Induction of membrane circular dorsal ruffles requires co-signalling of integrin–ILK-complex and EGF receptor

S. Babak Azimifar1, Ralph T. Böttcher1, Sara Zanivan2,*, Carsten Grashoff1, Marcus Krüger2,†, Kyle R. Legate1, Matthias Mann2 and Reinhard Fässler1,§

1Department of Molecular Medicine, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany
2Department of Proteomics and Signal Transduction, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany

*Present address: The Beatson Institute for Cancer Research, Cancer Research UK Beatson Laboratories, Bearsden, Glasgow G611BD, UK
†Present address: Biomolecular Mass Spectrometry, Max Planck Institute for Heart and Lung Research, Bad Nauheim 61231, Germany
§Author for correspondence (faessler@biochem.mpg.de)

Accepted 1 September 2011
Journal of Cell Science 125, 435–448
© 2012: Published by The Company of Biologists Ltd
doi: 10.1242/jcs.091652

Summary
Integrin and receptor tyrosine kinase signalling networks cooperate to regulate various biological functions. The molecular details underlying the integration of both signalling networks remain largely uncharacterized. Here we identify a signalling module composed of a fibronectin–αβ1-integrin–integrin-linked-kinase (ILK) complex that, in concert with epidermal growth factor (EGF) cues, cooperatively controls the formation of transient actin-based circular dorsal ruffles (DRs) in fibroblasts. DR formation depends on the precise spatial activation of Src at focal adhesions by integrin and EGF receptor signals, in an ILK-dependent manner. In a SILAC-based phosphoproteomics screen we identified the tumour-suppressor Cyld as being required for DR formation induced by αβ1 integrin and EGF receptor co-signalling. Furthermore, EGF-induced Cyld tyrosine phosphorylation is controlled by integrin–ILK and Src as a prerequisite for DR formation. This study provides evidence for a novel function of integrin–ILK and EGF signalling crosstalk in mediating Cyld tyrosine phosphorylation and fast actin-based cytoskeletal rearrangements.

Key words: ILK, Dorsal ruffles, Integrin, EGF, Cyld

Introduction
Cells are exposed to a wide variety of mechanical and chemical stimuli that must be integrated at the molecular level to achieve an appropriate biological response. The integration of distinct signalling pathways from different cell surface receptors into a common downstream response is referred to as signalling crosstalk. Such crosstalk occurs between integrins and receptor tyrosine kinases (RTKs) to control important biological processes such as cell differentiation, proliferation, survival, migration, innate immune response and angiogenesis (Cabodi et al., 2004; Chan et al., 2006; King et al., 2011; Loubaki et al., 2010; McCall-Culbreath et al., 2008; Ross, 2004; Somanath et al., 2009). However, the molecular details of how distinct signalling pathways arising from integrins and RTKs such as epidermal growth factor receptor (EGFR) can converge to regulate these processes remain largely unknown.

Integrins are heterodimeric transmembrane proteins that interact with extracellular matrix molecules to trigger intracellular signal transduction cascades leading to the reorganization of the actin cytoskeleton and activation of downstream signalling pathways (Hynes, 2002; Legate et al., 2009; Wiesner et al., 2005). Integrins assemble in different αβ subunit combinations that confer substrate and signalling specificity. Because integrins have short cytoplasmic domains that lack enzymatic and actin-binding activity, they depend on the assembly of adaptor proteins onto their cytoplasmic tails for signal transduction. More than 180 signalling and scaffolding molecules have been identified that can be recruited to large integrin-based signalling hubs called focal adhesions (FAs) (Legate and Fässler, 2009; Schiller et al., 2011; Kuo et al., 2011; Zaidel-Bar and Geiger, 2010). Among these molecules, integrin-linked kinase (ILK) is a key player that directly binds the β1 and β3 integrin cytoplasmic tails (Hannigan et al., 1996; Pasquet et al., 2002). ILK is a multifunctional protein that regulates various cellular processes by associating with regulatory and adaptor proteins such as Pinch, α- and β-parvins, IQGAP1 and paxillin (Bottcher et al., 2009; Lange et al., 2009; Wickstrom et al., 2011). The analysis of constitutive and conditional deletion of the Ilk gene in mice, Drosophila melanogaster and Caenorhabditis elegans revealed that ILK controls the organization of the F-actin cytoskeleton, cell polarity, differentiation and proliferation (Esfandiarei et al., 2010; Grashoff et al., 2003; Hannigan et al., 1996; Legate and Fässler, 2009; Lorenz et al., 2007; Mackinnon et al., 2002; Sakai et al., 2003; Wang et al., 2008; Zervas et al., 2001).

FAs serve as a signalling nexus to condense and direct numerous signalling molecules, including kinases. The proto-oncogene Src is one of the kinases that localizes to FAs. Src activity is regulated by both integrin and RTK signalling (Huvaneers and Danen, 2009; Yeatman, 2004), and precise spatiotemporal activation is important for its biological functions, including the regulation of FA stability, turnover and integrity (Fincham and Frame, 1998; Zou et al., 2002). Src also regulates F-actin cytoskeleton remodelling through activation of various effector proteins, including small GTPases (Huvaneers and Danen, 2009; Timpson et al., 2001), kinases such as Abl as
Fig. 1. ILK is required for DR formation. (A,B) Kinetics of DR formation in serum-starved ILK$^{+/+}$ and ILK$^{-/-}$ cells when treated with serum (A) or when seeded on FN and stimulated with EGF (B). DR formation is significantly decreased in ILK$^{-/-}$ cells. (C) Actin (green) and cortactin (red) staining of FN-seeded ILK$^{+/+}$ and ILK$^{-/-}$ cells after 3 minutes of EGF stimulation. Arrows indicate DRs in ILK$^{+/+}$ cells. Scale bars: 20μm. (D) Western blot analysis of EGF-triggered EGFR and ERK phosphorylation in ILK$^{+/+}$ and ILK$^{-/-}$ cells. ILK$^{-/-}$ cells expressed higher levels of EGFR, but downstream ERK signalling was not changed. (E) Western blot analysis of protein lysates from ILK$^{+/+}$ and ILK$^{-/-}$ cells that stably express ILK–FLAG or ANK–FLAG. Note the expression level of Pinch1 is rescued by expression of both ILK constructs. (F) Re-expression of ILK–FLAG, but not ANK–FLAG, rescued DR formation in ILK$^{-/-}$ cells; $n=4$ independent experiments. Data expressed as mean ± s.d. ***P<0.005 by Student’s t-test.
well as p120-catenin and cortactin (Castano et al., 2007; Chang et al., 1995; Plattner et al., 1999).

Circumolar dorsal ruffles or waves [also known as dorsal ruffles (DRs) or actin ribbons] are dynamic actin-based structures that assemble on the dorsal plasma membrane in response to a variety of growth factors (Abercrombie et al., 1970; Buccione et al., 2004; King et al., 2011; Schliwa et al., 1984). Growth factor stimulation activates a signalling cascade that starts with activation of master kinases such as Src and ends with transient cytoskeletal rearrangements regulated by cortical actin polymerization (Buccione et al., 2004). The exact function of DRs is still unclear, but they have been proposed to be important for macropinocytosis, trafficking of β3 integrin, sequestration and internalization of RTKs after ligand stimulation, and fast remodelling of the actin cytoskeleton during cell migration and invasion (Abella et al., 2010; Buccione et al., 2004; Dowrick et al., 1993; Gu et al., 2011; Krueger et al., 2003; Orth et al., 2006; Suetsugu et al., 2003).

In this study, we show that DRs are the result of cooperative signals emanating from integrin and RTK signalling pathways. We found that ILK is an essential component in the DR signalling cascade downstream of fibronectin (FN)–α5β1 integrins. ILK regulates the spatiotemporal activation of Src at FAs, which is required for tyrosine phosphorylation of the tumour-suppressor Cyld and the formation of DRs. The implications of these findings are discussed.

Results
ILK is crucial for DR formation

We generated ILK-floxed (ILK<sup>fl</sup>f) and ILK-deficient (ILK<sup>−−</sup>) fibroblasts to investigate the consequence of ILK deletion in vitro (Sakai et al., 2003). During our experiments we realized that stimulation of starved ILK<sup>fl</sup>f cells with media containing 10% fetal calf serum induced DRs in approximately 30% of ILK<sup>fl</sup>f cells, whereas ILK<sup>−−</sup> cells very rarely formed DRs (Fig. 1A). To study this effect under defined conditions in the presence of specific growth factors, we measured epidermal growth factor (EGF)-triggered DR formation in serum-starved ILK<sup>fl</sup>f and ILK<sup>−−</sup> fibroblasts that were seeded on FN-coated surfaces.

Consistent with our observation using 10% fetal calf serum, about 25% of ILK<sup>fl</sup>f cells formed DRs after EGF stimulation, whereas ILK<sup>−−</sup> cells showed very few ruffles (Fig. 1B,C). Similarly, ILK<sup>−−</sup> cells formed fewer DRs in response to platelet-derived growth factor (PDGF) stimulation (supplementary material Fig. S1A). This reduction in DR formation was not a clonal artifact because we consistently found a significant reduction of DRs in all ILK<sup>−−</sup> clones compared with their ILK<sup>fl</sup>f counterparts (supplementary material Fig. S1B). Immunostaining of ILK showed no localization to DRs after EGF stimulation (supplementary material Fig. S1C). The reduced DR frequency in ILK<sup>−−</sup> cells was not due to reduced EGF phosphorylation or ERK1/2 activation because their relative levels were similar in ILK<sup>fl</sup>f and ILK<sup>−−</sup> cells with the exception of phosphorylation of EGFR Tyr992, which was increased in ILK<sup>−−</sup> cells (Fig. 1D, supplementary material Fig. S1D). Similarly, EGF-induced Rac1 activation was similar in ILK<sup>−−</sup> and ILK<sup>fl</sup>f cells, although the activation was prolonged in ILK<sup>−−</sup> cells but the differences were not statistically significant (supplementary material Fig. S1E,F). ILK<sup>−−</sup> cells show spreading defect, raising the possibility that impaired DR formation is a consequence of the reduced spread area of these cells. However, when ILK<sup>−−</sup> cells were allowed to spread for longer time periods of up to 2 days they did not show a significantly increased frequency of DR formation despite a normal spread area (Fig. 1A; data not shown). Moreover, stable re-expression of FLAG-tagged ILK (Fig. 1E,F) or ILK-EGFP (supplementary material Fig. S1G) fully rescued the DR defect of ILK<sup>−−</sup> cells.

It has been suggested that ILK interconnects integrins with growth factor pathways through Pinch1. Additionally, ILK and Pinch1 are components of the ILK–Parvin (IPP) complex, whose members depend on complex formation for maintaining their stability (Legate et al., 2006). Western blot analysis showed that the level of Pinch1 expression is strongly reduced in ILK<sup>−−</sup> cells (Fig. 1E). To test whether the DR formation defect in ILK<sup>−−</sup> cells is caused by the diminished Pinch1 protein level, we stably re-expressed FLAG-tagged N-terminal ANK-repeats of ILK (ANK–FLAG) in ILK<sup>−−</sup> cells. The presence of

![Fig. 2. Functional consequence of aberrant DR formation in ILK<sup>−−</sup> cells.](image-url)

(A) Chemotaxis migration of ILK<sup>fl</sup>f, ILK<sup>−−</sup> and ILK–FLAG-rescued ILK<sup>−−</sup> cells towards EGF or PDGF. Chemotaxis migration to EGF and PDGF was significantly decreased in ILK<sup>−−</sup> cells; n=4 independent experiments. (B) EGF internalization after EGF stimulation was diminished in ILK<sup>−−</sup> cells; n=3 independent experiments. Data expressed as mean ± s.d. *P<0.05, ***P<0.005 by Student’s t-test.)
Fig. 3. See next page for legend.
ANK–FLAG stabilized Pinch1 expression to wild-type levels, but cells were still not able to form DRs (Fig. 1E,F). Hence, the reduced DR frequency in ILK1−/− cells was due not to reduced Pinch1 protein levels. Expression of ANK–FLAG had no effect on DR formation in ILK3 cells (supplementary material Fig. S1H, I). Conversely, Pinch1−/− cells showed strongly reduced ILK protein levels (Stanchi et al., 2009) and decreased DR formation (supplementary material Fig. S1J). Together, these data demonstrate that ILK plays an essential role in the induction of DRs.

ILK1−/− cells have defects in DR-related functions

The precise biological function of DRs is still uncertain, but various reports ascribe macropinocytosis, large-scale actin reorganizations prior to migration and growth factor receptor internalization as downstream consequences of DR formation (King et al., 2011; Orth et al., 2006). We found that ILK1−/− cells migrated towards a source of EGF or PDGF less efficiently than ILK3 cells or ILK-FLAG-rescued ILK1−/− cells (Fig. 2A). Furthermore, the internalization of activated EGF-R was significantly reduced in ILK1−/− cells, whereas the internalization of transferrin receptor remained unchanged (Fig. 2B, supplementary material Fig. S2). Reduced internalization of EGF-R is expected to result in prolonged signalling, and indeed the relative level of phosphorylation of EGF-R in ILK1−/− cells was increased, perhaps as a result of impaired downregulation through internalization (supplementary material, Fig. S1D). Therefore, ILK1−/− cells displayed phenotypic differences that are consistent with a reduction in the formation and number of DRs.

DRs are the result of α5β1 integrin and EGF-R co-signalling

The finding that ILK plays a crucial role in DR formation suggested a requirement for integrin signalling in the formation of these structures. To test whether integrin engagement is necessary for DR formation, we monitored EGF-induced DRs in ILK3 cells seeded on FN or poly-L-lysine (PLL). Whereas FN can be recognized by many integrin receptors, most notably α5β1 and αVβ3, PLL-mediated adhesion is integrin-independent. Only FN-seeded ILK3 cells formed DRs (Fig. 3A), and the rate of DR formation in ILK3 cells increased with the FN concentration (Fig. 3B).

To examine whether the formation of DRs depends on a specific integrin heterodimer, we evaluated DR assembly in a FN-free system by seeding serum-starved FN-null (FN−/−) fibroblasts on FN, vitronectin (VN), collagen1 (Col1), or PLL (Fig. 3C–E). FN−/− cells established a distinct morphology on each substrat and formed paxillin-rich focal adhesions on FN, VN and Col1 but not on PLL (Fig. 3E). Whereas about 25% of FN-seeded FN−/− cells formed DRs, cells adherent to PLL, VN or Col1 formed significantly fewer DRs (<5%, Fig. 3F). The spread areas of FN−/− cells on FN, VN and Col1 were comparable, indicating that differences in spreading do not contribute to altered DR formation (supplementary material Fig. S3A). In addition, when we limited the spreading time of FN-seeded FN−/− cells to 30 minutes, so that they covered the same spread area as PLL-attached cells, they still formed DRs normally (supplementary material Fig. S3B). Moreover, DR formation in FN−/− cells plated on FN was also dependent on ILK (supplementary material Fig. S3C,D).

Integrin-mediated cell adhesion to FN is mainly achieved through α5β1 and αVβ3 integrins, whereas VN is bound by αVβ3 but not α5β1 integrin (Hynes, 2002). Therefore, our results suggest that only α5β1 integrin signals trigger formation of DRs. To confirm this, we examined EGF-induced DRs in serum-starved FN-seeded integrin β1E15 and β1+/− fibroblasts (which lack α5β1 integrin but express αVβ3 integrin). In agreement with the previous experiments, about 30% of β1E15 cells formed DRs, whereas β1+/− cells showed significantly reduced DR formation (Fig. 3G). Re-expression of β1 integrin in β1+/− cells rescued DR formation in these cells (supplementary material Fig. S3E,F). These results suggest that DRs are the consequence of FN-α5β1-integrin–ILK and EGF-R co-signalling.

ILK affects active Src localization to FAs

Both integrin and RTK signalling stimulate Src tyrosine kinase activity, which is known to play a central role in DR formation (Chang et al., 1995; Huveneers and Danen, 2009). In line with these previous reports, ILK3 cells pretreated with a Src inhibitor (PP1) failed to form DRs (supplementary material Fig. S4A). Therefore we decided to investigate the role of Src in more detail. First, we investigated whether Src activation is impaired in ILK1−/− cells. Immunostaining with antibody against Tyr416-phosphorylated Src (pY416-Src) showed that active Src levels were dramatically reduced in FAs of FN-seeded ILK1−/− cells before EGF stimulation and remained reduced after EGF stimulation (Fig. 4A,B). Re-expression of ILK–EGFP in ILK1−/− cells rescued the level of active Src in FAs (supplementary material Fig. S4B–D). Western blot analysis showed that total Src levels were similar in ILK3 and ILK1−/− cells (Fig. 4C) and that non-adherent ILK3 and ILK1−/− cells showed a similar (twofold) increase in Src activity after EGF treatment (Fig. 4C,D). Plating cells on FN caused a basal increase in Src phosphorylation in ILK3 cells that did not manifest in ILK1−/− cells, but EGF treatment induced a similar activation of Src in both cell lines, resulting in a net decrease in active Src in ILK1−/− cells of about 20% (Fig. 4C,D). Co-immunoprecipitation of ILK with antibody against Src in ILK–FLAG-rescued ILK1−/− cells (Fig. 4E), and of Src with antibody against GFP in ILK–GFP-rescued ILK1−/− cells (Fig. 4F) indicated that Src and ILK form a complex in our fibroblast cell lines. However, a complex between Src and endogenous ILK was not easily detectable in our cells (data not shown).

Importantly, transient expression of constitutively active EGF-R-tagged Src-Y527A mutant was localized to FAs and rescued DR formation in ILK1−/− cells (supplementary material...
Fig. 4. ILK affects active Src levels at FAs during spreading on FN. (A) Tyr416-phosphorylated Src (pY416-Src) (green) and vinculin (pY416-vinculin) (red) immunostaining of serum-starved FN-seeded ILK^{ff} and ILK^{-/-} cells. Note the reduced pY416-Src staining in ILK^{-/-} cells. Scale bars: 10 μm. (B) Quantification of pY416-Src and pY416-vinculin intensity at FAs in cell stainings showed reduced active Src intensity at FAs of ILK^{-/-} cells. Data expressed as the mean + s.d.; n=3 independent experiments. (C) pY416-Src immunoblotting of serum-starved ILK^{ff} and ILK^{-/-} cells when seeded on FN or kept in suspension (S) and stimulated with EGF. (D) Measurement of pY416-Src band intensity in immunoblots indicated that FN-induced Src activation is impaired in ILK^{-/-} cells. Data expressed as the mean + s.e.m.; n=6 independent experiments. (E) ILK–FLAG was detected in anti-Src immunoprecipitates from ILK–FLAG cell lysates. (F) Src was detected in anti-GFP immunoprecipitates from ILK–EGFP-rescued ILK^{-/-} cells. *P<0.05, **P<0.01, ***P<0.005 by Student’s t-test.
Fig. S4E–G). Furthermore, a decreased level of active Src at FAs significantly correlated with decreased DR frequency, whereas Src activity and the number of DRs concomitantly increased in ILK^{ff} cells when seeded on increasing FN concentrations (supplementary material Fig. S4H; compare with Fig. 3B). Together, these experiments suggest that ILK affects DR formation through control of Src activity at FAs.

Fig. 5. Analysis of the ILK-dependent phosphoproteome. (A) Representation of the SILAC-based phosphoproteomics screening strategy (see Material and Methods). Non-stimulated ILK^{ff}, EGF-triggered ILK^{ff} and EGF-triggered ILK^{ff} cells were labelled with light, medium or heavy amino acids isotopes, respectively. These conditions represent cells with activated integrin–ILK (L), EGFR (M) or both (H) signalling pathways. (B) Panel of identified immunoprecipitated proteins after 2 minutes of EGF stimulation in ILK^{ff} cells (x-axis), and ILK^{ff} cells (y-axis). Positions of Cyld and Asap2 on the graph are indicated. (C) List of candidate proteins that were assayed for their involvement in DR formation after shRNA- or siRNA-mediated knockdown. (C,D) Quantification of DR formation in Cyld (D) or Asap2 (E) knockdown cells demonstrates their involvement in the DR pathway downstream of EGF stimulation; n=4 independent experiments. Data expressed as mean ± s.d. **P < 0.01, ***P < 0.005 by Student’s t-test.
Fig. 6. See next page for legend.
β1 integrin–ILK and EGFR co-signalling triggers tyrosine phosphorylation of proteins involved in DR formation

The kinase signalling cascade leading to DRs is mediated by β1 integrin–ILK and EGFR co-signalling, which activates Src in FAs. To identify potential ILK-dependent substrates for EGFR and Src, which are involved in DR formation, we compared the phosphoproteome of ILK<sup>+/+</sup> and ILK<sup>-/-</sup> cells treated with EGF for 30 seconds or 2 minutes by combining phosphorylated tyrosine immunoprecipitation and SILAC-based mass spectrometry (Fig. 5A). Candidate proteins involved in DR formation induced by β1 integrin and EGFR were defined as those that displayed increased phosphorylation upon EGF stimulation in ILK<sup>+/+</sup> cells, but not in ILK<sup>-/-</sup> cells. Our analyses identified and quantified more than 2000 proteins and 140 specific phosphorylation sites (supplementary material Tables S1 and S2) after excluding proteins that are expressed at different levels in ILK<sup>+/+</sup> and ILK<sup>-/-</sup> cells, identified in whole proteome SILAC-based mass spectrometry experiments (data not shown). The majority of proteins identified 2 minutes after EGF stimulation had the same SILAC ratio in ILK<sup>+/+</sup> and ILK<sup>-/-</sup> cells, although certain proteins were upregulated in an ILK-dependent manner (Fig. 5B).

To test the involvement of these proteins in DR formation, we performed short hairpin RNA (shRNA)- or short interfering (siRNA)-mediated knockdown in ILK<sup>+/+</sup> cells of candidate proteins that were consistently represented in four independent SILAC screens (Fig. 5C). Knockdown efficiency was evaluated using western blotting and quantitative RT-PCR (supplementary material Fig. S5A,B). DR frequency decreased significantly in Cyld- and Asap2-depleted cells (Fig. 5D,E). We decided to further analyse the involvement of Cyld in the β1 integrin–ILK and EGFR co-signalling pathway.

**Cyld tyrosine phosphorylation is essential for DR formation**

The tumour suppressor protein Cyld tunes several signal transduction pathways including NFkB, JNK and Wnt–β-catenin through its deubiquitylating (DUB) activity (Massoumi, 2010). We prepared Cyld<sup>-/-</sup> fibroblasts from Cyld-deficient mice (Massoumi et al., 2006) to corroborate the crucial role of Cyld in DR formation (Fig. 6A). Re-expression of FLAG–Cyld normalized DR formation in Cyld<sup>-/-</sup> fibroblasts (Fig. 6A; supplementary material Fig. S6A). Similarly, re-expression of a catalytically inactive Cyld mutant (Cyld C>S) (Brummelkamp et al., 2003; Trompouki et al., 2003) restored DR formation in Cyld<sup>-/-</sup> cells (Fig. 6A). Immunostaining of endogenous Cyld in wild-type cells (Fig. 6B) and overexpression of GFP–Cyld in Cyld<sup>-/-</sup> cells revealed that Cyld was recruited to DRs (supplementary material Fig. S6B–D). Rac1 activation was not impaired in Cyld<sup>-/-</sup> cells (supplementary material Fig. S6E,F), which was similar to the results using ILK<sup>-/-</sup> cells (supplementary material Fig. S1).

Because our phosphoproteomics screen enriched for tyrosine-phosphorylated proteins, we tested whether Cyld becomes tyrosine-phosphorylated when serum-starved cells are seeded on FN and stimulated with EGF. Fig. 6C shows that Cyld became tyrosine-phosphorylated within 2–4 minutes after EGF stimulation. This phosphorylation was abrogated when cells were treated with the selective EGFR inhibitor Gefitinib (Iressa), indicating that Cyld phosphorylation is indeed downstream of EGFR signalling (Fig. 6D). Cyld became tyrosine-phosphorylated in response to EGF stimulation when cells were plated on FN but not when they were seeded on PLL, VN or Coll1 (Fig. 6,E,F). Consistent with our phosphoproteomics data, Cyld phosphorylation was reduced in FLAG–Cyld-rescued Cyld<sup>-/-</sup> cells in which ILK was depleted (Fig. 7A). Similarly, Cyld phosphorylation was diminished in ILK<sup>-/-</sup> cells expressing FLAG–Cyld as compared with control ILK<sup>+/+</sup> cells (Fig. 7B), whereas localization of endogenous Cyld was not changed in ILK<sup>-/-</sup> cells upon EGF stimulation (supplementary material Fig. S7A). Finally, Src inhibition with PP1 abrogated EGF-induced Cyld tyrosine phosphorylation in FLAG–Cyld-rescued Cyld<sup>-/-</sup> cells, indicating that EGFR-induced Cyld tyrosine phosphorylation occurs downstream of Src (Fig. 7C).

Immunostaining of active Src in Cyld<sup>-/-</sup> cells expressing mCherry–Cyld suggested that cytoplasmic Cyld was not localized to FAs (Fig. 7D). Although phosphorylation of Cyld on serine has recently been reported (Hutti et al., 2009), this is the first report demonstrating Cyld tyrosine phosphorylation. To identify which tyrosine residue(s) are phosphorylated in response to EGF, we conducted a mutational analysis. Mutation of four tyrosine residues to alanine (FLAG–Cyld-<sup>6</sup>) identified in other phosphoproteomics experiments (data not shown; locations of these tyrosines are given in supplementary material Table S3) reduced neither EGF-induced tyrosine phosphorylation of Cyld nor DR formation (Fig. 6A; supplementary material Fig. S7B). The substitutions of additional tyrosine residues with alanine (FLAG–Cyld-9× and FLAG–Cyld-18× mutants, supplementary material Table S3) led to a significant decrease in EGF-induced tyrosine phosphorylation and DR formation (Fig. 6A, Fig. 7E).

Together, these data demonstrate that Cyld lies downstream of an integrin–ILK and EGFR co-signalling pathway leading to the formation of DRs in fibroblasts. Although the DUB activity of Cyld is dispensable for DR formation, tyrosine phosphorylation of Cyld is required, and this phosphorylation lies downstream of EGF-mediated and ILK-dependent Src activation.

**Discussion**

Previous studies have shown that dynamic, transient actin-based DRs form in response to a variety of growth factors, including EGF, PDGF and hepatocyte growth factor (HGF) (Buccione et al., 2004). In the present study, we report that integrin and RTK signalling pathways cooperatively control the formation of DRs.
The integrin-based signalling leading to DR formation emanates specifically from $\alpha_5\beta_1$ integrin through a signalling module containing ILK, Src and Cyld.

The specificity of the involvement of $\alpha_5\beta_1$ integrin in DR formation can be explained by the differential assembly of specific FA signalling complexes at the integrin tails that confers distinct signalling specificities to different $\alpha/\beta$ integrin subunit combinations (Humphries et al., 2009). For example, $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins have distinct effects on actin cytoskeletal regulation through different modulation of Rho GTPases (Danen
The absence of ILK strongly reduces the levels of active Src in mitogenesis, PDGF-activated Src outside of caveolae affects F-actin reorganizations (Bottcher et al., 2009; Legate et al., 2006; Reiley et al., 2004), which can be controlled by phosphorylation on Ser418 by ILK (Huveneers et al., 2009). However, the role of Cyld in DR formation is independent of its DUB activity. Rather, EGF stimulation resulted in Cyld tyrosine phosphorylation, which is necessary for DR formation. Cyld phosphorylation is dependent on Src activity and occurs downstream of a cooperative EGFR and integrin signalling network involving FN, α5β1 integrin and ILK. Because ILK does not localize to DRs and Cyld does not localized to FAs we propose a model whereby, upon EGF stimulation, activated Src localizes to ILK-containing FAs, where it activates substrates that either directly or indirectly phosphorylate Cyld, causing it to redistribute to DRs to exert its specific function.

Although we have identified Cyld as an important intermediary for DR formation, the precise function of Cyld tyrosine phosphorylation in this process has yet to be elucidated. Tyrosine phosphorylation might be required for the interaction of Cyld with as-yet-unknown binding partners, including proteins that can directly regulate actin dynamics. On the other hand, Cyld associates with α-tubulin and microtubules via its CAP-Gly domains and increases the levels of acetylated tubulin through an inhibitory interaction with histone deacetylase-6 (Gao et al., 2008; Wickstrom et al., 2010). Cyld tyrosine phosphorylation might control DR assembly by affecting the ability of Cyld to bind to microtubules and influence their dynamic instability, thereby controlling actin–microtubule crosstalk. We are currently addressing these possibilities to understand the role of Cyld in DR formation more precisely.

In conclusion, our work has identified Cyld as a key member of an integrin–ILK and EGFR co-signalling pathway. Interestingly, deregulation of each of these molecules has been implicated in cancer progression (Cabodi et al., 2010; Demchenko et al., 2010; Grandal and Madshus, 2008; Massoumi, 2010). Although the biological function of DRs is unknown, proposed functions such as RTK endocytosis and sites of localized matrix degradation could be important for tumourigenesis and metastatic behaviour. Future work to more precisely define how integrin–ILK and EGFR collaborate to activate Cyld, and how Cyld functions to enable rapid actin reorganizations leading to DRs, could provide novel insights into how deregulation of these signalling pathways promotes the formation and spread of cancer.

**Materials and Methods**

**Reagents and antibodies**

Human recombinant EGF and PDGF-BB were from Millipore; Boyden chambers were from BD Bioscience; PP1 inhibitor was from Cell Signaling Technology. Gefitinib (Iressa) was supplied by Selleck. The following antibodies were used: ILK, Rac1 and paxillin (BD Bioscience); EGFR, Tyr1173-phosphorylated EGFR, Tyr1068-phosphorylated EGFR, Tyr992-phosphorylated EGFR, Tyr845-phosphorylated EGFR and Tyr416-phosphorylated Src (Cell Signaling Technology); tyrosine-phosphorylated 4G10 and Lasp-1 (Millipore); β1 integrin and Cyld antibodies were homemade antibodies raised in rabbit; anti-FLAG, SHC2, and
ILK particles were used for infection of transiently transfected into human embryonic kidney (HEK293T) cells; viral generate stable cell lines cDNAs were cloned into pCLMFG retroviral vectors and Cells were transiently transfected with Lipofectamine 2000 (Invitrogen). To phosphorylated EGFP–ILK were amplified by PCR. The DUB-dead FLAG–cDNA constructs of FLAG–Cyld, FLAG–ILK, FLAG–ANK ILK and manufacturer's instructions (cat#A2220, Sigma). For other immunoprecipitations, inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma-Aldrich). Cell lysates were prepared by quickly washing cells in ice-cold PBS prior to addition of lysis buffer [50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma-Aldrich)]. FLAG-tagged protein immunoprecipitation was performed according to the manufacturer’s instructions (cat#A2220, Sigma). For other immunoprecipitations, cell lysates at 0.5−1 mg/ml were pre-cleared by centrifugation for 1 hour at 4 °C and incubated with antibody for 3 hours or overnight at 4 °C. Protein complexes were captured using protein A or G agarose beads for 1 hour at 4 °C. Protein complexes were captured for 15 minutes with an interval time of 90 seconds. We isolated data sets.

Statistical analysis
Results are expressed as means ± s.d. or means ± s.e.m. Statistical analysis was performed using GraphPad Prism (version 5.00, GraphPad Software) or Excel software. ANOVA or Student’s t-test were used for comparisons between different data sets.

Acknowledgments
We thank Rene Bernards for providing the DUB-dead Cyld C construct.

Funding
This work was supported by the Max Planck Society and the Tiroler Zukunftsstiftung.

Supplementary material available online at http://jcs.biologists.org/lookup/suppl;doi:10.1242/jcs.091652/-/DC1

References
Abella, J. V., Parachonik, C. A., Sangwan, V. and Park, M. (2010). Dorsal ruffle microdomains potentiate Met receptor tyrosine kinase signaling and down-regulation. J. Biol. Chem. 285, 24955-24967. Abercrombie, M., Heaysman, J. E. and Pegrum, S. M. (1970). The locomotion of fibroblasts in culture. II. “Ruffling”. Exp. Cell Res. 60, 437-444. Arthur, W. T., Peich, L. A. and Barridge, K. (2000). Integrin engagement suppresses Rho's activity via a c-Src-dependent mechanism. Curr. Biol. 10, 719-722.
Bass, M. D., Morgan, M. R., Roach, K. A., Settleman, J., Goryachev, A. B. and Humphries, M. J. (2008) p190RhoGAP is the convergence point of activation signals from alpha 5 beta 1 integrin and syndecan-4. *J. Cell Biol. 181*, 1013-1026.

Berdeaux, R. L., Diaz, B., Kim, L. and Martin, G. S. (2004). Active Rho is localized to podosomes induced by oncogenic Src and is required for their assembly and maintenance. *Cell 116*, 317-327.

Bottcher, T. R., Lange, A. and Fassler, R. (2009). How ILK and kindlins cooperate to orchestrate integrin signaling. *Curr. Opin. Cell Biol. 21*, 670-675.

Brummelkamp, T. R., Nijman, S. M., Dirac, A. M. and van Breemen, C. (2004). A dominant-negative Rac2/3 complex mediates actin reorganization in growth factor-stimulated cells. *Mol. Cell Biol. 14*, 1085-1095.

Kuo, J. C., Han, H., Hsiao, C. T., Yates, J. R. and Waterman, C. M. (2011). Analysis of the myosin-II-responsive focal adhesion proteme reveals a role for beta-Pix in negative regulation of focal adhesion maturation. *Nat. Cell Biol. 13*, 383-393.

Lai, F., Szeczdraz, M., Oelkers, J. M., Ladwein, M., Acconia, F., Benschet, S., Aninger, S., Fais, J., Smald, J. W., Poles, S. et al. (2009). Contactin promotes migration and platelet-derived growth factor-induced actin reorganization by signaling to Rho-GTPases. *Mol. Cell Biol. 20*, 3209-3223.

Lange, A., Wickstrom, S. A., Jakobsson, M., Zent, R., Sainio, K. and Fallgren, G. (2009). Integrin-linked kinase is an adaptor with essential functions during mouse development. *Nature 461*, 1002-1006.

Legate, R. K. and Fassler, R. (2009). Mechanisms that regulate adaptor binding to integrin cytoplasmic tails. *J. Cell Sci. 122*, 187-198.

Legate, R. K., Montanez, E., Kundack, O. and Fassler, R. (2006) ILK, PINCH and parvin: the tIIF of integrin signalling. *Nat. Rev. Mol. Cell Biol. 7*, 20-31.

Legate, R. K., Wickstrom, S. A. and Fassler, R. (2009). Genetic and cell biological analysis of integrin outside-in signalling. *Genes Dev. 23*, 397-418.

Legate, R. K., Takahashi, S., Besnard, N., Fahy, B., Boettiger, D., Zent, R. and Fassler, R. (2011). Adhesion integrin and force coupling are independently regulated by localised PtdIns(4,5)(2) synthesis. *EMBO J. 30*, 4539-4553.

Li, Y., Dai, C., Wu, C. and Liu, Y. (2007). PINCH–1 promotes tubular epithelial-to-mesenchymal transition by interacting with integrin-linked kinase. *J. Am. Soc. Nephrol. 18*, 2534-2543.

Lorenz, K., Grashoff, C., Torka, R., Sakai, T., Langbein, L., Bloch, W., Aumaule, M. and Fassler, R. (2007). Integrin-linked kinase is required for epidermal and hair follicle morphogenesis. *J. Cell Biol. 177*, 501-513.

Loubaki, L., Semlali, A., Boisvert, M., Jacques, E., Plante, S., Aoudjit, F., Mourad, W. and Chakir, J. (2010). Crosstalk between T cells and bronchial fibroblasts obtained from asthmatic subjects involves CD40L/alpha 5 beta 1 interaction. *Mol. Immunol. 47*, 2112-2118.

Mackinnon, A. C., Qadota, H., Norman, R. K., Moorman, D. G. and Williams, B. D. (2002). c-egens: PAT-4/ILK functions as an adaptor protein within integrin adhesion complexes. *Curr. Biol. 12*, 787-797.

Mann, M. (2006). Functional and quantitative proteomics using SILAC. *Nat. Rev. Mol. Cell Biol. 7*, 952-958.

Massoumi, R. (2010). CYLD: a deubiquitination enzyme with multiple roles in cancer. *Future Oncol. 7*, 285-297.

Massoumi, R., Chmielarska, K., Hennecke, K., Pfeifer, A. and Fassler, R. (2006) CYLD inhibits tumor cell proliferation by blocking Bcl-3-dependent NF-kappaB signaling. *Cell 125*, 665-677.

McCall-Culbreath, K. D., Li, Z. and Zutter, M. M. (2008). Crosstalk between the alpha 6beta 1 integrin and c-met/IGF-R regulates innate immunity. *Blood 111*, 3562-3570.

Orth, J. D., Krueger, E. W., Weller, S. G. and McNiven, M. A. (2009). A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization. *Cancer Res. 69*, 3630-3636.

Pasquet, J. M., Noury, M. and Norden, A. T. (2002) Evidence that the platelet integrin alphabeta 3 beta is regulated by the integrin-linked kinase, ILK, in a PI3-kinase dependent pathway. *Thromb. Haemost. 88*, 115-122.

Pear, W. S., Nolan, G. P., Scott, M. L. and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA 90*, 8392-8396.

Pfeifer, A., Kessler, T., Sillietti, S., Chersh, D. A. and Verma, I. M. (2000). Suppression of angiogenesis by lentiviral delivery of p53, a noncatalytic fragment of the metalloproteinase 2. *Proc. Natl. Acad. Sci. USA 97*, 12227-12232.

Plattner, R., Kadic, L., DeMalli, K. A., Kazlauskas, A. and Pendergast, A. M. (1999). c-Ab1 is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF. *Genes Dev. 13*, 2400-2411.

Reiley, W., Zhang, M. and Sun, S. C. (2004). Negative regulation of JNK signaling by the tumor suppressor CYLD. *J. Biol. Chem. 279*, 55161-55167.

Ross, R. S. (2004). Molecular and mechanical synergy: cross-talk between integrins and growth factor receptors. *Cardiovasc. Res. 63*, 381-386.

Sakai, T., Li, S., Docheva, D., Grashoff, C., Sakai, K., Kostka, G., Braun, A., Pfeifer, A., Yurchenco, P. D. and Fassler, R. (2003). Integrin-linked kinase (ILK) is required for polarizing the epithelium, cell adhesion, and controlling actin accumulation. *Genes Dev. 17*, 627-640.

Schiller, H. B., Friedel, C. C., Boulegue, C. and Fassler, R. (2011). Quantitative proteomics of the integrin adhesomes show a myosin II-dependent recruitment of LIM domain proteins. *EMBO Rep. 12*, 259-266.

Schliwa, M., Nakamura, T., Porter, K. R. and Euteneuer, U. (1984). A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. *J. Cell Biol. 99*, 1045-1059.

Sirent, A., Leroy, C., Boureux, A., Simon, V. and Roche, S. (2008) The Src-like adaptor protein regulates PDGF-induced actin dorsal ruffles in a Cbl-dependent manner. *Oncozinc 27*, 3459-3450.
