on <25% of normal IPP levels. It is, however, also possible that an ILK-parvin-paxillin scaffold within FAs is required for directional cell migration but not for adhesion and spreading. The inability to rescue the cell migration defect by overexpressing ILK-VT/GG supports the latter possibility. The paxillin-parvin complex has been crystallized, and the binding interface was precisely determined and mapped (22–24). A similar endeavor carried out with in vitro expressed ILK/paxillin proteins could show whether such a function is principally possible to occur in FAs.

How do the alterations we observed on molecular and cell level relate to the phenotype that we observed in vivo? ILK-VT/GG embryos had a less severe defect than ILK-null embryos, which die at peri-implantation due to defective cell adhesion, spreading, polarity, and F-actin distribution (13). These processes were not affected in ILK-VT/GG embryos and therefore implantation also proceeded normally. Instead, we observed a general developmental delay with an onset at around E8.5 and fully penetrant embryonic lethality by E12.5, which could be explained with the prominent defects in the yolk sac vasculature. Several mouse strains showing an impaired development of the yolk sac vasculature suffer from pleiotropic defects due to insufficient nutritional supply and die at around E12.5 (38–40). The ILK-VT/GG phenotype resembled the endothelial cell-specific deficit of the ILK gene, which also resulted in a lethal vasculogenesis defect (16). Vascular development critically depends on directed migration of endothelial precursor cells (36), which is also affected in ILK-VT/GG cells. Reduced cell migration directionality upon loss of ILK in keratinocytes was also previously shown to disrupt hair follicle morphogenesis (15). We can currently only speculate if and to what extent defective cell migration contributes to the observed general developmental delay in other tissues. When considering the large phenotypic overlap with other mutants experiencing defective yolk sac development, we believe that perturbed yolk sac vasculogenesis due to defective cell migration is likely the dominant cause for the embryonic lethality.

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Mutations in the PaxBS Destabilize ILK

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