Cellular force generation in focal adhesion maturation and extracellular matrix remodeling

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Abstract
The extracellular matrix (ECM) functions as a structural scaffold for tissues, but it also drives intracellular signaling by interacting with specific receptors and by regulating the bioavailability of growth factors. This unique combination of functions makes the ECM an important regulator of organ development and maintenance. The deposition and remodeling of the ECM into a precise configuration is a cell-dependent process that requires integrin adhesion receptors as well as generation of cellular forces. Integrin-linked kinase (ILK) is an essential adaptor protein that binds to β1- and β3-integrin cytoplasmic tails and links them to the actin cytoskeleton. Our work has uncovered functions of ILK in cellular force transduction and ECM remodeling and the role of these processes in cell fate regulation.

Interactions between cells with their neighbors and the environment not only provide tissues their shape and proper architecture, but also regulate fate decisions of individual cells. Cell-matrix interactions have the potential to propagate signals that regulate proliferation, differentiation, and migration, which ensure coordinated cell behaviors during development and tissue homeostasis (Wickström et al., 2011). Cells further actively remodel the extracellular matrix (ECM) thereby engaging in dynamic crosstalk with their environment (Daley and Yamada, 2013).

The ECM and integrins
The ECM is a complex non-cellular network composed mainly of fibrous proteins and proteoglycans that determine the biochemical and mechanical properties of the tissue. It serves as a physical scaffold, but also as a platform for intercellular communication and as a reservoir for growth factors. Therefore the composition and organization of the ECM needs to be tightly controlled (Frantz et al., 2010; Watt and Fujiwara, 2011; Gattazzo et al., 2014).
Integrins are the major ECM receptors in all metazoans. Accordingly, their main task is to facilitate the adhesion of cells to the ECM. In addition, intracellular coupling of integrins to the actin and intermediate filament cytoskeletons allows generation of traction forces and regulation of cell shape and mechanics. Finally, integrins are able to assemble large intracellular signaling platforms termed focal adhesions (FAs) that activate signaling cascades. These unique features make integrins essential for a large number of cellular processes (Legate et al., 2009; Wickström et al., 2011).
Integrins comprise of 18 α and 8 β subunits that assemble non-covalently into 24 distinct heterodimers (Hynes, 2002). The specific subunit combination determines their binding affinity and ligand specificity. A hallmark of integrin receptors is their ability to mediate bi-directional signaling. "Inside-out" signaling regulates the ligand binding properties of integrins and is induced by

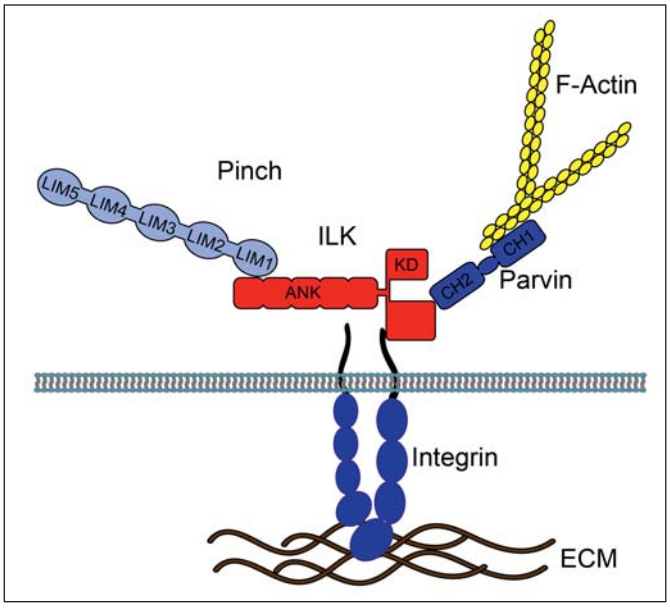

Figure 1: Integrin-linked kinase and its main binding partners
Integrin-linked kinase (ILK) is composed of two domains that are connected by a short linker: the N-terminal ankyrin repeat domain (ARD) and a C-terminal kinase domain (KD). The ARD binds to the LIM1 domain of particularly interesting Cys-His-rich protein (PINCH). The KD of ILK is a protein-protein interaction domain that binds among others to the CH2 domain of parvins and the cytoplasmic tails of β integrins. Parvins are capable of binding F-actin, thereby facilitating a direct link between integrins and the actin cytoskeleton. It is not clear, however, whether ILK binds β integrins directly in vivo and whether parvins can bind actin while bound to ILK (modified from Ghatak et al., 2013).

non-integrin mediated signaling pathways such as growth factor signaling. "Outside-in" signaling regulates cellular responses induced by ligand binding to integrin receptors that regulate cell spreading, migration and proliferation (Hynes, 2002). Upon ligand binding, integrins cluster at the plasma membrane and various integrin-binding proteins are recruited to their cytoplasmic tails to form FAs. These core FA components subsequently recruit a large number of actin-modulatory proteins and signaling molecules allowing actin stress fiber formation, FA maturation, and propagation of intracellular signaling cascades (Legate et al., 2009; Wickström et al., 2011).

Integrin-linked kinase – a pseudokinase with adaptor function

ILK is a central component of β1- and possibly also of β3-integrin adhesion complexes. It is ubiquitously expressed, consists of 452 amino acids, and has a molecular weight of 52 kDa. ILK was originally identified in a yeast two-hybrid screen as a direct binding partner of β1-integrin (Hannigan et al., 1996). Due to its sequence homology to protein kinases as well as in vitro observations showing that ILK is capable of phosphorylating substrates such as GSK-3β and PKB/AKT and β1-integrin, it was initially reported to be a serine/threonine kinase (Hannigan et al., 1996).

ILK is composed of five N-terminal ankyrin repeat (ANK) domains, followed by a pleckstrin homology (PH)-like sequence and an N-terminal kinase domain (KD). Together the five ANK domains form a superhelical spiral that serves as a binding domain for PINCH, another important integrin adaptor (Chiswell et al., 2008). The PH-like sequence of ILK is integrated into the ILK-KD and is in fact not capable of binding the second messenger phosphatidylinositol 3-phosphate (PIP3). The KD of ILK functions as a protein-protein interaction domain that binds among others the calponin homology 2 (CH2) domain of parvin (Fukuda et al., 2009; Stiegler et al., 2013). In cells, ILK, PINCH and parvin are mostly present in a ternary complex within FAs (Fig. 1).
Despite initial in vitro observations of kinase activity, inspection of the KD sequence already raised doubts about its catalytic activity. During phosphotransfer, the DFG (Asp-Phe-Gly) motif that is conserved in most eukaryotic kinases mediates the alignment of the γ-phosphate, but in ILK this motif is replaced by DVK (Asp-Val-Lys). Phosphotransfer further requires the proton acceptance from the hydroxyl group catalyzed by the aspartate residue in the HRD (His-Arg-Asp) motif that is also lacking in ILK (Wickström et al., 2010a). It is generally presumed that the presence of both DFG and HRD motif is required for kinase activity, and no kinase with reported activity lacks both of the motifs (Boudeau et al., 2006).

Definitive proof for the lack of catalytic activity was provided by the crystal structure of the ILK-KD bound to the CH2 domain of α-parvin, one of the 3 mammalian parvin isoforms. It revealed that ILK-KD folds into a typical bilobial kinase structure but it has a dramatically degenerated catalytic core compared to known kinases. The P-loop structure that is essential for ATP-binding contains a non-flexible motif in ILK, which is unable to receive non-transferable phosphates of ATP. Due to the unusual DVK motif the γ-phosphate is abnormally aligned and lies far away from the putative catalytic site. Hence, bound ATP remains in an unhydrolyzed state (Fukuda et al., 2009). It was further demonstrated that kinase activity can be detected in impure protein preparations of recombinant ILK, but this activity is lost upon further purifications steps (Fukuda et al., 2009; Fukuda et al., 2011).

Genetic studies provided further evidence for ILK being a pseudokinase with adaptor function. Deletion of ILK in D. melanogaster leads to embryonic lethality with failure in muscle attachment. Expression of ILK containing a mutation in the kinase domain (E359K) (a reported kinase dead mutant ILK), in ILK-deficient flies completely rescues the phenotype, indicating that ILK fulfills its function independent of kinase activity (Zervas et al., 2001). Similarly, the knockout of pat-4 (paralyzed, arrested elongation at two-fold; C. elegans homolog of ILK) in C. elegans impairs actin and myosin filament recruitment in embryonic muscle, and this can be rescued by the expression of a kinase dead ILK (Mackinnon et al., 2002).

The constitutive deletion of ILK in mice is embryonic lethal (Sakai et al., 2003b). The embryos succumb during peri-implantation due to a failure in epiblast polarization. This is caused by impaired F-actin rearrangement and basement membrane remodeling (Sakai et al., 2003b). In stark contrast, knock-in mice that carry either a R211A mutation within the PH-domain leading to a kinase-dead ILK, or specific mutations within the putative autophosphorylation site leading to a kinase-dead (S343A) or hyperactive (S343D) ILK are viable and healthy and show no differences in phosphorylation of the reported ILK substrates AKT or GSK-3β (Lange et al., 2009), supporting the kinase-independent function of ILK. Knock-in mice with mutations in the ATP-binding site, K220A or K220M, die shortly after birth due to renal dysgenesis (Lange et al., 2009). This mutation in ILK destabilizes the KD and thereby interferes with its ability to bind to α-parvin, demonstrating that ILK-α-parvin interaction is crucial for the function of ILK. α-parvin knockout mice develop similar kidney phenotype as that observed in ILK K220A/M mutants (Lange et al., 2009). Together, all these studies confirm the non-catalytic, adaptor function of ILK.

ILK regulates force generation, adhesion maturation, and actin dynamics

The assembly and remodeling of integrin adhesion complexes is a highly dynamic process that requires the recruitment of adaptor proteins and myosin II-containing actin networks to adhesion sites (Vicente-Manzanares and Horwitz, 2011). Upon cell attachment, integrins bind to the underlying substrate and focal complexes (FCs; also termed nascent adhesions) assemble at the contact site of the cell with the ECM. The maturation of small FCs (~100 nm in size) into large FAs (~1 μm) is driven by active myosin II that enables further recruitment of adhesion-associated proteins with actin-binding or modulatory activity, such as vinculin or paxillin, along polymerizing actin. The integrin-actin connection is subsequently strengthened leading to formation of stress fibers, antiparallel myosin II-containing actin bundles (Zamir and Geiger, 2001; Vicente-Manzanares and Horwitz, 2011).

A large number of studies implicate that the central cellular function of ILK is to regulate adhesion maturation and to establish and maintain the integrin-actin linkage. Mammalian cells lacking ILK display defects in actin reorganization and FA maturation (Sakai et al., 2003b). ILK itself lacks actin-binding properties and the precise molecular details of how ILK regulates actin engagement at FAs are not clear. A possible adaptor linking ILK to actin is parvin that was shown to bind actin through its two in-tandem CH-domains (Olski et al., 2001) (Fig. 1). ILK has also been reported to impact the activity of small GTPases such as RhoA and Rac that modulate actin dynamics but the detailed molecular mechanism of this regulation is not known (Boulter et al., 2006; Kogata et al., 2009; Blumbach et al., 2010). Interestingly, ILK also regulates the architecture and stability of the microtubule network, which might have implications on intracellular signaling as well as on the regulation of GTPase activity (Wickström et al., 2010b).

During FA maturation, the connection between integrin ligands and the actin cytoskeleton is strengthened and myosin II-dependent actin stress fiber formation facilitates cell contraction (Schiller and Fässler, 2013). ILK-deficient fibroblasts display large FAs at the cell edges but absence of nascent FCs and fibrillar adhesions (FBs), a specialized type of adhesion involved in ECM remodeling (Stanchi et al., 2009; Radovanac et al., 2013) (Fig. 2A). It has been proposed that ILK collaborates with α-parvin to segregate α5β1 integrins from FAs, thus allowing the recruitment of tensin and maturation of FBs (Stanchi et al., 2009). Furthermore, the actin cytoskeleton of ILK-deficient fibroblasts is disorganized and poorly linked to the abnormal FAs (Sakai et al., 2003b). As a consequence, these cells are severely compromised in their ability to generate traction forces and to exert force on the underlying ECM (Radovanac et al., 2013) (Fig. 2B, C). Collectively these data indicate that ILK is important for adhesion maturation by acting as an adaptor to establish and maintain the integrin-actin linkage.

ILK is essential for ECM remodeling

During FA maturation fibronectin (FN)-bound integrins such as α5β1 are segregated along the actin cytoskeleton. The subsequent generation of cellular forces and recruitment of additional adaptor proteins such as tensin induces FB formation (Pankov et al., 2000). The cellular force that is applied on FN leads to its conformational changes and self-assembly, resulting in FN fibrillogenesis (Zamir et al., 2000; Ohashi et al., 2002). FB maturation is a prerequisite for subsequent FN fibrillogenesis. Although the expression of FN in ILK-deficient fibroblasts is unaltered compared to controls, FN fibrillogenesis is absent (Radovanac et al., 2013) (Fig. 2D). As the affinity of integrin α5β1 to its ligand FN

is not altered in ILK-deficient cells (Vouret-Craviari et al., 2004), the defect in matrix assembly most likely results from the impairment in force generation and the failure to form FBs.

Interestingly, it was recently shown that the recruitment of most proteins to adhesion sites is dependent on myosin II activity (Schiller and Fässler, 2013). However, ILK recruitment to FA sites occurs independent of myosin II activity (Schiller and Fässler, 2013), suggesting that ILK might act upstream and be involved in myosin II-dependent recruitment of other FA components. Our finding that ILK is required for the generation of traction forces provides further functional evidence for this notion (Fig. 2B). This inability to generate force is very likely to affect further recruitment of FB-associated proteins, leading to the observed failure in ECM remodeling in ILK-deficient fibroblasts (Fig. 2D). Besides driving α5β1 integrin segregation along the actin cytoskeleton, ILK could additionally be involved in mediating force-dependent conformational changes in α5β1 integrins, which occur during fibrillogenesis (Clark et al., 2005).

Although α5β1 integrin is the primary FN receptor (Huveneers et al., 2008), integrin αvβ3 (Wennerberg et al., 1996), α4β1 (Sechler et al., 2000) and αIIbβ3 (Olorundare et al., 2001) have been shown to be involved in FN fibrillogenesis in vitro. Knockout studies in mice suggest overlapping as well as independent functions for α5- and αv-class integrins in this process and only the double knockout of α5- and αv- integrins in mice results in loss of fibrillogenesis (Yang et al., 1999). It is tempting to speculate that ILK, through its ability to bind both to β1- and β3-integrins, might be involved in their differential engagement and thereby in fine-tuning the forces required for FN fibrillogenesis.

It has been proposed that the assembly state of FN fibers plays an important role in regulating cell behavior by acting as a checkpoint signal for subsequent ECM remodeling (Schwarzbauer and DeSimone, 2011). For instance, only the precise ratio of FN fibril assembly ensures epithelial branching morphogenesis during cleft formation (Sakai et al., 2003a). Furthermore, FN fibrillogenesis regulates fibrillin-1 microfibril assembly (Kinsey et al., 2008) and could thereby impact the ability of these

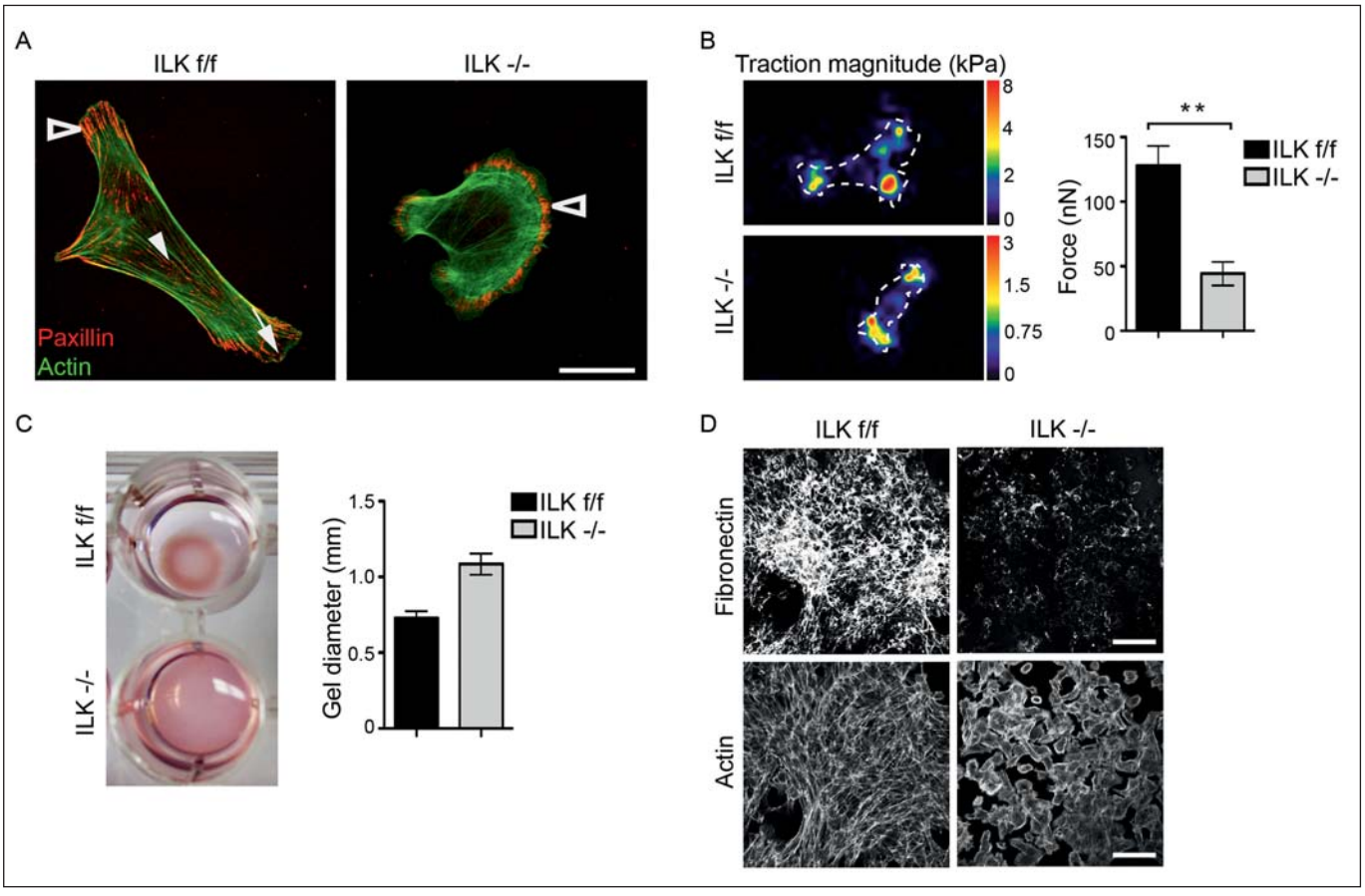

Figure 2: Integrin-linked kinase is required for focal adhesion maturation, cellular force generation and extracellular matrix deposition
A. Immunofluorescence analysis of paxillin as a marker for focal adhesions and F-actin in fibroblasts. Wild type fibroblasts display small focal complexes (arrow), focal adhesions (open arrowhead), and fibrillar adhesions (arrowhead), which are tightly connected to the actin cytoskeleton. Note lack of focal complexes and fibrillar adhesions, reduced amount of actin stress fibers, and accumulation of large peripheral focal adhesions (open arrowhead) in ILK-deficient cells. Scale bar 25 µm. B. Left panel shows a heat-scale map of traction stress magnitudes obtained using traction force microscopy. The color code indicates local traction in kPa. Cell outlines are indicated by dotted lines. Right panel shows the quantification of total cellular traction forces (mean ± SEM, n>30, **p=0.0011). Deletion of ILK severely compromises the ability of fibroblasts to generate traction forces. C. Collagen gel contraction assay with ILK f/f and -/- fibroblasts. Deletion of ILK impairs the ability of fibroblasts to contract collagen gels. D. Immunofluorescence staining of the fibronectin matrix and the actin cytoskeleton (phalloidin) to visualize cell area. Note decreased matrix deposition in ILK -/- cells. Scale bar 100 µm (modified from Radovanac et al., 2013).

structures to bind and regulate growth factor bioavailability. Therefore it is becoming clear that the ECM-remodeling function of ILK is central to its role as an essential regulator of cell and tissue behavior. It will be of great interest to evaluate how force-induced fibril assembly driven by ILK impacts these processes and what is the role of ECM remodeling in the various phenotypes of ILK-deficient mice.

ILK in dermal tissue repair and fibrosis

ECM deposition is an indispensible, but transient and reversible process during wound healing. When disturbed, it can convert tissue repair into a progressive and irreversible fibrotic response, leading to hypertrophic scarring, keloids or fibrosis. This results in destruction of normal tissue architecture and compromised organ function (Gabrielli et al., 2009; Hunzelmann and Krieg, 2010; Wynn and Ramalingam, 2012). ILK has important functions both during physiological tissue repair as well as in fibrosis. Deletion of ILK in dermal fibroblasts in mice results in impaired myofibroblast generation during wound healing, compromising matrix remodeling and subsequently tissue repair (Blumbach et al., 2010; Vi et al., 2011).

Interestingly, ILK, due to its kinase fold, is a substrate of the chaperone Heat shock protein 90 (Hsp90) that is required to stabilize ILK. Inhibition of Hsp90 activity in fibroblasts therefore leads to degradation and subsequent depletion of ILK protein, inducing a cellular phenotype closely resembling ILK-deficient cells (Radovanac et al., 2013). Consequently, blocking Hsp90 activity severely attenuates myofibroblast generation and the development of skin fibrosis in mice (Radovanac et al., 2013). This might provide a potential therapeutic strategy to treat fibrotic disease.

Interestingly, rigidity and mechanical stability of the matrix, in conjunction with the key profibrotic mediator transforming growth factor β1 (TGFβ1), act as the primary stimulus for myofibroblast differentiation and persistence (Tomasek et al., 2002; Nakamura-Wakatsuki et al., 2012). Furthermore, myofibroblasts can activate latent TGFβ1 that is bound to the ECM using integrin-mediated contraction, suggesting that activation of TGFβ1 is driven by a mechanical mode of action. In this respect it is interesting to note that ILK-deficient fibroblasts show impaired differentiation into myofibroblasts, and decreased release of TGFβ1 (Blumbach et al., 2010; Vi et al., 2011; Radovanac et al., 2013).

Concluding remarks

To conclude, recent work from our lab and others has identified mechanisms by which integrins, through adaptors such as ILK, transduce traction forces that are central to adhesion maturation, regulation of cell shape and migration as well as ECM remodeling. In addition, this work has revealed exciting new mechanisms by which kinase domains can be utilized as specific protein-protein interaction domains by pseudokinases. An important aim for future work is to understand the precise mechanisms by which cellular behavior is regulated by the dynamic crosstalk between cells and their immediate ECM microenvironment.

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