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**Molecular dissection of the crosstalk between
integrins/ILK- and EGFR-signalling pathways**

Seyed Babak Azimifar

aus

Ahvaz, Iran

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Erklärung

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Ehrenwörtliche Versicherung

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Seyed Babak Azimifar

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|--------------|---------------------------------|
| 1. Gutachter | Prof. Dr. Reinhard Fässler |
| 2. Gutachter | Prof. Dr. Christian Wahl-Schott |

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to my parents

and my love

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Aim of the thesis

Integrin and growth factor receptor (GFR) signalling crosstalk controls critical biological processes such as cell proliferation, differentiation, migration, spreading and immune response. The crosstalk occurs through a pool of signalling molecules that link integrin and growth factor signalling pathways. The molecular players of the integrin-GFR signalling crosstalk and the way they function at the mechanistic level have been remained largely uncharacterized. The overall goals of this thesis were:

- I. Identification of novel molecule(s) that intersect integrin and GFR signalling pathways.
- II. Analysis of the molecular mechanisms by which the identified intersector(s) mediate integrin and GFR signalling crosstalk.

To identify molecular players that link integrin-GFR signalling pathways, we established a screening strategy that combined SILAC-based quantitative proteomics followed by siRNA-mediated depletion of candidate proteins. The readout of our screening assay was circular dorsal ruffles, which are transient actin-based structures induced through integrin-GFR co-signalling. Subsequently, we used different biochemical approaches to investigate the mechanistic role of the novel proteins.

Summary

Integrin and growth factor receptor (GFR) signalling can converge to conduct a vast range of biological processes. The integrin-GFR crosstalk is achieved through activation of numerous signalling molecules that can operate ubiquitously or in a cell type- and/or developmental stage-specific manner. The molecular details underlying the integration of both signalling networks remain largely uncharacterized. We identified a signalling axis consisting of a fibronectin/ $\alpha 5\beta 1$ integrin/ILK complex that, in concert with epidermal growth factor receptor (EGFR) cues, controls the formation of transient actin-rich circular dorsal ruffles (DRs) in fibroblasts. Using DRs as readout and a SILAC-based phosphoproteomics screen, we identified two novel interectors of integrin/EGFR signalling crosstalk; one is Cyld and the other we termed Babak-2 (Plekhh2).

We showed that EGFR triggers Cyld tyrosine phosphorylation which requires integrin/ILK and c-Src, and that the Cyld phosphorylation is a prerequisite for DR formation (**publication I**). We also identified Babak-1 and -2 (Plekhhs) a novel family of focal adhesion (FA) molecules that localize to integrin adhesion sites and punctate structures in the cytoplasm of fibroblasts and Hela cells. We show that the recruitment of Babaks to FAs is dependent on their interaction with paxillin. The paxillin binding sequence (PBS) in Babak-1 and -2 is present in the N-terminal half of the protein. Analysis of expression pattern of Babak-2 and Babak-1 *in vivo* revealed that both genes are widely expressed in different tissues. Babak-2-deficient mice are fertile and did not show any phenotype, suggesting redundancy of Babak-2 function by Babak-1 (**publication II**). This study provides evidence that Cyld and Babak-2 link integrin/ILK and EGFR signalling pathways to induce rapid F-actin reorganisations.

Publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-III):

I. Azimifar S. Babak, Böttcher RT, Zanivan S, Grashoff C, Krugel M, Legate K, Mann M and Fässler R. **The induction of membrane circular dorsal ruffles requires integrin/ILK and EGF receptor co-signalling.** Journal of Cell Science. (in press) 2011.

II. Moritz Widmaier, Emanuel Rognoni, Korana Radovana, **S. Babak Azimifar**, and Reinhard Fässler. **Integrin-linked kinase at a glance.** Journal of Cell Science (in press) 2011.

III. Azimifar S. Babak, Mandoph Wang and Fässler R. **Babak-1 and Babak-2 are novel focal adhesion proteins that bind paxillin and regulate the assembly of membrane circular dorsal ruffles.** (Manuscript in progress).

Abbreviations

Abl	Abelson leukemia protein tyrosine kinase
ADAM	a disintegrin and metalloproteinase
ADP	adenosine diphosphate
Akt	RAC-alpha serine/threonine protein kinase
ANK	Ankyrin homology domain
AMIDAS	adjacent metal-ion-dependent adhesion site
ARF6	ADP-ribosylation factor 6
Arp2/3	actin-related protein 2/3 complex
ATP	adenosine triphosphate
Bcl3	B-cell CLL/lymphoma 3
CAP-Gly	cytoskeletal-associated protein Glycine-rich
Cdc42	cell division cycle 42
Cadherin	calcium-dependent adhesion molecule
CH	calponin homology
Cre	cyclization recombinase
Crk	CT-10 related kinase
DNA	deoxyribonucleic acid
DR	circular dorsal ruffle
DUB	deubiquitinase or
E-cadherin	epithelial calcium-dependent adhesion molecule
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELMO	engulfment and cell motility
ER	endoplasmic reticulum

ErbB2	erythroblastic leukemia viral oncogene homolog
ERK	extracellular signal-regulated kinase
F-actin	filamentous actin
FA	focal adhesion
FAT	focal adhesion targeting domain
FAK	focal adhesion kinase
FB	fibrillar adhesions
FC	focal complex
FERM	4.1, ezrin, radixin, moesin domain
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FN	fibronectin
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GFR	growth factor receptor
GPGL1	P-selectin glycoprotein ligands1
GPCR	G protein coupled receptor
Gsk3 β	glycogen synthase kinase-3 β
HDAC6	histone deacetylase 6
HGF	hepatocyte growth factor
HGFR	hepatocyte growth factor receptor
I-EGF	integrin-cysteine-rich epidermal growth factor repeats
ICAM	intercellular adhesion molecule
IGFR	insulin-liked growth factor receptor
IKK	inhibitor of ' κ -light-chain-enhancer' of B-cells (I κ B) kinase complex
IKK γ	inhibitor of ' κ -light-chain-enhancer' of B-cells (I κ B) kinase subunit γ
ILK	integrin-linked kinase
IPP	ILK/PINCH/Parvin

JNK	c-jun N-terminal kinase
LAP	latency-associated peptide
LARG	leukemia-associated RhoGEF
LC-MS/MS	liquid chromatography followed by tandem MS/MS
Lck	lymphocyte-specific protein tyrosine kinase
LIM	Lin-11, Isl-1 and Mec-3 homology domain
LTBP	latent TGF β binding protein
MADCAM1	mucosal vascular addressin cell adhesion molecule 1
MAPK	mitogen-activated protein kinase
MD	membrane distal
MEK	MAP kinase-ERK kinase
MIDAS	metal-ion-dependent adhesion site
MLCK	myosin light chain kinase
MMP2	matrix metalloproteinase 2
MP	membrane proximal
MS	mass spectrometry
MT	microtubule
mTOR	mammalian target of rapamycin
NFA	nascent focal adhesion
N-WASP	neuronal Wiskott–Aldrich Syndrome protein
NEMO	NF- κ B essential modulator
NF- κ B	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NTHi	nontypeable haemophilus influenzae
OH	Hydroxyl group
p130Cas	p130 Crk-associated substrate
PAK1	P21-associated kinase 1
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor

PECAM1	platelet/endothelial cell adhesion molecule 1
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidyl-inositol-3,4,5-trisphosphate
PIP2	phosphatidyl-inositol-4,5-bisphosphate
PINCH	particularly interesting new Cys-His rich protein
PIPKI γ	phosphatidyl-inositol phosphate Kinase Type I γ
PIX	PAK-interacting exchange factor
PK	pseudokinase domain
PKC	protein kinase C
PKL	ArfGAP Paxillin kinase linker
PDK	3-phosphoinositide-dependent kinase-1
PSI	plexin-sempahorin-integrin domain
PTB	phosphotyrosine binding domain
PTM	post-translational modification
RGD	arginine, glycine, aspartate
Rho	Ras homology gene family
RhoA	Ras homology gene family, member A
RhoGTPase	Rho guanine triphosphate hydrolase
RIAM	Rap1-GTP-interacting adaptor molecule
ROCK	Rho-associated protein kinase
Ron	receptor d'origine nantais
SDS	Sodium dodecyl sulfate
SEM	scanning electron microscopy
Ser	serine
SFK	Src family kinase
SH2	Src homology domain 2
SH4	Src homology domain 4

SILAC	Stable isotope labeling by amino acids in cell culture
Src	Rous sarcoma oncogene
TAK1	TGF β -activated kinase
TEM	transmission electron microscopy
Thr	threonine
Tie-2	tyrosine kinase with Ig and EGF homology domains 2
TGF β	transforming growth factor β
TM	transmembrane
TNF	tumor necrosis factor
TRAF2	tumor necrosis factor receptor (TNFR)-associated factor 2
TRIP	TRAF interacting protein
Tyr	tyrosine
VCAM	vascular cell-adhesion molecule
VEGFR	vascular endothelial growth factor receptor
WAVE1	WASP family Verprolin-homologous protein 1

Introduction

1. Integrins

1.1. Integrin family of cell surface receptors

The evolution of multicellularity from their unicellular ancestors crucially relied on the appearance of novel proteins that can orchestrate and maintain integrity of tissues and organs. Special classes of these proteins mediate cell-cell and cell-extracellular matrix (ECM) adhesions. Integrins are the major family for cell-ECM adhesion. They are heterodimeric transmembrane cell surface receptors that represent "integrators" as they integrate extracellular (ECM) with intracellular (F-actin cytoskeleton) polymers at focal adhesions (FAs), hemidesmosomes and cell-cell adhesion sites. They consist of α and β subunits both of which are transmembrane type I proteins (see section 1.2). They are ubiquitously expressed by almost all cell types of higher organisms (Barczyk et al., 2010; Hynes, 2002; van der Flier and Sonnenberg, 2001).

Before integrins became functional and diversified in higher biological systems, their structures evolved from earlier organisms. Sequence-based searches revealed that a cyanobacterium (*Trichodesmium erythraeum*) is expressing a protein with partial homology with the β integrin ectodomain (Johnson et al., 2009). A clear α/β integrin heterodimer is present in a *Metazoan*, the sponge *Geodia cydonium*, which is more than 600 million years distant from mammals (Brower et al., 1997). A recent study showed that 'primitive' integrins able of mediating cell-ECM adhesions emerged much earlier than *Metazoans*, in a filose amoeboid (*C. owczarzaki*) which expresses 4 α and 4 β integrin subunits (Sebe-Pedros et al., 2010). In the nematode *Caenorhabditis elegans*, two integrin heterodimers are assembled through combination of two α integrins (Ina-1 and Pat-2) and one β integrin (Pat-3) (Gettner et al., 1995; Johnson et al., 2009). Fruit flies (*Drosophila melanogaster*) have five integrins made of five α subunits (α PS1 to 5) and one β subunit (β PS) (Brabant and Brower, 1993; Roote and Zusman, 1995; Zaidel-Bar, 2009).

adhesions (see section 3) through binding to ECM extracellularly and to actin in the cytoplasm (Hynes, 2002). Integrins mediate cell-cell adhesions by binding to counter receptors that are present on the cell surface of other cells such as ICAM (Bella et al., 1998; Diamond et al., 1991; Staunton et al., 1990), VCAM (Klemke et al., 2007), MadCAM-1 (Gorfu et al., 2009; Wagner et al., 1996), PECAM-1 (Piali et al., 1995), ADAM (Bridges et al., 2005; McGinn et al., 2011), E-cadherin (Taraszka et al., 2000) and bacterial or viral surface antigens (Flaherty et al., 1997; Ruiz-Saenz et al., 2009).

Integrins transduce signals bidirectionally across the plasma membrane (Hynes, 2002; Legate et al., 2009). Moreover, their signals are integrated with other cell surface receptors cues such as growth factor receptors (GFRs; see section 4), calcium-dependent adhesions (Cadherins) and Syndecans. This signalling integration regulates a broad range of biological events including actin cytoskeletal rearrangement, cell adhesion, spreading, polarity, migration, survival, proliferation, differentiation, immune response and morphogenesis (Barczyk et al., 2010; Huveneers and Danen, 2009; Hynes, 2002).

1.2. Integrin structure

Integrins are type I transmembrane (TM) heterodimers that consist of non-covalently associated α and β glycoprotein subunits. Each subunit consists of three regions: a large extracellular domain (also called ectodomain composed of ~ 800 amino acids (aa)), a TM helix (~ 20 aa) and a short cytoplasmic tail (13 to 70 aa; except the $\beta 4$ tail with 1018 aa). Some integrin genes come in different splice variants that further increase the diversity of integrins. The largest part of integrins is their ectodomain with approximately 700 (β subunits) to 1000 (α subunits) aa. Integrin ectodomains form a globular head containing the ligand-binding pocket and an elongated flexible stalk that undergoes huge conformational changes during integrin activation.

The globular head of α subunits is composed of seven β -propeller motifs followed by three β sandwich domains (tight, Calf -1 and Calf-2) of stalk region (fig. 3). An additional $\alpha A/\alpha I$ domain insertion present in the head regions of 9 out of 18 different α subunits is composed of a Rossman fold with 7 α helices around 5 β -sheets.

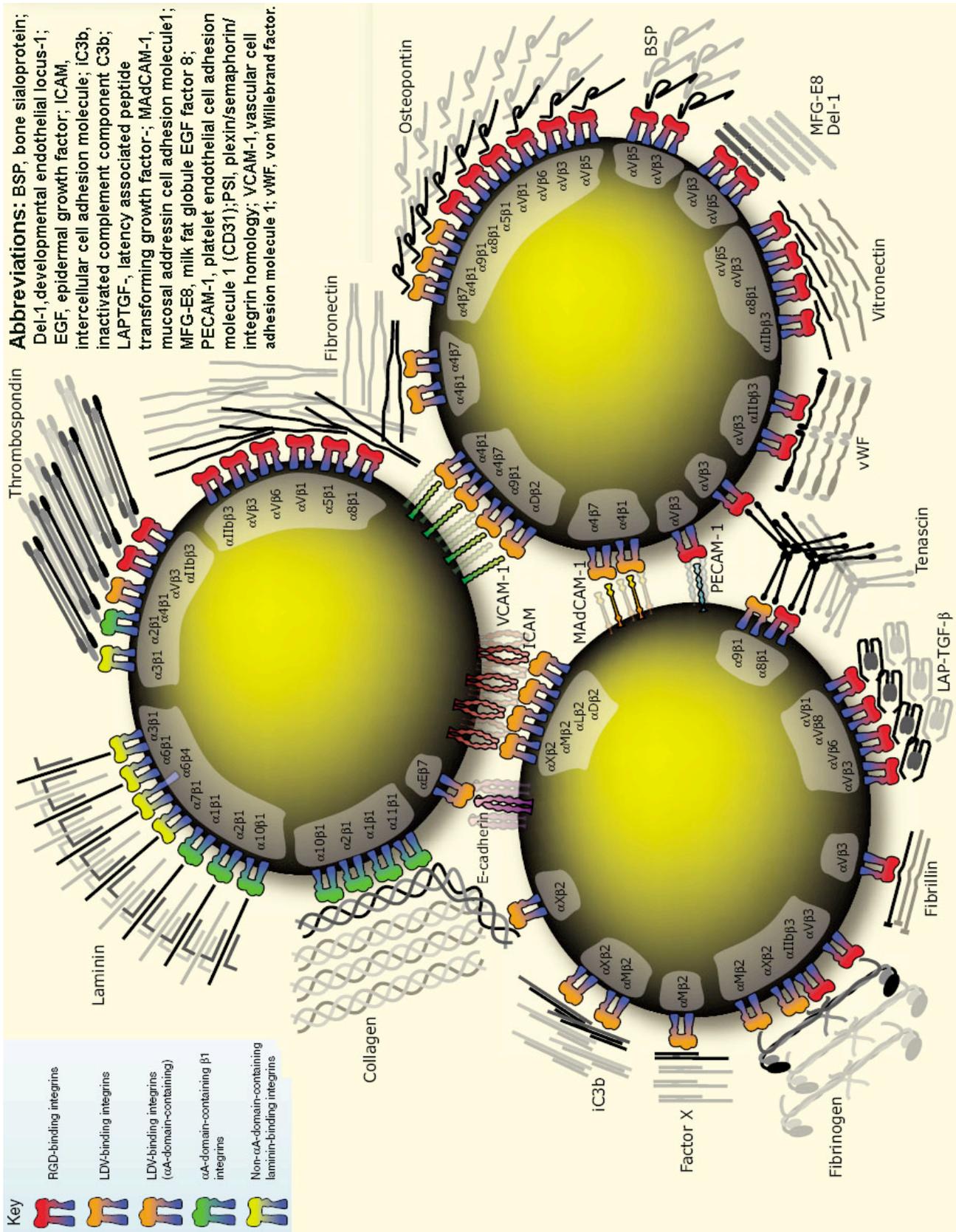


Figure 2. Integrin ligands. Adopted from (Humphries et al., 2006).

The β subunit head consists of a Plexin-Semaphorin-Integrin (PSI) domain, a β I domain which is embedded in a Hybrid domain and four integrin-cysteine-rich epidermal growth factor (I-EGF) repeats in the integrin stalk. Several metal-ion-dependent adhesion sites (MIDAS) are found in the integrins ectodomain that affect ligand-binding affinity (Fig. 3). α I domains contain Mg^{++} binding MIDAS, while β I domain have Mg^{++} coordinating MIDAS and an adjacent to MIDAS (AMIDAS) motif, which promotes activation or inhibition of integrins by binding to Mn^{++} or Ca^{++} , respectively.

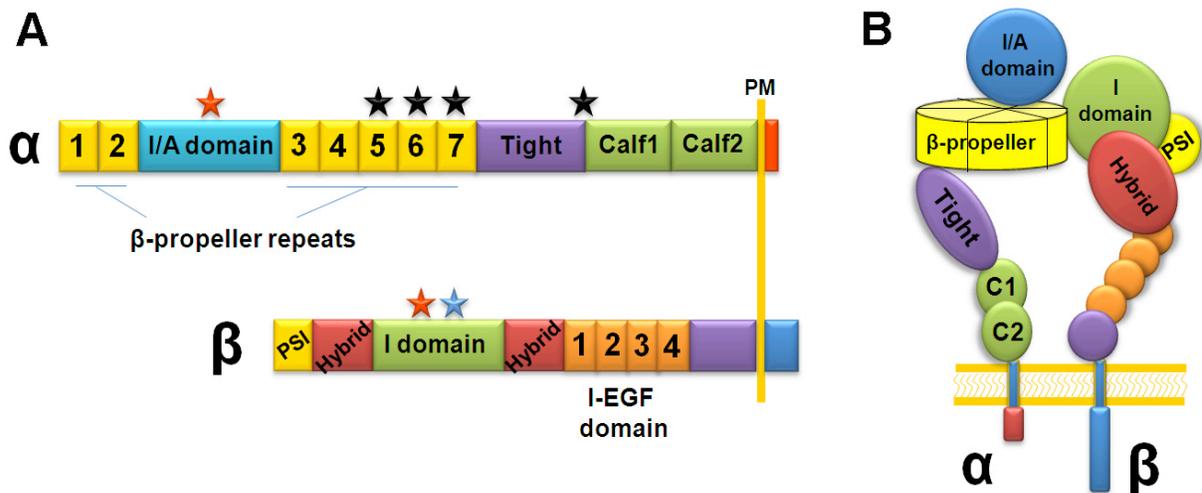


Figure 3. The structure of integrin heterodimers. (A) Structural motifs of α and β subunits. The position of Mg^{++} MIDAS (red stars), Ca^{++} MIDAS (black stars) and Mn^{++}/Ca^{++} AMIDAS (blue star) are indicated. (B) Schematic view of integrin heterodimer. α I/ α A motifs are only present in α subunits of collagen-binding and leukocyte-specific integrins.

One of the recent evolutionary diversification of α integrins was the appearance of the α I ectodomain in α subunits. Out of 9 mammalian α I domain-containing α subunits, four α subunits bind fibrillar collagen while the remaining α subunits dimerize with β 2 or β 7 subunits to form the leukocyte integrins of immune system (Fig. 4A) (Hughes, 2001; Johnson et al., 2009; Sebe-Pedros et al., 2010). The late emergence of α I ectodomain in α integrins might represent an adaptation to novel cell-ECM interactions which was necessary during the evolution of cartilage, bone and immune system. β integrin subunits can be divided into three major phylogenetic groups: two vertebrate β 1 and β 3 integrin families and one invertebrate β integrin family (Fig. 4B). An exception is β 4 integrin that stays as an outlier in the vertebrate cluster and shows no homology with any other β integrin subunits present in the phylogeny with its long cytoplasmic tail (about 1018 aa).

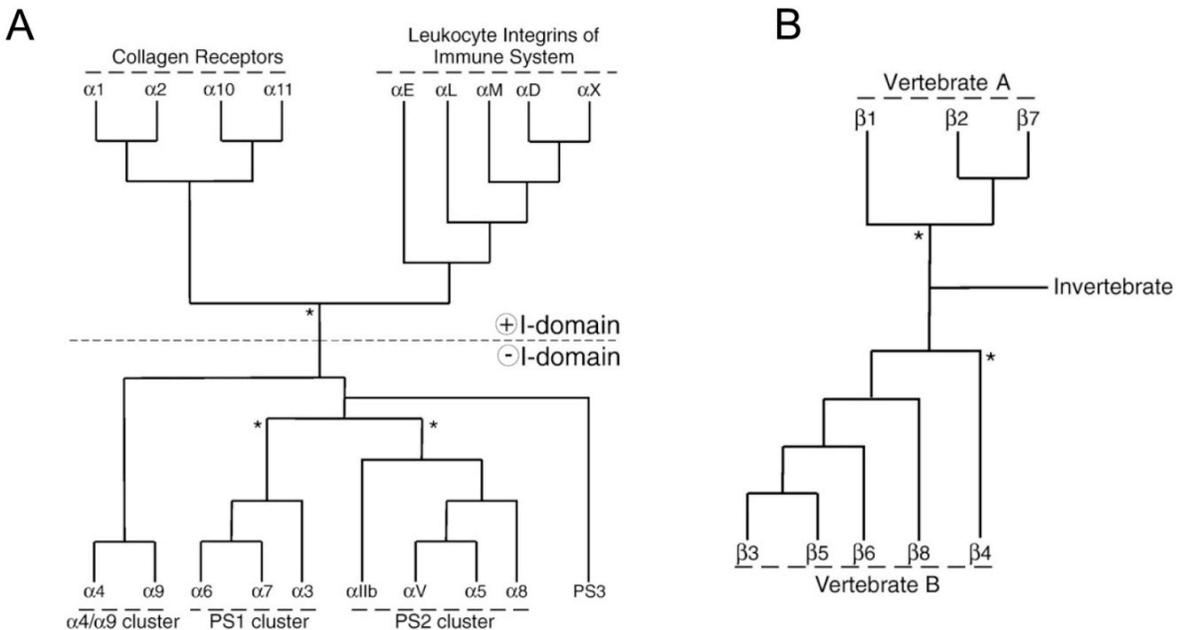


Figure 4. Generalized evolutionary tree for the family of human integrins. (A) human α subunits divided into two big groups based on existence of α I motif that emerged very early in vertebrates. **(B)** β subunits showed no obvious homology to invertebrates β integrins and can be classified into A and B groups. An expectation is β 4 subunit that contains a long cytoplasmic tail and clusters as an outlier of group B. Adopted from (Johnson et al., 2009).

The TM region of integrin subunits consists of right handed coil-coil α helices that prolong to a membrane proximal motif to the cytoplasmic tail (Wegener and Campbell, 2008). The structure of the TM region is not well defined due to the lack of a high resolution structure. Among all integrins, the TM region of α IIB β 3 integrin has been studied most extensively (Fig. 5A). The TM of β 3 integrin has 30 residues and is supposed to be tilted within the plasma membrane. The sequences of the TM regions are well conserved among integrins, suggesting that the packing interactions between consensus reciprocal groups on neighboring α and β helices are essential for integrin heterodimer formation. NMR analysis of TM domains of α IIB β 3 integrins proposed that the association of α IIB and β 3 subunits in the TM regions is mediated by the interaction of conserved Glycine residues (α IIB G972, α IIB G976 and β 3 G708) forming the so called outer membrane clasp (Fig. 5B and D) as well as a hydrophobic interaction of α IIB F992-F993 residues and the electrostatic bridge between α IIB R995- β 3 D723 residues (Fig. 5C-D) forming the inner membrane clasp (Berger et al., 2009; Lau et al., 2009; Ulmer, 2010; Yang et al., 2009).

1996), cell adhesion and migration (Imai et al., 2008; Sakai et al., 2001), the disruption of salt bridge in $\beta 1$ (D759A) integrin resulted in no obvious phenotype in mice (Czuchra et al., 2006). Perhaps, other inter-subunit interactions in TM regions (Berger et al., 2009; Luo et al., 2004) or binding of uncharacterized proteins to integrins cytoplasmic tail have a much more prominent effect on restraining integrins in their inactive conformation.

Human β_1	709	IIPIVAGVVAGIVLIGLALLLIWKLMIIDHRRREFAKFEKEKMNAKWDGTGENPIYKSAVTTVVNPKYEGK	778
Mouse β_1	709	IIPIVAGVVAGIVLIGLALLLIWKLMIIDHRRREFAKFEKEKMNAKWDGTGENPIYKSAVTTVVNPKYEGK	778
Human β_2	679	IAAIVGGTVAGIVLIGILLVIVKALIHLSDLREYRRFEKEKLKSQWNN-DNPLFKSATTVMNPKFAES	747
Mouse β_2	680	VAAIVGGTVVGVVLLIGVLLVIVKALTHLTDLREYRRFEKEKLKSQWNN-DNPLFKSATTVMNPKFAES	748
Human β_3	693	ILVVLLSVMGAILLIGLAALLIWKLLITIDHRRREFAKFEERARAKWDANNPLYKEATSTFTNITYRGT	762
Mouse β_3	693	ILVVLLSVMGAILLIGLATLLIWKLLITIDHRRREFAKFEERARAKWDANNPLYKEATSTFTNITYRGT	762
Human α_2	1103	TGVIIGSIIAGILLLLALVAILWKLGFKKRKYKMTKNPDEIDETTELSS	1152
Mouse α_2	1103	TGVIIGSIIAGILLLLAMTAGLWKLGFKKRQYKMGQNPEMDETTELNS	1152
Human α_4	945	IVIISSSLLGLIVLLISYVMWKAGFFKRQYKSILQENRRDSWSYVNSKSNDD	999
Mouse α_4	945	IIIITISLLGLIVLLISCVMMKAGFFKRQYKSILQENRRDSWSYVNSKSNDD	999
Human α_{11b}	966	IWWVLVGLGGLLLLTILVLMWVKGFFKRNRPPLEEDDEEGE	1008
Mouse α_{11b}	961	VWVVLVGLGGLLLTLLVLMWVKGFFKRNRPPLEED-EEEE	1002
Human α_v	964	VWVILAVLAGLLLAIVLVFVYRMGFFKRVRPPQEEQEREQLQPHENGEENSET	1018
Mouse α_v	960	VWVILAVLAGLLLAIVLVFVYRMGFFKRVRPPQEEQEREQLQPHENGEENSET	1014
Human α_l	1065	LYLYVLSGIGGLLLLLIFIVLYKVGFFKRNLKEKMEAGRGVNGIPAEDSEQLASGQEAGDPGCLKPLHEKDSSEGGGD	1145
Mouse α_l	1063	LHVYVLSGIGGLVLLFLIFLALYKVGFFKRNLKEKMEADGGVNGSPPEDDTDLAVPGEETKDMGCLPEPSGRVTRTKA	1140
Human α_m	1090	LPLIVGSSVGLLLALITAAIYKLGFFKRQYKDMMESEGGSPGAEPQ	1136
Mouse α_m	1091	VPLIVGSSIGGLVLLALITAGLYKLGFFKRQYKDMMEAAAPQDAPPQ	1137
Human α_x	1087	TPLIVGSSIGGLLLALITAVLYKVGFFKRQYKEMMEANGQIAPENGTQTPSPPEK	1144
Mouse α_x	1096	VPLIVGSSVGLLLALITAILYKAGFFKRQYKEMLEANGQFVSDGTPTPQVAQ	1150
Human α_d	1085	IPIIMGSSVGLLLALITATLYKLGFFKRHYKEMLEDKPEDTATFSGDDFSCVAPNVPLS	1145
Mouse α_d	1082	VFLMVFSSVGLLLALITVALYKLGFFKRQYKEMLDLPSADPDPAQADSNHETPPHLTS	1142

Figure 6. Sequence alignment of leuko-integrin cytoplasmic tails. Cytoplasmic tails of β subunits contain a MP and a MD consensus NXXY motif (highlighted blue) that mediates integrin signalling. The formation of a salt bridge occurs through the interaction of D and E residues in the β subunit HDRKE sequence with an R residue in the α subunit GFFKR sequence (highlighted yellow).

The intracellular domain of integrins is very short (>70 aa; except $\beta 4$ subunit) and lacks enzymatic activity. The cytoplasmic tail of integrins has been the focus of many studies as it transduces bidirectional signaling, i.e. first, inside-out signaling leading to conformational changes and second, ligand binding and outside-in signalling (Hynes, 2002; Moser et al., 2009; Shattil et al., 2010). Despite the high divergence of cytoplasmic tails of α subunits they only show homology in the membrane proximal GFFKR sequence. The intracellular tails of β subunits have 3 motifs in common; HDR (R/K)E, membrane proximal NPXY and membrane distal NXXY (Fig. 6). The NPXY and NXXY motifs mediate interactions that are essential for Clathrin-mediated internalization of integrins (see section 1.3; (Pellinen et al., 2008)) as well as integrin signalling (see section 2.1 and 2.2).

The conformational changes in the TM and the cytosolic tails of integrins are key points in understanding the molecular mechanism of integrin activation. Our knowledge about integrin structure is limited to $\alpha 11b\beta 3$ integrin and it is not clear to what extent other

integrin heterodimers follow the same structure-function mechanism. In addition, the models that have been proposed so far are controversial due to lack of high resolution structure for different affinity states of native integrins in complex with the plasma membrane. The development of new crystallization methods for integrin-macromolecule complexes and novel high resolution microscopy techniques will provide answers to some of these questions in future.

1.3. Integrin expression, maturation and trafficking

Mammalian integrins have 26 different integrin genes. In human, the loci of some integrins cluster near each other in the same chromosomal arm (e.g. $\alpha 1$ and $\alpha 2$ at 5q11.2; αL , αM , αD and αX at 16p11.2), suggesting that integrins evolved from an ancestral gene by gene duplication followed by molecular evolution (Table 1). Almost all cells in man express integrins with a specific integrin expression profile which varies depending on cell type and the stage of development. The most common integrin subunit is $\beta 1$ that forms 12 different integrin heterodimers and is highly expressed in most cells including embryonic stem cells.

Integrin mRNAs are transferred to rough endoplasmic reticulum (ER), where they are expressed and released into the lumen of the ER. The splice variants of integrins expand the diversity of integrin functions in different tissues during development (van der Flier and Sonnenberg, 2001). Most of the integrins form one isoform (Table 1). The ubiquitously expressed $\beta 1$ subunit has 5 different isoforms (NCBI database), most likely due to its divergent role with α subunits in different cell types. For example, the $\beta 1A$ isoform is present in all tissues, while it is replaced by $\beta 1D$ isoform during the differentiation of cardiac and skeletal muscles (van der Flier et al., 1997; van der Flier and Sonnenberg, 2001). Notably, subunits of the $\alpha 6\beta 4$ integrin are forming the highest number of isoforms, suggesting that simplicity at gene level is compensated by diversity at transcriptional and translational levels.

	$\beta 1$	$\beta 2$	$\beta 3$	$\beta 4$	$\beta 5$	$\beta 6$	$\beta 7$	$\beta 8$
Size (amino acid)	798	769	788	1822	799	788	798	769
Gene locus	10p11.2	21q22.3	17q21.32	17q25	3q21.2	2q24.2	12q13.13	7p21.1
Isoform (#)	5	1	3	5	1	1	2	1
M.W. (KD)	88.5	84.8	87	202	88	85.9	86.9	85.3

	$\alpha 1$	$\alpha 2$	$\alpha 10$	$\alpha 11$	αE	αL	αM	αD	αX
Size (amino acid)	1179	1181	1167	1188	1179	1086	1152	1161	1163
Gene locus	5q11.2	5q11.2	1q21	15q23	17p13	16p11.2	16p11.2	16p11.2	16p11.2
Isoform (#)	1	1	2	1	1	1	1	1	1
M.W. (KD)	130.8	129.3	127.6	133.5	130.2	119.2	127.2	126.8	127.8

	$\alpha 4$	$\alpha 9$	$\alpha 6$	$\alpha 7$	$\alpha 3$	αIIb	αV	$\alpha 5$	$\alpha 8$
Size (amino acid)	1032	1035	1130	1200	1066	1039	1048	1049	1063
Gene locus	2q31.3	3p21.3	2q31.1	12q13	17q21.33	17q21.32	2q31-q32	12q11-q13	10p13
Isoform (#)	1	1	9	1	2	3	2	1	1
M.W. (KD)	114.9	114.5	126.6	130.8	118.8	113.4	116	114.5	117.5

Table 1. Characteristics of human integrin α and β subunits. Clusters of integrin loci are indicated by same colors. Data retrieved from NCBI database (<http://www.ncbi.nlm.nih.gov>).

Chaperon proteins, such as Calnexin, mediate the correct folding and dimerization of nascent integrin subunits in the ER (Isaji et al., 2006; Mitchell et al., 2006). The exact mechanism of dimerization in the ER is not well understood. However, it is clear that only properly folded integrins are heterodimerized, transferred to the Golgi and routed to the cell surface. Integrin subunits undergo N-glycosylation that initiates before heterodimerization in the ER and is followed by further glycosylation and Sialylation in the Golgi (Janik et al., 2010). The N-glycosylation and Sialylation of integrins is essential for integrin dimerization, maturation and their ligand binding affinity (Isaji et al., 2006; Janik et al., 2010). The mature integrin heterodimers are transported from the Golgi apparatus to the plasma membrane where they engage in different adhesion and signalling complexes (Fig. 7). The mature integrins, which reach the cell surface, can be internalized and recycled to the plasma membrane by complex highly dynamic endosomal pathways (Caswell et al., 2009; Wickstrom and Fassler, 2011).

Interestingly, integrins themselves can affect endosomal trafficking of other receptors both positively and negatively (Caswell et al., 2009; Collinet et al., 2010; Pelkmans et al., 2005).

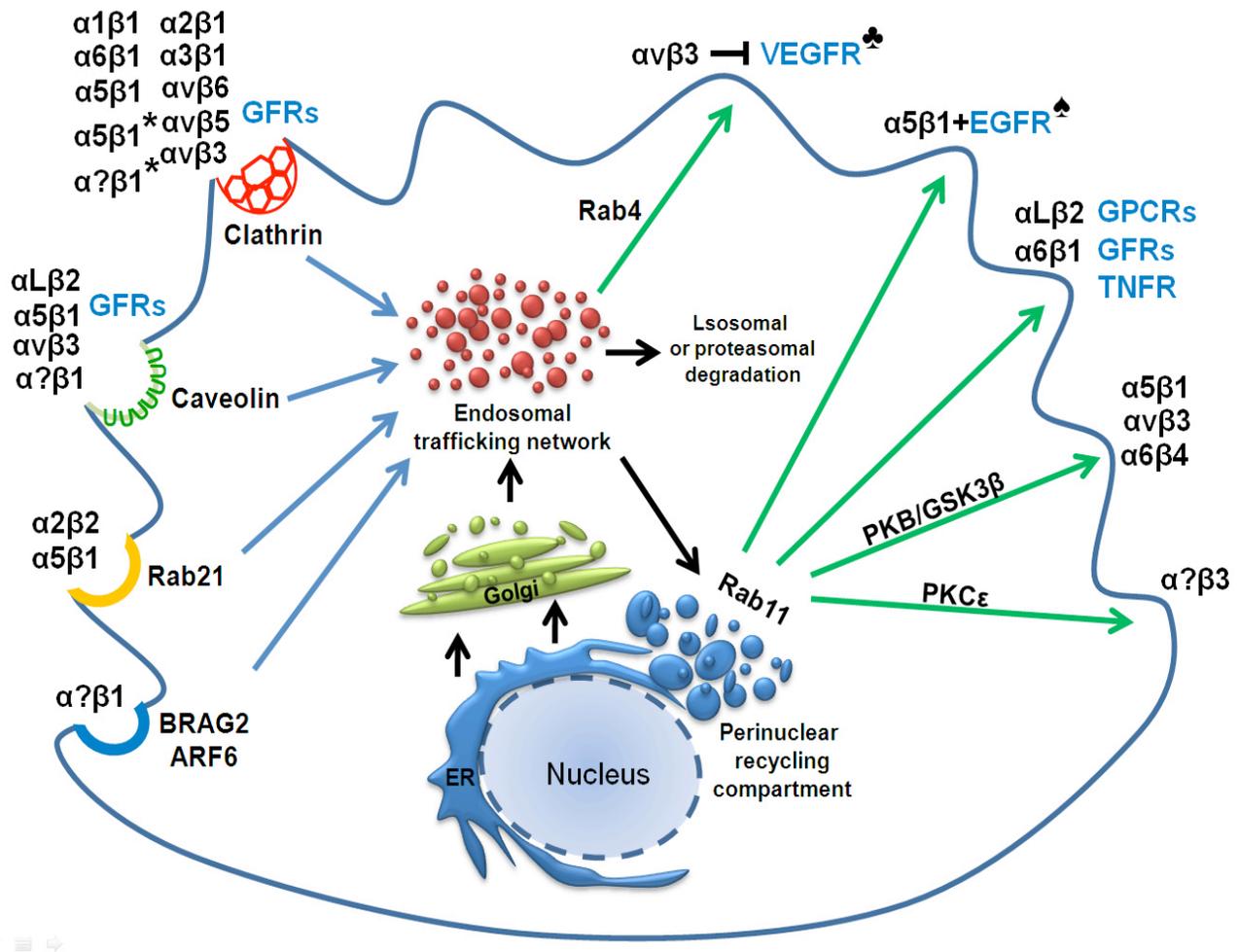


Figure 7. Integrin trafficking. Integrins are internalized (blue arrows) via at least four different endocytotic routes. Internalized integrins join a complex endosomal trafficking network, before recycle to the cell surface or guided to a proteasomal/lysosomal degradation route. Nascent integrins are expressed and heterodimerize in the endoplasmic reticulum (ER), undergo further maturation in Golgi apparatus and traffic to the cell surface via endosomal trafficking network. Internalized integrin recycle back to the cell surface (green arrows) via short Rab4-mediated- ($t_{1/2} \sim 3\text{min}$) or long Rab11-mediated- ($t_{1/2} > 10\text{min}$) trafficking routes. (*) indicates that internalized integrin was reported in an active conformation. (♣) Integrin signals control trafficking of GFRs, for example both $\alpha v\beta 3$ and VEGFR2 traffic to the cell surface by Rab4. Blocking of $\alpha v\beta 3$ integrin suppresses VEGFR2 degradation and promotes VEGFR2 recycling to the cell surface and tumor angiogenesis. Moreover, (♣) integrins share trafficking routes with GFRs, for example $\alpha 5\beta 1$ and EGFR colocalize in Rab11-positive endosomes before trafficking to the cell surface. The colocalization of integrin and GFRs in trafficking endosomes might facilitate integrin-GFR complex formation and cosignalling in endosomes. Abbreviations are GFRs: growth factor receptors; VEGFR2: vascular endothelial growth factor receptor; GPCR: G protein coupled receptor and ARF6: ADP-ribosylation factor 6. Data retrieved from (Caswell and Norman, 2006; Caswell et al., 2009).

The internalization of integrin heterodimers can be classified into 4 endocytotic routes (Fig. 7). 1) Clathrin-dependent integrin internalization mediated by adaptor proteins such as AP2 that interact with NXXY motif in the cytoplasmic tail of β integrin subunits or through

integrin-associated proteins such as DAB2 and Numb (Caswell et al., 2009). 2) Caveolin-dependent pathway shown to be responsible for endocytosis of $\alpha v\beta 3$, $\alpha L\beta 2$ and $\alpha 5\beta 1$ integrins that, in the case of $\alpha 5\beta 1$, is mediated by direct interaction of protein kinase C α (PKC α) with cytoplasmic tail of $\beta 1$ subunit (Caswell et al., 2009; Ng et al., 1999; Shi and Sottile, 2008). 3) The Rab21 route, a Clathrin-independent pathway, starts by direct binding of Rab21 to cytoplasmic tails of α subunits (Pellinen et al., 2006; Pellinen et al., 2008). 4) there are a few uncharacterized integrin internalization mechanisms that are mediated through proteins such as JAM-A or BRAG2 (Cera et al., 2009; Dunphy et al., 2006).

Like integrin internalization, integrin recycling to the plasma membrane is mediated through distinctive routes that require Rab family guanine triphosphate hydrolases (GTPases) such as Rab4 (Roberts et al., 2001; Vukmirica et al., 2006; Woods et al., 2004), Rab5 (Torres and Stupack, 2011), Rab11 (Eva et al., 2010; Skalski and Coppolino, 2005), Rab21 (Pellinen et al., 2006; Pellinen et al., 2008) and Rab25 (Caswell et al., 2007). For example, $\alpha v\beta 3$ integrin is transported from early endosomes to the plasma membrane through Rab4-dependent pathway in platelet-derived growth factor (PDGF)-triggered cells (Fig. 7), while the exocytosis of $\alpha v\beta 3$ integrin in non-stimulated cells and of $\alpha 5\beta 1$ integrin are carried out by Rab11 and Arf6 from a perinuclear recycling compartment to the leading edge of the cell (Roberts et al., 2001).

Integrin trafficking exerts crucial effects on different biological functions that are regulated by integrins including cell division, adhesion, migration and tumorigenesis. However, the precise molecular mechanism of integrin recycling remains unknown. Like GFRs, some internalized integrins adopt an active conformation in endosomes and potentially could mediate signalling. Moreover, as the formation of integrin-GFR complexes at the plasma membrane is one of the mechanisms of underlying integrin-GFR cosignalling (see section 4.1), the colocalization of integrins with growth factors in trafficking vesicles might further facilitate signalling events (Caswell et al., 2008). The power of the proteomics technology in combination with advanced microscopy techniques and photoactivatable molecules will further improve our understanding of novel molecular players, dynamic and signalling outputs of trafficking integrins.

2. Integrin signalling

In all organisms the cell fate is determined by a labyrinthic signalling network which is programmed by inherited machines that are activated in response to environmental challenges. Consequently, sensing various extracellular stimuli is crucial for cells to properly coordinate their signalling homeostasis. Cells achieve such coordination through ultrasensitive cell surface receptors that transduce signals across the plasma membrane barrier. In higher organisms, a large variety of cell surface receptors emanate signals upon interaction with extracellular molecules in order to induce and orchestrate cellular actions in tissues at different developmental stages. Among them, integrins play a decisive role in tuning cell signalling network in several ways:

Firstly, integrins can trigger signals bidirectionally across the plasma membrane (Hynes, 2002). Secondly, integrins are the core components of cell adhesions, the highly dynamic signalling platforms where other signalling molecules are recruited, interact with each other and trigger additional cues (Zaidel-Bar and Geiger, 2010; Zaidel-Bar et al., 2007). Thirdly, integrins interaction with ECM molecules facilitates receptor-ligand binding (e.g. transforming growth factor β (TGF β) receptor) and signalling of other cell surface receptors (Margadant and Sonnenberg, 2010; Wipff and Hinz, 2008). Fourthly, integrins regulate endosomal trafficking which could potentially affect signalling of all endosomal trafficking components including GFRs (Caswell et al., 2009; Wickstrom and Fassler, 2011). Finally, integrins signals crosstalk with signals of other cell surface receptors (e.g. GFRs and Syndecans) to control various biological events in different cell types (Cabodi et al., 2004; Huvneers and Danen, 2009).

Classically, integrin signal transduction has been classified into two classes based on the direction and position of cues that they transduce across the plasma membrane. 1) Integrin inside-out signalling refers to the intracellular signalling events that modulate the integrin binding affinity to extracellular ligands. 2) Integrin outside-in signalling assigns the signalling process that starts extracellularly by binding of integrins to ligands which, in turn, trigger various integrin signalling pathways inside the cell (Fig. 8). Based on recent studies, a third class of integrin signals might be considered as 3) intracellular integrin signalling events that occurs by trafficking integrins, far away from the plasma membrane. It has been shown that NPXY motif of integrin cytoplasmic tails signals intracellularly

during trafficking of endosomal integrin to the cell surface (Caswell et al., 2009; Collinet et al., 2010). In addition, internalized endosomal integrins, could still maintain in their active conformation and therefore, like active internalized GFRs, could potentially elicit signals (Caswell et al., 2009; Wickstrom and Fassler, 2011).

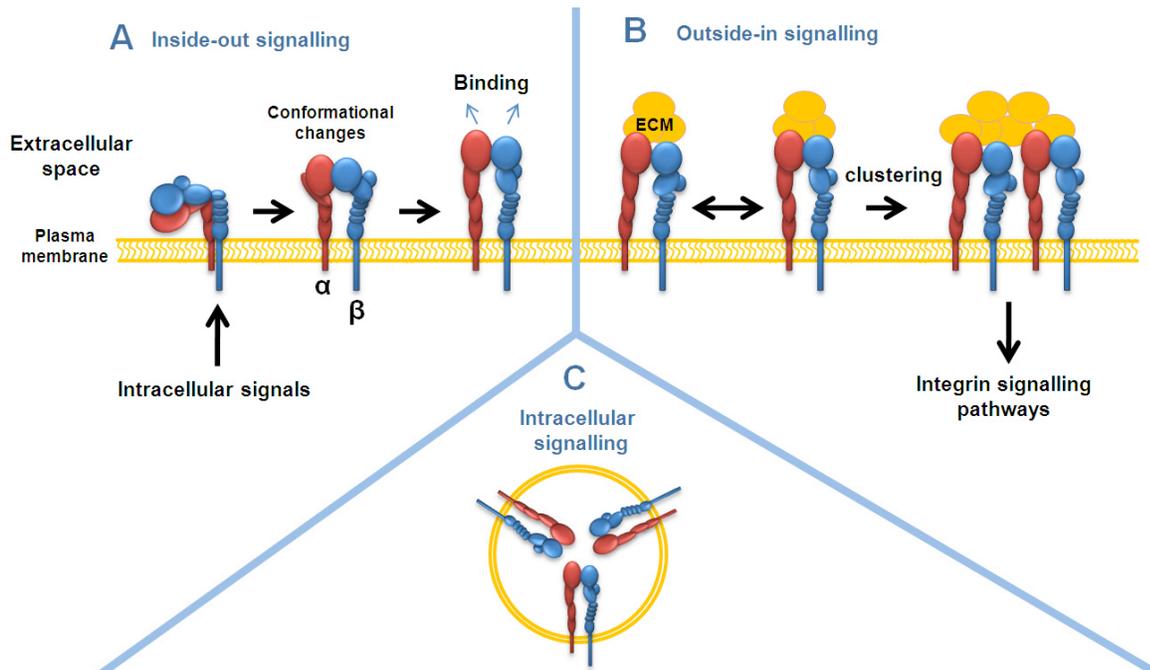


Figure 8. Integrin signalling. Integrin cues can be divided into (A) inside-out and (B) outside-in signalling pathways. Based on recent studies, an emerging class of integrin signals can be considered eliciting from (C) intracellularly active integrins that localized to endosomes.

In this section, classical integrin inside-out- and outside-in- signalling pathways and emerging concepts of integrin mechanotransduction will be discussed.

2.1. Integrin inside-out signalling

It is essential for physiological function of different cell types to tightly regulate integrin activation process, particularly for leukocytes and platelets that circulate in different tissues and therefore, should keep their integrins in a low affinity and inactive conformation. The main integrin of platelets is α IIB β 3 (Fig. 5), which has long been known for its role in platelet spreading on ECM and blood coagulation. A broad range of different agonists, including thrombin, collagen, adenosine diphosphate (ADP), trigger different cell surface receptors in platelets and mediate complex intracellular signals that finally activate α IIB β 3

integrin (Kasirer-Friede et al., 2007). Activated α IIb β 3 integrin can bind to Arginine, Glycine and Aspartate (RGD)-containing ligands including fibrinogen, thrombospondin, Von Willebrand factor, fibronectin (FN) and vitronectin. Subsequently, the bound platelets release coagulants (e.g. fibrinogen, thrombospondin, von Willebrand factor, FN) from their α -granules that leads to further platelet aggregation (Kasirer-Friede et al., 2007).

Leukocytes integrins are α 4 β 1 (VLA-4), α 4 β 7, α L β 2 (LFA-1) and α M β 2 (MAC-1) that maintain in low affinity state before their rapid activation via integrin inside-out signalling pathway (Fig. 9). The activation process is started by capturing and rolling of lymphocytes on endothelial cells and mainly achieve through shear-induced partial activation of VLA-4 integrin and the interaction between L-selectin and P-selectin of inflamed endothelium and P-selectin glycoprotein ligands1 (PSGL1) of leukocytes (Alon and Shulman, 2011; Ley et al., 2007).

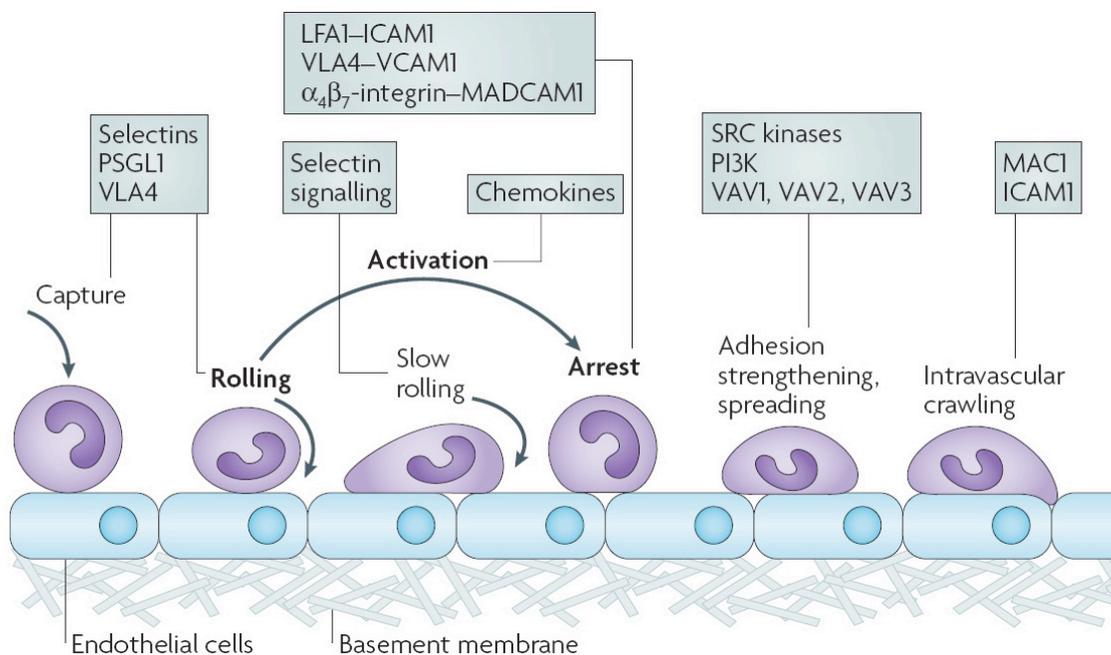


Figure 9. Leukocyte adhesion signalling cascade. Integrin activation plays a crucial role in mediating leukocyte rolling and adhesion on endothelial cells.

The chemokines on the surface of inflamed endothelial cells bind to G-protein coupled protein receptor (GPCR) on lymphocytes which leads to the activation of leuko-integrins through intracellular signalling pathways. This causes lymphocytes to roll slower, arrest, strengthen their adhesions and make diapedesis (Alon and Shulman, 2011; Ley et al., 2007).

The molecular mechanism of inside-out integrin activation has been studied extensively. The critical step of integrin activation is binding of talin and kindlin molecules to cytoplasmic tail of integrins (Moser et al., 2009; Nieswandt et al., 2009). Talin is a 270 kD protein, composed of an N-terminus 47 kD globular FERM domain with four subdomains (F0 to F3) and a 220 kD C-terminal rod domain (Fig. 10). F3 sub-domain of talin is a PTB domain and binds to membrane-proximal NPxY motif of $\beta 1$ or $\beta 3$ integrin tails (Moser et al., 2009). In addition, talin has multiple vinculin and F-actin binding sites as well as a second integrin binding site in the C-terminal flexible rod domain that is critical for connecting integrins to actin cytoskeleton (Fig. 10).

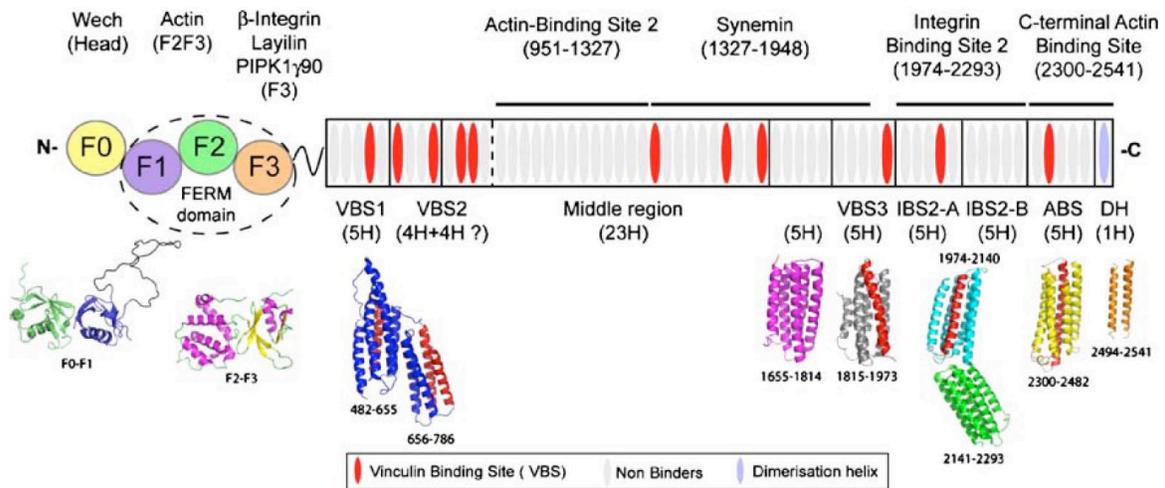


Figure 10. Domain structure of talin. N-terminal globular head of talin is composed of a FERM domain with four subdomains (F0 to F3) that is followed by a 220 kD C-terminal rod domain. Talin connects actin cytoskeleton to β integrin cytoplasmic tails through a F-actin binding site and vinculin binding sites (red). Adopted from (Roberts and Critchley, 2009).

Talin-integrin interaction alone is insufficient to trigger inside-out integrin signalling. Recent studies showed that the interaction of the PTB-like subdomain of kindlin FERM domain with membrane-distal NxxY motif of $\beta 1$, $\beta 2$ and $\beta 3$ integrin tails is essential, but not sufficient for mediating integrin activation (Montanez et al., 2008; Moser et al., 2009; Moser et al., 2008). The cooperative interaction of talin and kindlin with the cytoplasmic tail of integrins causes separation of integrins heterodimer tails and leads to conformational changes in intracellular, transmembrane and ectodomain of integrins. While, conformational changes of the ectodomain are well demonstrated, the structural details of integrin transmembrane domains remain unknown. The transition from resting to active

integrin conformation has been proposed by two main models; Switchblade (Luo et al., 2007) and Deadbolt (Xiong et al., 2003). Both models suggest conformational change of head domain during activation, while integrin ligand-binding occurs before (deadbolt) or after (switchblade) extension of integrin ectodomain (Arnaout et al., 2007). Structural studies proposed at least three main conformations for integrins: resting low affinity integrins with bent ectodomain conformation, intermediate state(s) and active high affinity integrins with extended extracellular domain (Fig. 11).

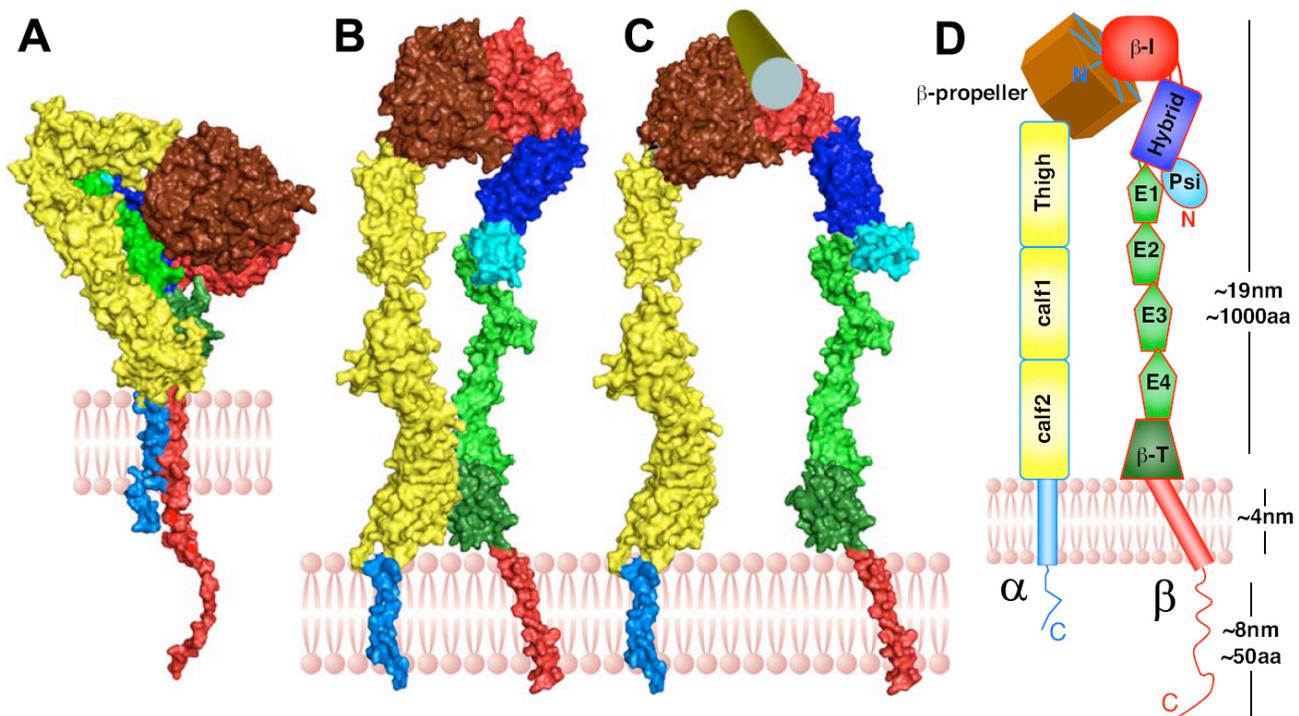


Figure 11. Integrin conformations. (A) Resting low affinity, (B) intermediate state(s) and (C) active high affinity integrins with extended extracellular domain. (D) Domain structure of integrins represented with the same color codes in A to C. The cytosolic tails are truncated in B and C panels. Adopted from (Anthis and Campbell, 2011).

The precise molecular mechanism of integrin activation remains unknown. It is clear that the activation is organized by series of signaling events including activation of agonist receptors and engagement of talins, kindlins and Rap1-GTP-interacting adaptor molecule (RIAM). The interaction of talin and kindlin with cytoplasmic tail of β integrins is tightly regulated at the molecular level (Fig. 12). NMR studies revealed that talin exist in activated and autoinhibited states. The inactive talin conformation is characterized by intramolecular autoinhibitory interaction of the C-terminal rod domain and the PTB-like subdomain

covering the integrin binding site. The autoinhibitory conformation of talin is disrupted through interaction with the Rap1-RIAM complex that becomes activated through GPCR signaling and binding to phosphatidylinositol phosphate Kinase Type-I γ (PIPKI γ)/phosphatidylinositol-4,5-bisphosphate (PIP₂). The talin head can also be released from its inhibitory interaction by Calpain cleavage. Finally, integrins undergo conformational changes and activation through binding of F3 subdomains of talins and kindlins to membrane proximal NPXY and membrane distal NXXY motifs of β integrin tails, respectively (Fig. 12).

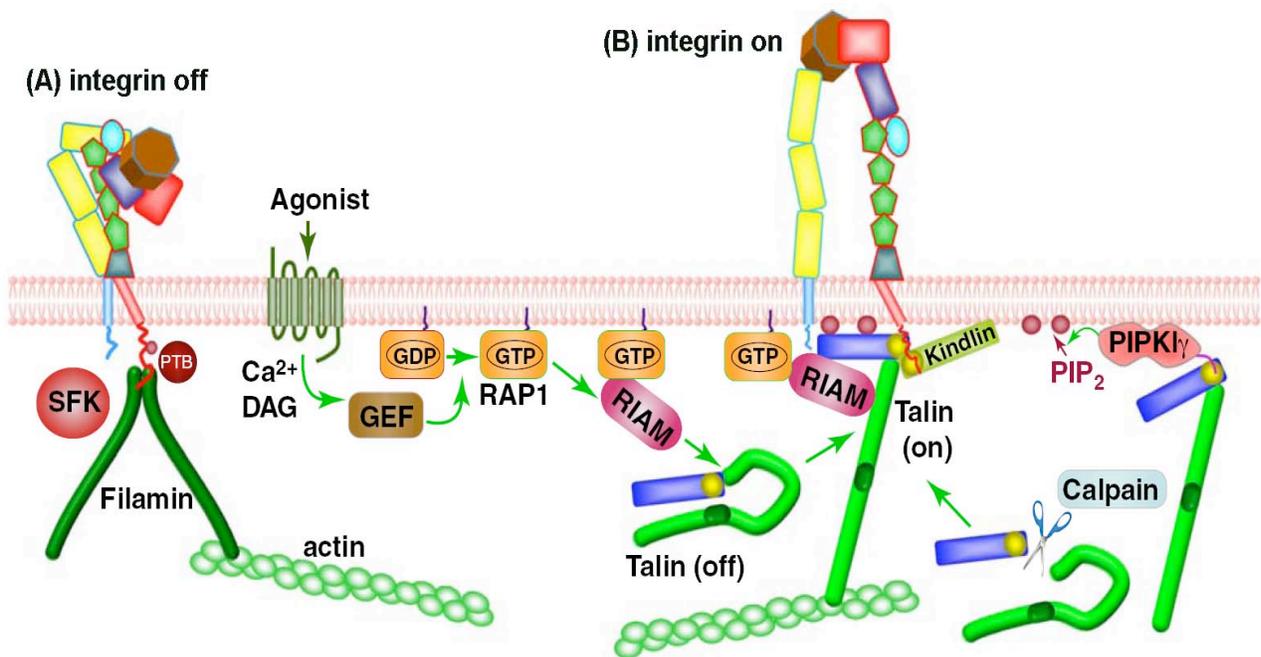


Figure 12. Integrin inside-out signalling pathways. (A) Integrin resting state can be stabilized by binding of PTB-containing proteins or filamin and tyrosine phosphorylation with SFKs. (B) Cooperative interaction of talin and kindlin leads to integrin activation. Agonist stimulation, Calpain cleavage and interaction with PIPKI γ /PIP₂ trigger talin binding to β integrins cytoplasmic tail. Talin and kindlin F3 subdomains (yellow circles) bind to membrane proximal NPXY and membrane distal NXXY motifs of β integrin cytoplasmic tails, respectively. This causes separation of integrin cytoplasmic tails, transfer of conformational changes from tails to the ectodomain and integrin activation. Adopted from (Anthis and Campbell, 2011).

The low affinity resting conformation of integrins is stabilized by binding to Filamin or PTB-containing proteins such as Dock1 and ICAP. Dock1 binding to β integrin tails causes integrin inactivation by Src family kinase (SFK)-dependent tyrosine phosphorylation of integrin NPXY and NXXY motifs, suggesting that posttranslational modifications can regulate integrin activation at the receptor level (Anthis and Campbell, 2011). Although,

the levels of $\beta 1$ integrin tail tyrosine phosphorylation is very low, even in presence of v-Src, several studies indicated that SFK-dependent tyrosine phosphorylation of $\beta 1$ integrin on NPXY and NXXY motifs (Tyr783 and Tyr795) causes integrin inactivation by blocking talin and kindlin binding to $\beta 1$ integrin. Mutating both $\beta 1$ integrin Tyr783 and Tyr795 to phenylalanine (but not either one) decreases adhesion strength and tumorigenesis, only when Src activity is low. However, in present of high Src activity, a Src-FAK signalling complex (see section 2.2) promotes tumorigenesis downstream of $\beta 1$ integrin and independent of $\beta 1$ integrin tyrosine phosphorylation (Meves et al., 2011).

2.2. Integrin outside-in signalling

Integrins mainly traffic to the cell surface in a resting inactive state. Based on the switchblade model, the binding of ECM ligands to integrin ectodomain modifies integrin heterodimer conformation to an extended active state and leads to separation of α - and β -integrin cytoplasmic tails (Arnaout et al., 2007). Consequently, about 200 different proteins are recruited to integrin cytoplasmic tails, where they interact and regulate activity of each other and trigger a broad range of different signalling pathways. Integrin outside-in signalling pathways are referred to signalling events which are triggered intracellularly upon integrin binding to ECM ligands. The diversity of ECM molecules and integrin heterodimers as well as cell type-/tissue-specific expression of FAs proteins increases the complexity and variety of integrins outside-in signalling pathways (Fig. 13).

Integrins outside-in signalling events can be divided chronologically into: immediate early, short term and long term effects (Legate et al., 2009). The early effects of integrin activation are fast phosphorylation of FAs proteins and increase of lipid kinases activity (Fig. 13A). Phosphatidylinositol 3-kinase (PI3K) and PIPKI γ cause a local increase in phosphatidyl-inositol-3,4,5-trisphosphate (PIP3) and PIP2 concentrations, which in turn, affect recruitment of many adhesion molecules such as talin, α -actinin, tensin and Shc (Legate et al., 2009). Phosphorylation also triggers immediate early signals upon integrin activation by modifying conformation, activity and binding properties of FA molecules. Short term effects are the activation of signalling pathways such as extracellular signal-regulated kinase (ERK1/2) and c-jun N-terminal kinase (JNK), elevation of active Ras homology protein (Rho) family of small GTPases levels and modulating the activity of

proteins that play a role in actin cytoskeleton rearrangements. The final consequence of these signaling events are long-term effects of integrin signals, a wide range of diverse biological functions, including proliferation, differentiation and survival (Fig 14.A) (Legate et al., 2009).

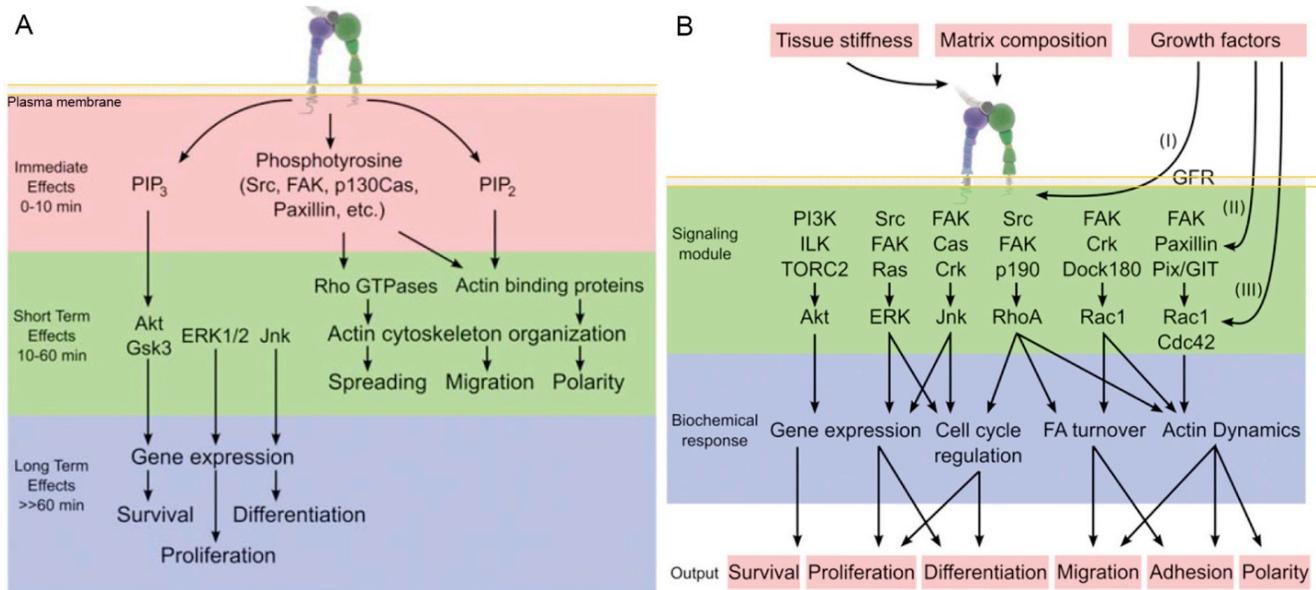


Figure 13. Integrin outside-in signalling. (A) Chronological events of integrin activation. (B) Examples of signalling modules downstream of integrin activation. From (Legate et al., 2009).

Integrin activation through binding to ECM ligands triggers a broad range of diverse and often distinct biological consequences in cells, tissues and developmental stages. This diversity and complexity increases even more, since integrin signals cooperate with different type of cell receptors including GFRs (see section 4).

Integrin activation enhances PI3K activity and promotes its recruitment to FAs (Fig. 13B). Activated PI3K elevates PIP₃ levels at plasma membrane, which in turn, causes RAC- α serine (Ser)/threonine (Thr) protein kinase (Akt) phosphorylation on Thr308 through PIP₃-dependent recruitment of 3-phosphoinositide-dependent kinase1 (PDK1). Akt can be also phosphorylated on Ser407 in presence of mammalian target of rapamycin (mTOR) and integrin linked kinase (ILK). The correlation of ILK expression with Akt phosphorylation on Ser407 may occur through the influence of ILK on mTOR2 activity (McDonald et al., 2008).

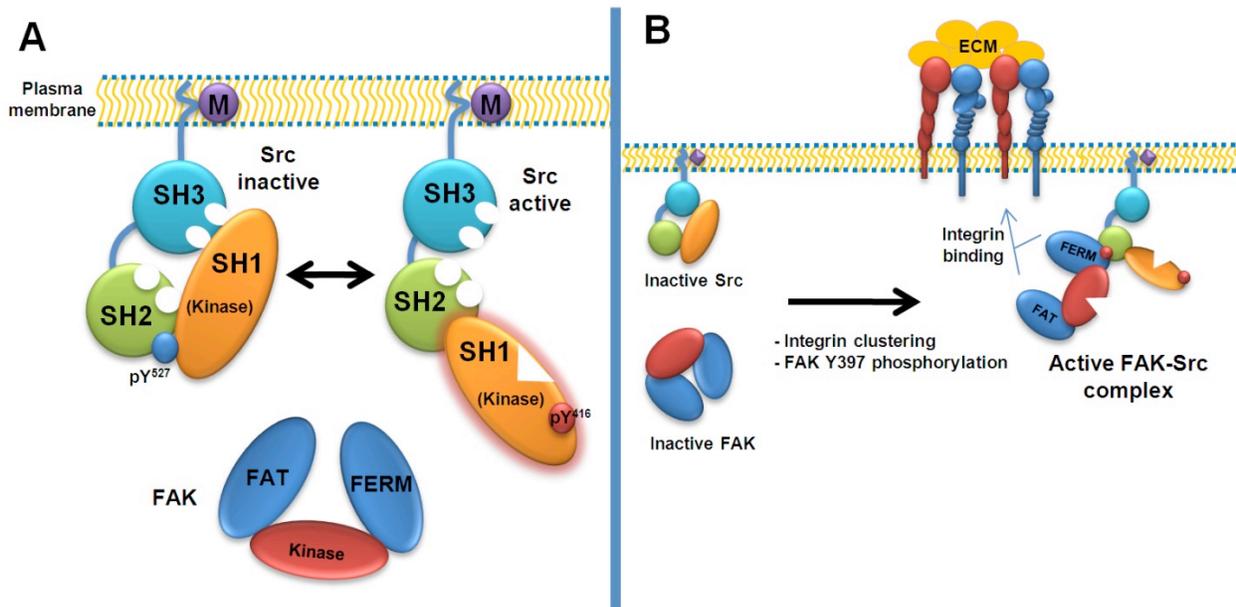


Figure 14. Assembly of c-Src-FAK complex. (A) c-Src has four Src homology domains (SH): SH1 or kinase domain, SH2, SH3 and SH4 domains. Immediately after c-Src expression, myristoylation of the SH4 domain anchors c-Src to the plasma membrane. Transition from catalytically inactive bent c-Src to active c-Src is tightly controlled by a molecular switch that is composed of an inhibitory phospho-Tyr527 and an activatory phospho-Tyr416. FAK is composed of an N-terminal FERM domain, kinase domain and a C-terminal focal adhesion targeting (FAT) domain. (B) Plasma membrane-associated c-Src adopts an inactive conformation. The c-Src-FAK complex forms by (*trans*-*auto*-) phosphorylation on FAK Tyr397 upon integrin clustering followed by binding of FAK Tyr397 to SH2 domain of Src. Finally, auto-phosphorylation of c-Src Tyr416 causes its full activation. Abbreviations are ECM: extracellular matrix; FERM: 4.1, ezrin, radixin, moesin domain; FAT: focal adhesion targeting domain ; M: myristoylation.

c-Src belongs to the Src family of non-receptor tyrosine kinases (SFK), of which Src, Fyn and Yes are widely and often co-expressed in different tissues during all stages of development. c-Src achieves an inactive conformation through interaction of the Src homology domain 2 (SH2) domain with the C-terminus phosphorylated tyrosine (Tyr) 527 (Tyr530 in human) (Fig. 14A). Upon integrin activation and clustering, focal adhesion kinase (FAK) is activated through (*trans*-*auto*-) phosphorylation on Tyr397. Phosphorylated Tyr397 binds to Src SH2 domain and disrupts c-Src inhibitory conformation (Fig. 14). The disruption of c-Src inhibitory conformation also occurs through the interaction of c-Src SH2 domain with phospho-Tyrs of activated GFRs or, c-Src SH3 domain with proline-rich motif of p130 Crk-associated substrate (p130Cas) (Arias-Salgado et al., 2003; Huvneers and Danen, 2009; Mitra et al., 2005). c-Src is then fully activated by auto-phosphorylation at Tyr416 (Tyr419 in human). In turn, activated c-Src further promotes FAK activity by phosphorylation of FAK on several Tyr residues. Activated c-Src is a small proportion of

the total c-Src that associated with the plasma membrane and endosomal compartments (Huveneers and Danen, 2009; Yeatman, 2004).

The signalling outcomes of c-Src-FAK complexation are vast including activation of Ras-MEK-MAPK/ERK as well as phosphorylation of Paxillin and p130Cas (Fig. 13B and Fig. 15A). Modulation of the Ras-MEK-MAPK/ERK signalling axis by integrins regulates cyclin D1 expression and cell cycle progression, while the activation of Paxillin and p130Cas has distinctive signalling outputs depending on the cellular spreading phase (Fig. 15A) (Legate et al., 2009).

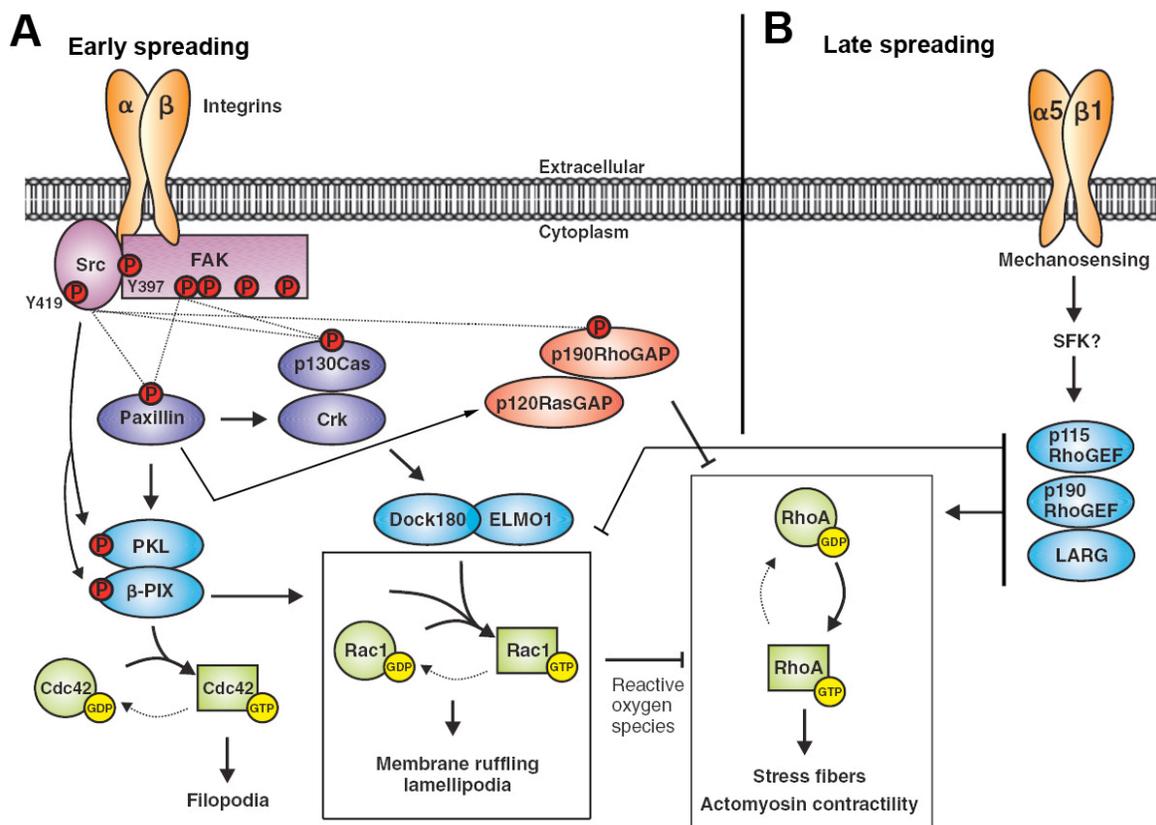


Figure 15. Integrin regulation of small GTPases. (A) During early spreading, the c-Src-FAK complex activates Rac/Cdc42 through multiple signalling pathways. (B) At late phases of spreading, integrin signals promote Rho actomyosin contractility through several Rho GEFs. Modified from (Huveneers and Danen, 2009).

Both Paxillin and p130Cas can bind to the adaptor molecule CT-10 related kinase (Crk) upon its phosphorylation (Fig. 15A). During early spreading, phosphorylated Paxillin on Tyr31 and Tyr118 can bind to p120Ras-GTPase activating protein (GAP), release p190RhoGAP from its inhibitory complex with p120RasGAP and inhibit Rho activity

(Huveneers and Danen, 2009). Moreover, phosphorylated Paxillin recruits Arf GAP Paxillin kinase linker- β -PAK-interacting exchange factor (PKL- β -PIX), a complex that additionally can be activated by c-Src to enhance Rac/Cdc42 activity (Fig. 15A). Activated p130Cas promotes cell migration and spreading through up-regulation of Rac activity via the Dock180-Engulfment and Cell Motility (ELMO) complex (Siesser and Hanks, 2006). This scenario shifts gradually into the reverse direction during late phase of spreading, when integrin signals promote RhoGTPase activity, formation of stress fibers and maturation of FAs. $\alpha 5 \beta 1$ integrin of FN-seeded cells plays an effective role during late phase of spreading by up-regulation of leukemia-associated RhoGEF (LARG), p190RhoGEF and p115RhoGEF, probably through SFKs (Huveneers and Danen, 2009).

2.3. Integrin mechanotransduction

Cells sense and react to mechanical environments through activation of a variety of receptors including cell-cell and cell-ECM adhesion molecules. The linkage of the ECM to the F-actin cytoskeleton by integrins mediates bidirectional transmission of mechanical forces. Integrin-mediated conversion of ECM mechanical stimuli to biochemical signals as well as actomyosin forces to ECM molecules are referred as integrin mechanotransduction (Huveneers and Danen, 2009; Papusheva and Heisenberg, 2010).

Local tension and ECM stiffness facilitate integrin activation and clustering as well as maturation of FAs through tension-dependent modification of conformation, composition and signalling properties of adhesion molecules (Papusheva and Heisenberg, 2010). The formation of nascent FAs (NFAs) is driven by myosin light chain kinase (MLCK) and non-muscle myosin II produces protrusions at the cell edge (Giannone et al., 2007). NFAs and further mature actin-linked focal complexes (FCs) are enriched with $\beta 3$ integrin and adhesion molecules such as talin and vinculin (Fig. 16A). Force-induced stretching of talin exposes cryptic vinculin binding sites on talin and promotes formation of talin-vinculin complexes, which are molecular clutches that mediate integrin-F-actin cytoskeleton connection (del Rio et al., 2009). The maturation of FCs and low density FAs in cell protrusions into big FAs and $\alpha 5 \beta 1$ integrin-rich fibrillar adhesions (FBs) proceeds by ROCK-dependent actomyosin forces (Ballestrem et al., 2001).

It proposed that $\alpha V\beta 3$ and $\alpha 5\beta 1$ integrins may differentially mediate the activation of RhoA GTPases (Fig. 16B). While $\alpha V\beta 3$ integrin-rich FCs and low density FAs in cell protrusions trigger Rac/Cdc42 activation, $\alpha 5\beta 1$ integrin-rich FAs and FBs in central region of the cell promote RhoA-mediated formation of stress fibers and contractility (Huveneers and Danen, 2009). Recently, the precise spatiotemporal dynamics of active RhoGTPases was monitored by photoactivatable molecules. RhoA is predominantly active in a narrow band at the leading edge of cells and in cell central regions, while active Rac/Cdc42 is present in cell protrusion (Papusheva and Heisenberg, 2010).

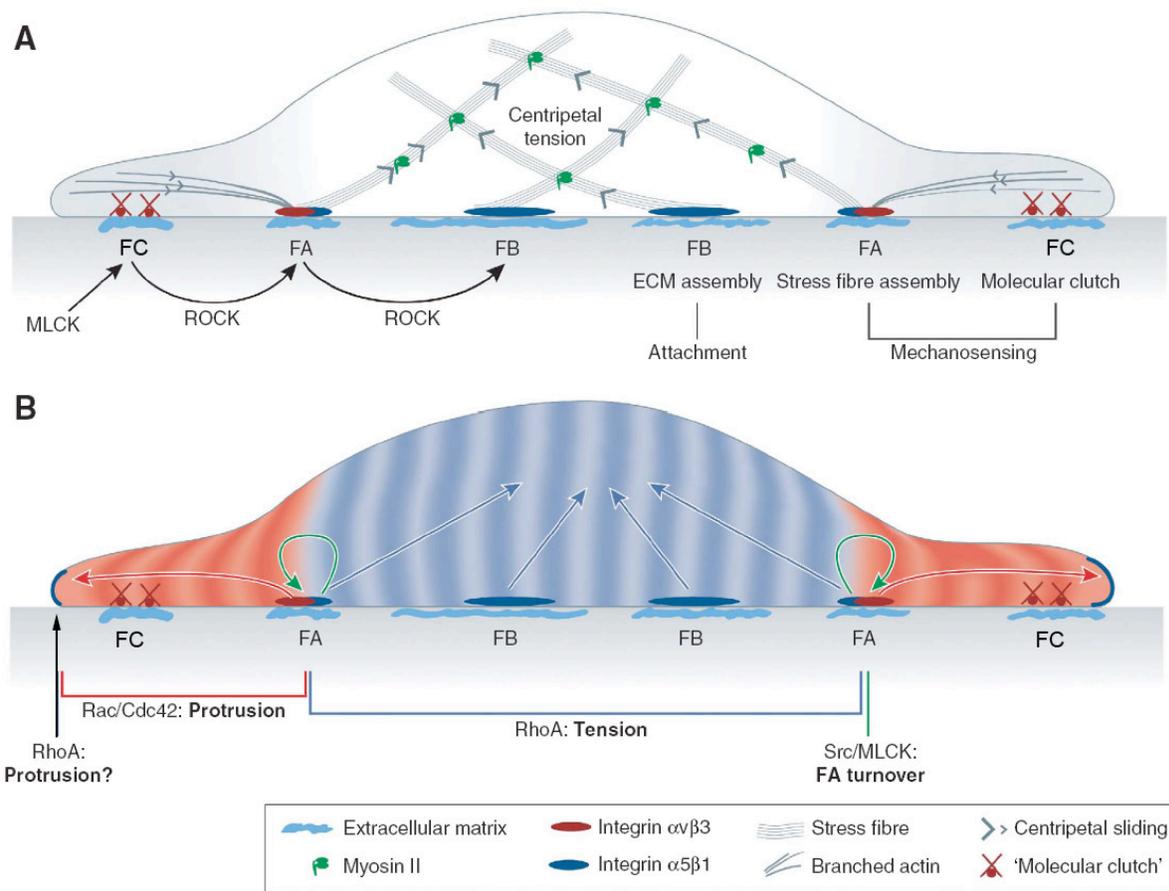


Figure 16. Tension-induced remodeling of adhesion composition and cell signalling. (A) NFAs and FCs assemble in cell protrusions by myosin light chain kinase (MLCK)-induced forces and mature to FAs and FBs via actomyosine forces. (B) Coordination of RhoA GTPases through molecular composition of cell-ECM adhesions. RhoA is activated in a narrow band at the cell edge by an unknown mechanism. $\alpha V\beta 3$ containing adhesions in cell protrusions induce Rac/Cdc42 activation (red area) that are essential for the stabilization of the protrusions, while central $\alpha 5\beta 1$ integrin-rich adhesions elevate active RhoA levels (blue area). From (Papusheva and Heisenberg, 2010).

Integrin mechanotransduction is also crucial for assembly and organization of ECM molecules. $\alpha 5\beta 1$ integrin binds to soluble FN more efficiently (Huveneers et al., 2008) and promotes FN fibrillogenesis through cytoskeletal force-induced stretching of soluble FN dimers. Integrins also facilitate assembly of collagen matrix, directly, through collagen-binding integrins and, indirectly, via assembly of FN-matrix (Huveneers and Danen, 2009). Moreover, FN fibrillogenesis by $\alpha 5\beta 1$ integrin-dependent mechanical tensions, indirectly, facilitates latent TGF β binding protein (LTBP) binding to FN fibrils during assembly of latent TGF β complex. The binding of $\alpha V\beta 3$, $\alpha V\beta 5$, $\alpha V\beta 6$ and $\alpha V\beta 8$ integrins to the RGD site in the latency-associated peptide (LAP) of latent TGF β complex followed by actomyosine-mediated contractions release TGF β in ECM and trigger numerous signalling pathways (Worthington et al., 2011).

Matrix rigidity can cause modifications of integrin signals via change in expression, conformation and activation of integrins. Increasing ECM stiffness during tumorigenesis has been considered to be essential for regulating tumor growth. Increased substratum stiffness stimulates activation of RhoA GTPases and differentiation of breast epithelial cells. In addition, elevation of ECM remodeling by increased RhoA-dependent cytoskeletal contractility promotes tumor cell proliferation in stiff matrices (Huveneers and Danen, 2009). Indeed, integrin mechanotransduction has a pivotal role in tissue morphogenesis and *in vivo* signal transduction, although little is known about physiological context of integrin mechanosensing *in vivo*. It requires further efforts to reveal how composition of adhesion molecules drives distinctive signalling pathways in different tissues and how integrins nano-mechanosensory properties in different cell types orchestrate overall tissue morphogenesis.

3. Integrin-dependent cell adhesions

The biogenesis of integrin-dependent cell adhesions starts with the interaction of the glycocalyx embedded extracellular domain of integrins with ECM ligands. The interaction causes assembly of integrin-mediated adhesions with distinctive morphology, molecular

composition and dynamics, even in the same cell. Initially, activated ligand-bound integrins cluster to an early form of dot-shape integrin-mediated adhesions known as nascent FAs (NFAs; $<0.1\text{-}0.5\ \mu\text{m}^2$), that mainly assemble at the leading edge of migrating cells (Geiger and Yamada, 2011). They mature to FCs and FAs ($\sim 1\text{-}5\ \mu\text{m}^2$) by further integrin clustering and recruitment of actin-linker multiprotein complexes to the cytoplasmic platform of NFAs (Fig. 17). FAs that contain $\alpha 5\beta 1$ integrin and are under intracellular actomyosine tensions can further develop into elongated fibrillar adhesions (FBs; $>5\ \mu\text{m}$ long) which are located in the central areas of cells.

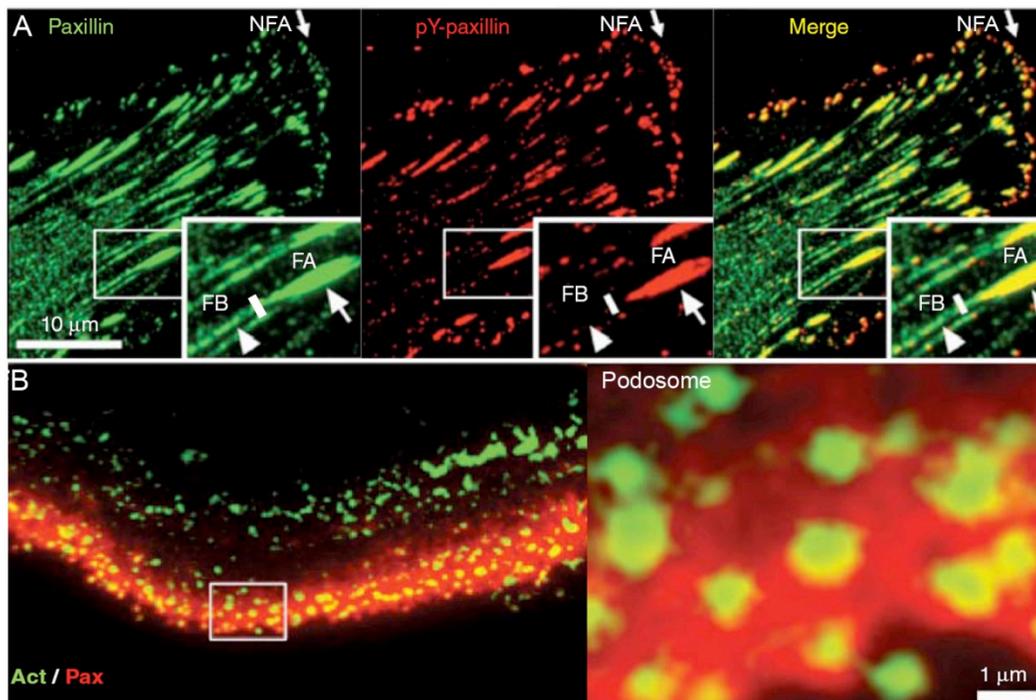


Figure 17. Different type of integrin-based adhesions. (A) Nascent Focal adhesions (NFA), focal adhesions (FA) and fibrillar adhesions (FB) are visualized by Paxillin (green) and pY-Paxillin (red) immunostaining. (B) Podosomes are visualized by Paxillin (red) and actin (green) immunostaining in osteoclasts. From (Geiger and Yamada, 2011).

Podosomes are another type of integrin-based adhesion that is found in smooth muscle cells, endothelial cells and cell types of monocytic origin (osteoclasts, macrophages and dendritic cells). They are ring-shaped adhesions that assemble around an F-actin core and contain matrix degrading enzymes. Podosomes are important for cell adhesion and degradation of matrixes such as bone by osteoclasts (Linder and Kopp, 2005).

So far, only a few *in vivo* integrin-mediated adhesions have been characterized including FAs of endothelial cells at sites of fluid shear stress in blood vessels and smooth muscle

dense plaque (Geiger and Yamada, 2011). The morphology, composition and signalling properties of integrin-based adhesions are considered to be different in 2D cell cultures and *in vivo* environments (Fig. 18). Fibroblastic cells in 3D environment of tissue show distinct morphology and are more biologically efficient during migration and proliferation when compared with 2D-matrix cell cultures (Cukierman et al., 2001).

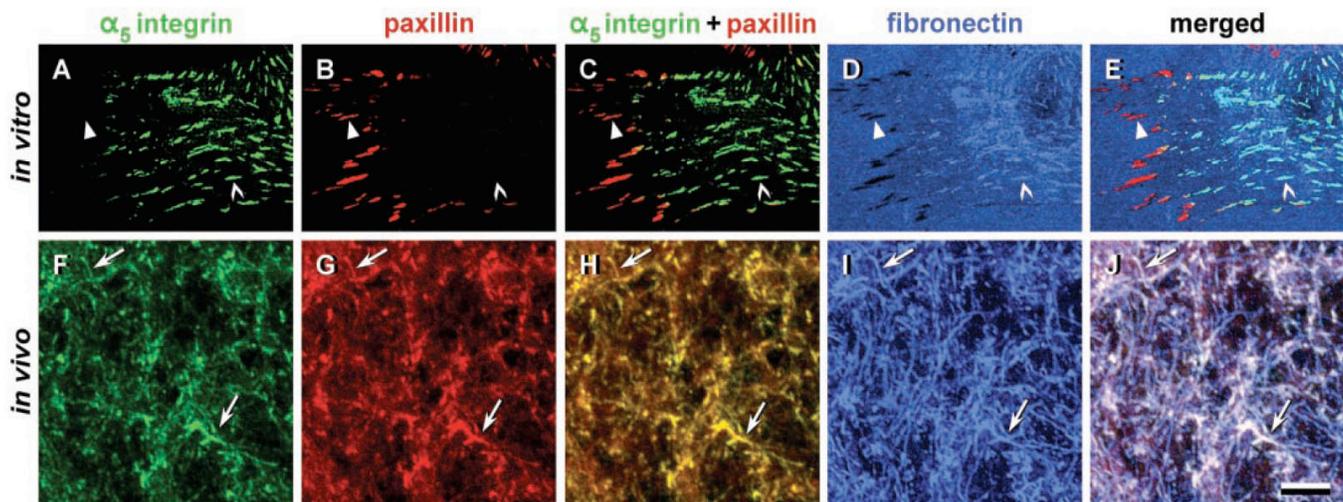


Figure 18. *In vivo* integrin-based cell adhesions. Immunostaining of Paxillin (red) and α_5 integrin (green) showed distinctive localization *in vivo* and *in vitro*. FAs (filled arrowheads) and FBs (open arrowheads) of (A-E) FN-seeded NIH-3T3 mouse fibroblast in cell culture compared with (F-J) 3D-matrix adhesion (arrows) of craniofacial mesenchymes from transverse cryosections of E13.5 mouse embryo. Scale bar 5 μ m. From (Cukierman et al., 2001).

Recently, the ultra-structure -architecture of FAs has been revealed by cyro-electron tomography (Fig 19A-C). Patla et al. (2010) showed that the interaction of the plasma membrane with cytoskeleton in FAs of fibroblasts is mediated through hundreds of donut-like particles with a diameter of 20-30 nm. F-actin connections to particles were in different orientations (about 25 different structural classes; Fig 19D) proposing diversity in the molecular composition within FAs (Patla et al., 2010). It has been shown that many of FA proteins recruited to FAs bind integrin tails directly (Zaidel-Bar and Geiger, 2010). As cytoplasmic tails of integrins are short, the simultaneous interaction of all these molecules with the same integrin heterodimer is not possible and integrin tail-bound multiprotein complexes peobaly contain different composition in the same adhesion site.

FA proteins can be divided into four classes based on their binding/scaffolding properties:

- 1) Integrin-associated proteins that directly bind to F-actin cytoskeleton, such as talin, filamin and α -actinin;
- 2) Integrin-associated proteins that indirectly bind to/regulate the F-

actin cytoskeleton, such as Paxillin, FAK, ILK and kindlins; 3) Actin-binding proteins that are not associated with integrins directly, such as vinculin and Parvins; and 4) scaffolding/signalling molecules that tune integrin signals but are not associated with integrin/actin (Legate and Fassler, 2009).

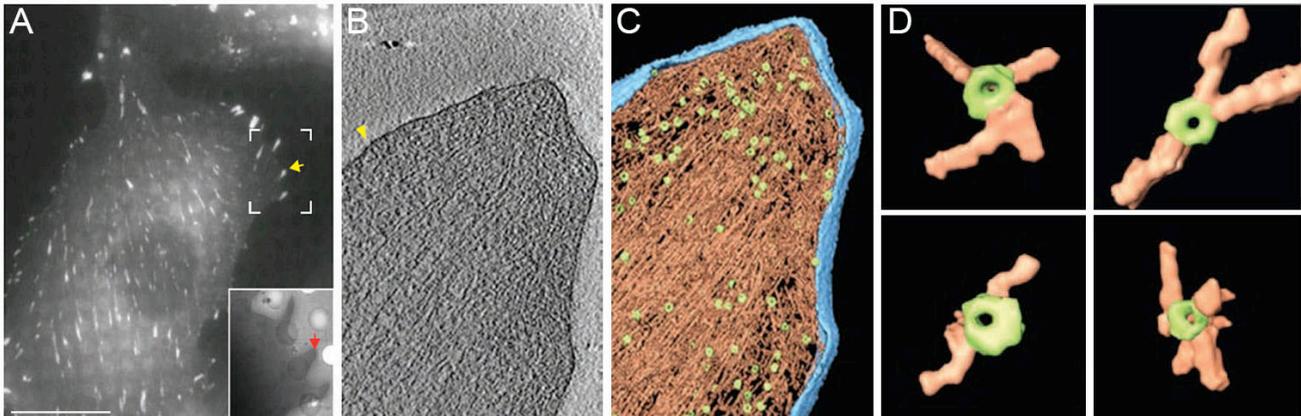


Figure 19. Ultra-structure of FAs. Correlated microscopy, combining fluorescence microscopy and cryo-electron tomography. **(A)** Fibroblasts expressing YFP-Paxillin to visualize FAs (yellow and red arrows). Scale bar is 20 μm . **(B)** A cryo-tomogram slice of the same FA in panel A (yellow arrow) and **(C)** its surface rendering view with F-actin (brown), plasma membrane (blue) and donut-like adhesion-related particles (green). **(D)** Higher magnification of adhesion-related particles (20-30 nm diameters; green) linked to differentially oriented F-actin (brown). Adopted from (Patla et al., 2010).

3.1. Integrin-linked kinase (ILK)

ILK was initially found in a yeast-two hybrid screen as a direct binding partner of $\beta 1$ and $\beta 3$ integrin tails (Hannigan et al., 1996; Pasquet et al., 2002). ILK consists of five N-terminal Ankyrin homology domains (ANK), a Plekstrin homology (PH) domain and a C-terminal pseudokinase (PK) domain. ILK predominantly localizes to FAs, although there have been reports on ILK recruitment to nucleus, centrosomes and cell-cell adhesions (Widmaier et al, 2011). ILK forms an ILK-Pinch-Parvin (IPP) complex in the cytoplasm by interacting with Lin-11, Isl-1 and Mec-3 (LIM) domain of Pinch and the second Caplonin homology (CH) domain of Parvins (Widmaier et al, 2011). The formation of the IPP complex prevents the proteasomal degradation of its components. IPP serves as a signalling platform that recruits many other molecules. Post translational modification of IPP components and alternative assembly of Pinch and Parvin isoforms give rises to distinctive signalling outputs that are cell type-, tissue- and developmental stage-specific.

ILK was originally considered to be a Ser/Thr protein kinase; however, X-ray crystallography and genetic studies in *D. melanogaster*, *C. elegans* and mice demonstrated that the ILK kinase domain is catalytically inactive (Wickstrom et al., 2010). ILK controls cell polarity, proliferation, differentiation, stability of microtubule tips at the cell cortex and the organization of the F-actin cytoskeleton through signalling crosstalks with growth GFRs (Grashoff et al., 2003; Lange et al., 2009; Legate et al., 2006; Lorenz et al., 2007; Mackinnon et al., 2002; Wang et al., 2008; Wickstrom et al., 2011). However, it remains unclear how ILK co-operates with GFR to control F-actin remodeling and downstream signalling pathways. For more information the reader is referred to a review article presented in the appendix of this thesis (Widmaier et al, 2011).

4. Integrins and growth factors signalling crosstalk

Cells must maintain their physiological homeostasis in response to a wide variety of environmental challenges with cell surface receptors that transduce extracellular stimuli into biochemical signals. Single cells and, particularly, multicellular organisms must have been adapted a mechanism to orchestrate the signals of different receptors to achieve an appropriate biological response. The integration of distinct signals from different receptors into a common downstream effector/response is known as signalling crosstalk.

Integrin and GFR signalling crosstalk is responsible for a wide range of different biological processes such as cell proliferation, survival, differentiation, migration, innate immune response, angiogenesis and tumor cell growth (Cabodi et al., 2004; McCall-Culbreath et al., 2008; Moro et al., 1998; Ross, 2004; Somanath et al., 2009; Soung et al., 2010; Veevers-Lowe et al., 2011). The mode of integrin-GFR cosignalling could be reciprocal or unidirectional. In reciprocal integrin-GFR cosignalling, both integrin and GFR activation trigger common downstream effectors observed with α V β 3-insulin-like GFR (IGFR) and α V β 3-PDGF signalling. In the unidirectional integrin-GFR crosstalk, one receptor induces activation of the other receptor. β 1 integrin-dependent activation of epidermal GFR (EGFR) through c-Src and EGFR-/c-Met-dependent activation of α 6 β 4 integrin are

examples of unidirectional integrin-GFR cosignalling (Cabodi et al., 2004; Soung et al., 2010).

Integrin-GFR signalling crosstalk can be achieved through several mechanisms (Fig. 20). 1) Integrins form complex with GFRs at plasma membrane (Fig. 20A-B). 2) Integrin signals can activate GFRs at receptor levels (Fig. 20C-D). 3) GFRs can activate integrins and modulate their signaling output (Fig. 20E-F). 4) Integrins propagate ligand-mediated signals of GFRs or synergize on parallel pathways to achieve full biological responses (Fig. 20G-H).

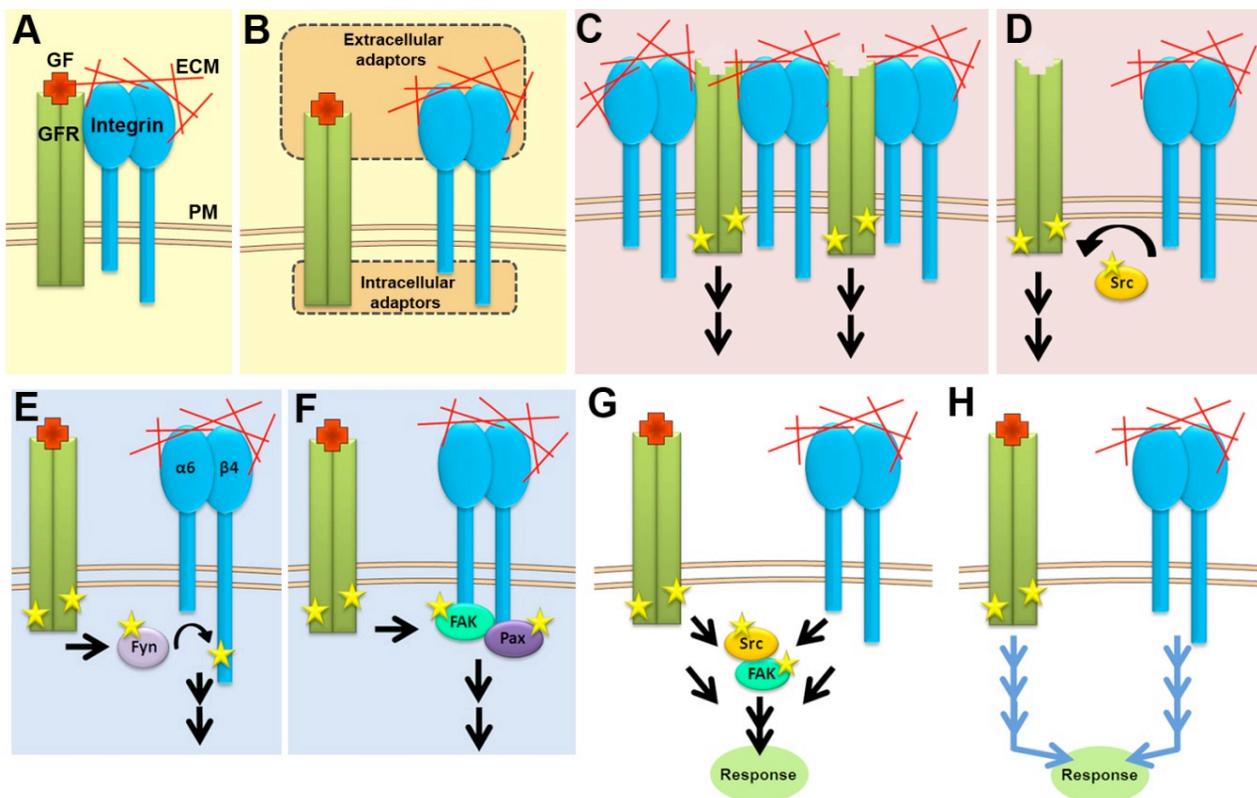


Figure 20. Mechanisms of integrin-GFR cosignalling. (A) Integrins form a complex with GFRs directly or (B) indirectly through extra-/intra-cellular adaptor molecules. Integrin-induced GFR activation through (C) integrin clustering and (D) integrin-induced c-Src activation. (E) GFR-dependent phosphorylation and activation of $\alpha 6 \beta 4$ integrin via Fyn Tyr kinase. (F) GFR-induced phosphorylation of many FA molecules such as FAK and Paxillin, affects the integrin signalling pathway. (G) Propagative integrin-GFR cosignalling through common downstream effectors such as Src and FAK. (H) Integrin and GFR signals can synergize on parallel pathways to achieve the same biological response. Yellow stars indicate phosphorylation. For details refer to text.

4.1. Integrin-growth factor receptor complexation

Integrins and GFRs can become physically linked at the plasma membrane (Table 2; Fig. 20A-B). Integrin-GFR complex formation occurs directly through the interaction of GFRs and integrins, or indirectly via extracellular or intracellular adaptor/crosslinking molecules. $\alpha V\beta 3$ integrin directly binds to fibroblast GFR (FGFR) and IGFR (Mori et al., 2008; Saegusa et al., 2009). In FN-seeded mesenchymal stem cells, the association of $\alpha 5\beta 1$ integrin with phosphorylated PDGF receptor- β (PDGFR- β) is essential for migration (Veevers-Lowe et al., 2011). A $\beta 1$ -N-wasp-PDGFR complex has been also shown to be crucial for fibroblast chemotactic migration (King et al., 2011). In endothelial cells hepatocyte GFR (HGFR/c-Met) forms a hetero-complex with both $\alpha V\beta 3$ and $\alpha 5\beta 1$ integrins through association of HGF with FN and vitronectin in the ECM (Rahman et al., 2005).

Integrin	GFR	Function
$\alpha V\beta 3$	IGFR (direct) ¹ , FGFR-1 (direct) ² , HGFR (c-Met) ³ , PDGFR ⁴ , VEGFR2 ⁴ and Tie-2 ⁵	GFR signalling ^{1, 2, 4} Angiogenesis ^{3, 4} Integrin internalization ⁵
$\alpha 5\beta 1$	HGFR (c-Met) ³ , Tie-2 ⁴ , PDGFR- β ⁶ and EGFR ⁷	Angiogenesis ^{3, 4} Migration ⁶ GFR signalling ⁷
$\alpha 6\beta 4$	HGFR (c-Met) ³ , ErbB2 ⁴ , EGFR ⁴ and Ron ⁴	Angiogenesis ³ Metastasis and invasion ⁴
$\alpha 2\beta 1$	PDGFR- β ⁸ and EGFR ⁹	Smooth muscle cell proliferation ⁸ Cell-cell contact signalling ⁹

Table 2. Examples of integrin-GFR complexation. IGFR: Insulin-like growth factor receptor (GFR); FGFR-1: Fibroblast GFR-1; HGFR: Hepatocyte GFR; PDGFR: platelet-derived GFR; VEGFR: Vascular Endothelial GFR; EGFR: Epidermal GFR; ErbB2: Erythroblastic Leukemia Viral Oncogene Homolog; Tie-2: Tyr kinase with immunoglobulin and EGF homology domains 2; Ron: Receptor d'origine nantais. From 1 (Saegusa et al., 2009), 2 (Mori et al., 2008), 3 (Rahman et al., 2005), 4 (Soung et al., 2010), 5 (Thomas et al., 2010), 6 (Veevers-Lowe et al., 2011) and 7 (Liu et al., 2009), 8 (Hollenbeck et al., 2004) and 9 (Yu et al., 2000).

Local concentration of integrins at GFR-rich lipid rafts promotes integrin-GFR cosignalling in breast carcinoma cells. PKC- α -induced phosphorylation of a Ser residue in $\beta 4$ integrin cytoplasmic tail causes disassembly of $\alpha 6\beta 4$ integrin from hemidesmosomes. $\beta 4$ integrin is then recruited to lipid rafts upon palmitoylation on a Cys residue in membrane proximal motif of $\beta 4$ cytoplasmic tail (Soung et al., 2010). The complexation of integrin-GFR at

plasma membrane, not only affects integrin and GFR activities at receptor level, but also creates a microenvironment, where many integrin-/GFR-tail-bound signalling molecules can interact and tune downstream signalling pathways.

4.2. Integrin-dependent activation of growth factor receptor signalling pathway

Integrin engagement can directly mediate GFRs activation and phosphorylation. Activation of GFRs by integrin clustering at the plasma membrane has been reported in several studies (Fig. 20C). Integrin ligand occupancy and clustering using FN-coated beads promotes EGFR, PDGFR- α or- β and FGFR aggregation (Miyamoto et al., 1996). Moreover, antibody-mediated crosslinking of $\alpha 6\beta 4$ integrin was sufficient to promote RhoA activation through cell-surface clustering of EGFR in breast carcinoma cells (Gilcrease et al., 2009). In the same manner, $\alpha V\beta 3$ integrin induces growth factor-independent activation of IGFR, VEGFR2 and PDGFR (Ivaska and Heino, 2011).

Many αV -containing integrin heterodimers ($\alpha V\beta 3$, $\alpha V\beta 5$, $\alpha V\beta 6$ and $\alpha V\beta 8$) directly mediate activation of TGF β receptors by releasing TGF β from its inactive latent TGF β complex in the ECM (Worthington et al., 2011). Another very well known example is ligand-independent EGFR phosphorylation by integrins (Moro et al., 1998). Although, such an integrin-induced EGFR phosphorylation is much weaker than ligand-mediated EGFR activation, it is sufficient for adhesion-mediated cell survival (Cabodi et al., 2004). The process requires c-Src-induced binding of p130Cas to EGFR (Moro et al., 2002) and probably aggregation and transactivation of EGFR at plasma membrane through integrin clustering (Fig. 20D).

4.3. GFR-dependent modulation of integrin signalling pathway

GFRs can tune integrin signals through phosphorylation of integrin cytoplasmic domains and FA proteins (Fig. 20E-F). Activated EGFR induces tyrosine phosphorylation of $\beta 4$ integrin cytoplasmic tail via Fyn and Yes tyrosine kinases, leading to disassembly of hemidesmosomes in carcinoma cells (Mariotti et al., 2001). PDGF-mediated activation of PKC leads to $\alpha V\beta 3$ integrin activation and increases matrix binding and proliferation of

oligodendrocytes (Soung et al., 2010). Additionally, many FAs proteins undergo GFR-induced phosphorylation that modifies their binding and signalling properties. For example FAK phosphorylation can be triggered by growth factors directly or via GFR-induced c-Src activation (Siesser and Hanks, 2006).

4.4. Synergistic/propagative integrin-growth factor receptor cosignalling

Integrins and GFRs can intersect through multiple downstream signalling molecules for propagating each other signals (Fig. 20G) or synergizing on parallel pathways to finally achieve a full biological response (Fig. 20H). For example, integrins and GFRs share Ras-MAPK, PI3K-Akt and RhoGTPases signalling pathways (Assoian and Schwartz, 2001; Ivaska and Heino, 2011). As integrin and GFRs have many common downstream effectors, the evidence for propagative integrin-GFR cosignalling is vast. Integrins and GFRs cooperatively control Cyclin D1 expression and thereby promote cell cycle progression through intersecting with the ERK signalling pathway at several points. Two important convergent points of integrin-GFR signalling pathways are FAK and c-Src, as both of them can be phosphorylated and activated by GFRs or integrins and trigger many downstream signalling molecules (see section 2.2). There are examples of synergistic integrin-GFR cosignalling, in which integrin and GFRs triggers signals on parallel pathways. β 1 integrin induces GFR-independent phosphorylation of Akt on Ser473 and Thr308, while EGFR can trigger adhesion-independent Akt activation through c-Src and FAK (Ivaska and Heino, 2011).

Recent studies indicate that dimensions of integrin-GFR cosignalling are much broader than what has been considered before, as both integrin and GFR, negatively or positively, influence trafficking as well as expression levels of each other in different cell types and tissues. Although, the integrin-GFRs cosignalling plays a crucial role in regulating cellular behaviors, its molecular players and underlying mechanisms have not been comprehensively studied due to lack of appropriate readouts and technologies.

5. Circular dorsal ruffles

Circular dorsal ruffles/waves (also known as dorsal ruffles (DR) or actin ribbons) are transient actin-rich structures that form on the dorsal plasma membrane in response to growth factor stimulation. Although it might have been observed in cell cultures earlier, the term of DRs was used to describe morphological changes of fibroblasts as early as 1970 (Abercrombie et al., 1970; Chhabra and Higgs, 2007). DRs have been observed in different cell types (e.g. fibroblasts, macrophages, keratinocytes, smooth muscle cells and epithelial cells) in response to stimulation with various growth factors such as EGF, PDGF and HGF (Buccione et al., 2004). Morphologically, DRs are short-lived condense actin-based structures that usually appear 2-5 minutes after growth factor stimulation and disappear within 10-30 minutes. By scanning electron microscopy (SEM), DRs appeared as wave-shape arcs of uniformly sized bumps that protrude out of dorsal plasma membrane, perhaps due to upward pushing forces of newly polymerized F-actin (Fig. 21A). The sections of transmission electron microscopy (TEM) revealed that DRs are full of actin bundles and membranous tubules (~100-200 nm diameter) extending along with the DR crest (Fig. 21B), indicating that DRs are active site of membrane trafficking (Buccione et al., 2004; Orth et al., 2006).

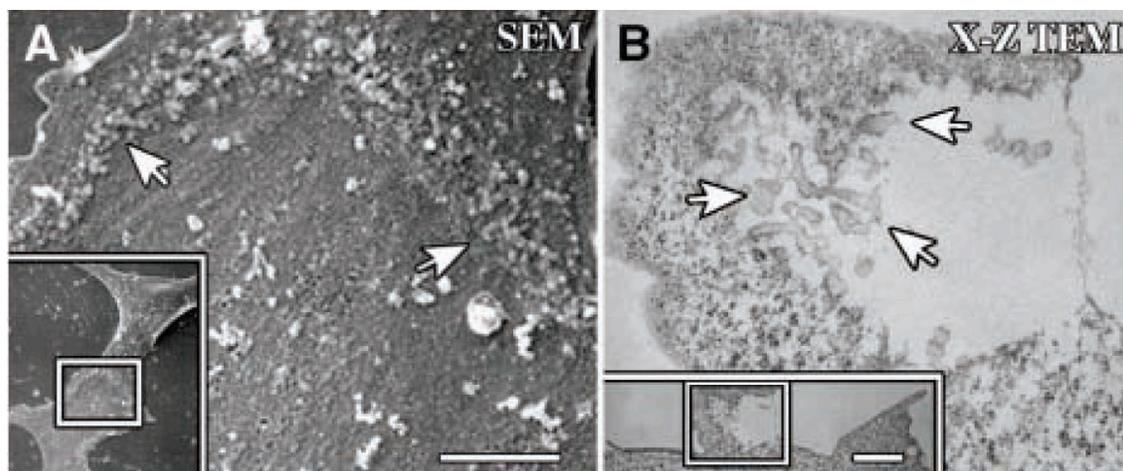


Figure 21. Ultrastructure of DRs. (A) Scanning electron microscopy of DRs showed that dorsal ribbons composed of numerous uniformly sized bumps. (B) Transmission electron microscopy revealed that DR cytosolic extension is rich of membranous tubules. From (Orth et al., 2006).

5.1. Signalling cascade to DRs

DRs are the result of a kinase signalling cascade that starts with GFR dimerization and transactivation followed by activation of few master kinases including c-Src and PI3K (Fig. 22). c-Src non-receptor tyrosine kinase plays a crucial role in DR formation through activation of other kinases including: PI3K and Abelson leukemia protein tyrosine kinase (Abl). c-Src also contributes in DR signalling cascade by elevation of active small GTPase levels as well as phosphorylation and activation of actin-binding adaptor molecules such as neuronal Wiskott-Aldrich Syndrome protein (N-WASP) and WASP family Verprolin-homologous protein 1 (WAVE1) (Buccione et al., 2004). PI3K activates P21-associated kinase 1 (PAK1) and potentially can recruit other signalling molecules to the plasma membrane by modifying membrane lipids. Activated PAK1 promotes DR formation via phosphorylation of p41Arc, a subunit of the Arp2/3 complex and actin. Using the PH domain of Akt, it was shown that enriched PIP3 in DRs directly affects recruitment and activation of gelsolin and dynamin to ruffling plasma membrane (Fig. 22).

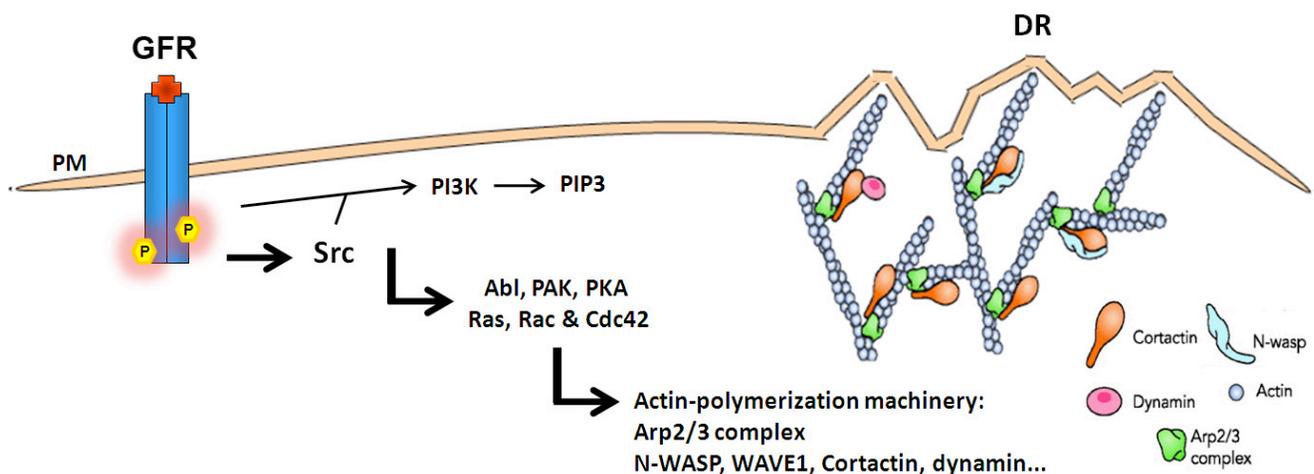


Figure 22. DR signalling cascade. GFRs trigger activation of a few master kinases such as Src and PI3K. Src also can activate PI3K, which in turn modifies lipids at plasma membrane (PM) and controls recruitment of lipid binding signalling molecules. c-Src activates many downstream effectors including: kinases, small GTPases and actin binding adaptors that eventually turn on the actin polymerization machinery. Consequently, DRs appear as bumps due to upward pushing of nascent actin filaments into the dorsal plasma membrane.

5.2. Putative function of DRs

Several physiological functions have been attributed to DRs including fast remodeling of actin cytoskeleton during migration and metastasis, sequestration and internalization of GFRs and macropinocytosis. DRs facilitate transition of static adherent cells to polarized motile cells by controlling fast integrin trafficking and disassembly of stress fibers (Buccione et al., 2004; Gu et al., 2011). A complex of $\beta 1$ integrin-N-WASP-PDGFR at DRs has been shown to be essential for chemotactic migration of fibroblasts toward a PDGF gradient (King et al., 2011). DR might also involve in metastasis process by facilitating ECM degradation. Suetsugu et al. (2003) showed that matrix metalloproteinase 2 (MMP2) are recruited to the tip of DRs and, therefore, proposed that DRs facilitate 3D-migration *in vivo* by degrading ECM components.

It has been shown that DRs have endocytic activity for fast sequestration and internalization of GFRs upon growth factor stimulation (Orth and McNiven, 2006). Orth et al. (2006) showed that such internalization occurs through EGFR-positive membranous tubules extending along DRs perimeter. GFP-tagged EGFRs are actively localized and internalized by EGF-induced DRs in NIH/3T3 cells (Krueger et al., 2003). Recently it was shown that other GFRs such as PDGFR and HGFR are also internalized by DRs (Abella et al., 2011; King et al., 2011).

The trafficking of both GFRs and integrins (Gu et al., 2011) through DRs suggests that DRs might be a signalling compartment for integrin and GFR interaction and cosignalling. Moreover, DRs may also play a role as a buffering mechanism to neutralize extra GFR signals as well as the signals of activated kinases and small GTPases through ruffling actomyosin machinery to protect stressed serum-starved cells.

During closure of DR rings a large amount of growth media is internalized by macropinosomes, suggesting another potential role for DRs (Buccione et al., 2004; Gu et al., 2011). Perhaps, macropinocytosis via DRs provide a mechanism to supply cell with enough metabolic material that is required for cell cycle progress upon GFR stimulation of serum-deprived cells. Since WAVE1-null cells, which are not able to form DRs, have normal macropinocytosis, DRs can mediate but are not essential for macropinocytosis (Suetsugu et al., 2003).

Despite the initial proposed role for DRs in promoting invasiveness (Suetsugu et al., 2003), recent evidences showing DR-mediated suppression of GFR signals suggest that DRs exert an important anti-cancer mechanism. It would be interesting to investigate therapeutic effect of DR formation on controlling tumor cell growth in parallel with other tumor suppressor proteins (see Cyld in publication I).

6. Dissection of signalling networks by SILAC-based proteomics

The biology of single cells and multicellular organisms regulated through a labyrinthic signalling network which engages a large number of molecules acting in parallel. Therefore, understanding the mechanism of signalling systems demands comprehensive information at all molecular levels. Antibody-based analysis of signalling events are limited to only a few signalling molecules and therefore give a limited view of what really happening in biological systems. Such limitation can be overcome by powerful large-scale proteomics approaches that are able to accurately identify and quantify thousands of proteins and their post translational modifications during signalling events.

The capacity of proteomics techniques for analysis of biological samples have been dramatically enhanced through improvement of mass spectrometry (MS) instruments, sample preparation techniques and data processing (Choudhary and Mann, 2010). Coupling liquid chromatography to tandem MS/MS (LC-MS/MS) in modern linear ion trap-orbitrap instruments improved number of identified proteins without loss of speed and accuracy. Many obstacles of feeding different samples into the MS pipeline have been overcome during recent years. For example, filter-aided sample preparation method uses the advantages of Sodium dodecyl sulfate (SDS)-based solubilization to identify 7093 proteins from HeLa cell lysates in a single experiment (Wisniewski et al., 2009). In parallel, computational proteomics revolutionized mass spectrometry approaches by open source softwares such as MaxQuant, which directly allows high peptide identification rate from raw MS data (Choudhary and Mann, 2010).

Quantitative proteomics is an attractive method in cell signalling research as it provides highly accurate quantitative comparison of thousands of proteins between a given control

and experiment samples. In simple label-free quantitative proteomics, the intensities of a given peptide is normalized by calculating the differences in peptide intensity of each sample in separate LC-MS/MS runs. As the variation in peptide intensities from replicates could be high, the method is less accurate, but useful for samples whose labeling is impossible or difficult (Fig. 23). To accurately quantify two distinct proteomes, stable-isotopic molecules which are analogous of natural isotopes have been introduced usually by chemical modification or metabolic labeling. Chemical strategies can be used for different type of cell lysates; however, they restrict measurement sensitivity by chemical side products and introducing experimental errors, as labeling is performed after a few steps of sample purification and fractionation. Metabolic labeling allowed direct mixing of samples from beginning, therefore leads to more accurate quantitative data by treating samples similarly during all steps of preparation (Walther and Mann, 2010).

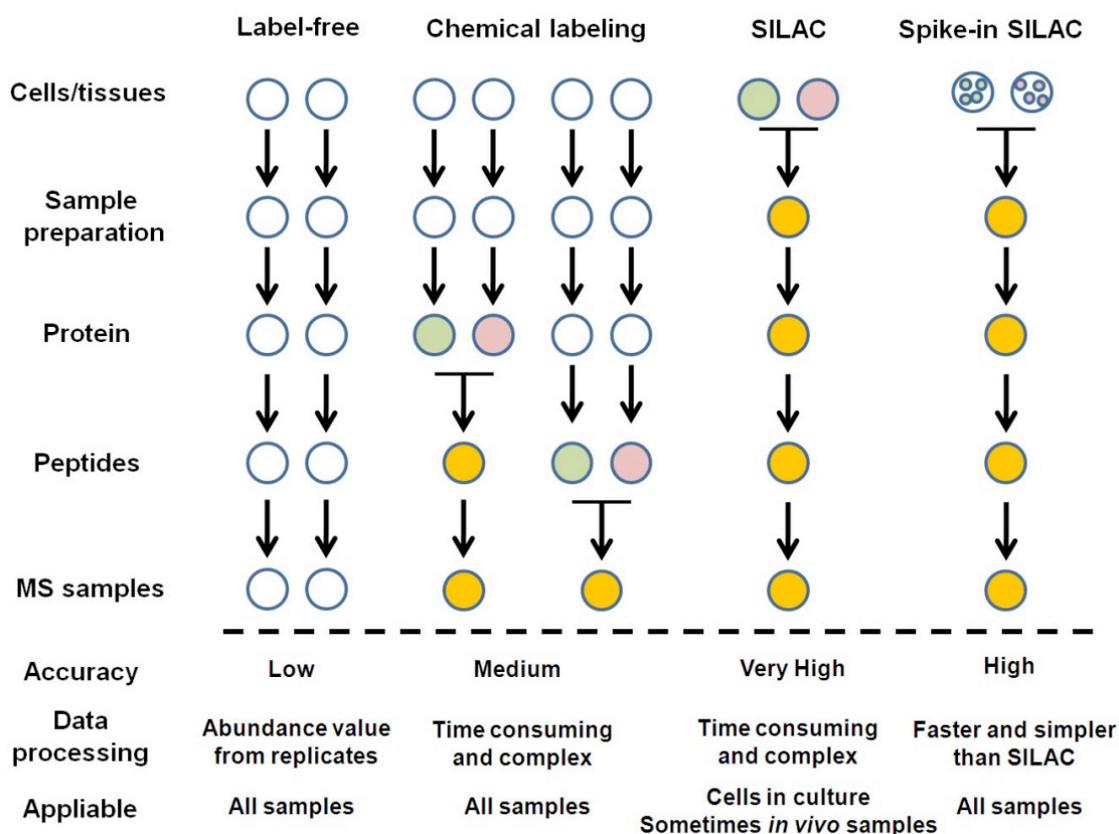


Figure 23. Quantitative proteomics. The accuracy of comparisons is high in SILAC-based proteomics as samples are treated similarly during preparation. However SILAC is usually expensive and limited to cells growing in culture. The new developed Spike-in SILAC strategy, uses isotopically labeled lysates that are mixed with samples from the beginning and used for quantification as internal (Spike-in) standard.

Stable isotope labeling by amino acids in cell culture (SILAC) is a simple and powerful metabolic labeling approach that widely applied to quantitative proteomics (Ong et al., 2002). Cells are metabolically labeled by incorporation of Arg and Lys essential amino acids that contain certain isotopic H, C and N elements. Up to three SILAC labeling media (light, medium and heavy) are available using combination of H, C and N isotopes in Arg and Lys structures (Fig. 24). The complete labeling of a proteome requires passaging of cells several times (about 2 weeks) in cultures supplied with SILAC media, and therefore SILAC technology is limited to cells growing in cell culture (Ong et al., 2002).

In recent years, researchers tried to expand SILAC applications from cell culture to the *in vivo* environments. Mice, that are entirely labeled over 4 generation, provide an appropriate source to apply quantitative proteomics to tissues and cell types that cannot be grown in culture (Kruger et al., 2008b). Moreover, using an accurate internal Spike-in SILAC standard (or super-SILAC mixture), quantitative proteomics analysis is now possible for human tissues that cannot be labeled (Geiger et al., 2010; Geiger et al., 2011).

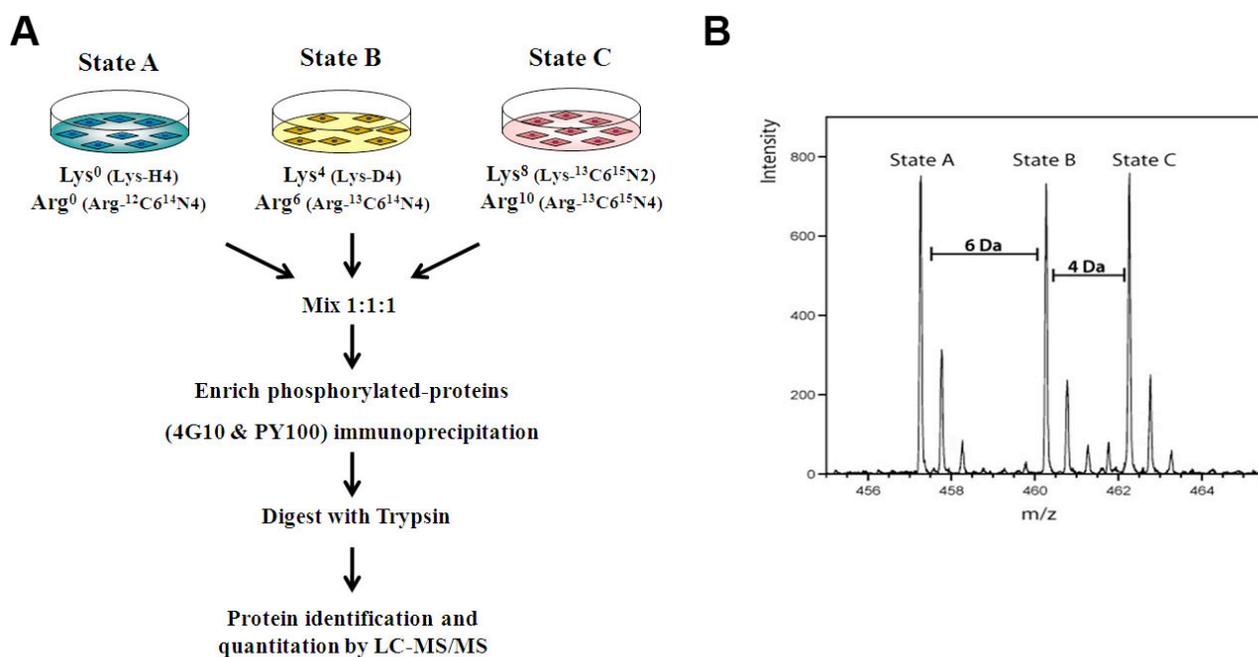


Figure 24. SILAC-based proteomics of phosphotyrosine signalling pathways. (A) Cells metabolically label in culture with essential amino acids that contain isotopic H, C and N elements. Cell lysates are mixed 1:1, tyrosine phosphorylated proteins are enriched by affinity purification, digested by trypsin and entered to LC-MS/MS pipeline. (B) Direct quantification of intensities are possible as tryptic peptides from each labeling condition shows distinctive MS/MS spectra due to difference in the mass of isotopic elements.

Signal transduction is derived by synchronized, highly dynamic molecular modifications at different levels including signal-induced expression, protein-protein interaction and particularly post-translational modification (PTM). PTMs usually affect only a low amount of proteins by modification of peptide biochemical structure through phosphorylation, ubiquitylation, acetylation, methylation, glycosylation, etc. Consequently, analysis of PTMs by MS is problematical due to low quantity and complex MS/MS spectra of modified peptides which, in turn, require more data processing to predict all possible modification sites on a given peptide (Choudhary and Mann, 2010). Phosphorylation usually occurs in eukaryotic organisms through transfer of a phosphate (PO₄³⁻) group from ATP to a Hydroxyl (OH) group of Ser, Thr or Tyr residues. Among them, phosphotyrosine-based signal transduction has evolved to conduct many crucial signalling events such as GFRs, cell-ECM and cell-cell signalling pathways (Lim and Pawson, 2010). Phosphotyrosine signalling machinery is composed of tyrosine kinases, tyrosine phosphatases and SH2-/PTB-containing proteins that allow fast and dynamic writing, erasing and reading of cellular signals at molecular levels.

SILAC-based proteomics have been applied as a powerful technique to study phosphotyrosine signals in a quantitative, system-wide and site-specific fashion. Affinity enrichment of phosphotyrosine proteins is required, as the quantity of tyrosine phosphorylated proteins are very low. SILAC-based analysis of EGFR phosphotyrosine proteome revealed 31 novel EGFR effectors and kinetics of their tyrosine phosphorylation site (Blagoev et al., 2004). The same strategy was used to dissect insulin receptor tyrosine phosphoproteome (Kruger et al., 2008a). In an interesting study 6600 phosphorylation sites (Ser, Thr and Tyr), identified and quantified in lysates of EGF-induced Hela cells. The study provides new insight on differential frequencies and upregulation profiles of phospho-Ser/Thr and -Tyr sites (Olsen et al., 2006). Recently, the number of pinpointed phosphosites in a single project increased to 20000 unique phosphopeptides out of 100 000 estimated sites in human (Olsen et al., 2011).

So far, quantitative proteomics have been supported deep mapping of signalling networks in large scale and with high accuracy. Analysis of cell type- and tissue-specific proteomes from different developmental stages in combination with new progress in proteomics technology will help us to further improve our knowledge of how signalling networks control the biology of cells.

7. The tumor suppressor CYLD

The cylindromatosis gene (*Cyld*) has been originally identified as a tumor suppressor whose germline mutations are associated with familial cylindromas (Bignell et al., 2000). *Cyld* is composed of two N-terminal cytoskeletal-associated protein Glycine-rich (CAP-Gly) domains which bind to microtubules (MTs), two proline-rich motifs, a NF- κ B essential modulator (NEMO)-binding CAP-Gly domain and a C-terminal deubiquitinase (DUB) domain that contains a Zink-finger-like B-box domain (Fig. 25). *Cyld* is a signalling molecule that is involved in numerous signalling events through its DUB activity, MT-binding capability and phosphorylation (Massoumi, 2010).

7.1. *Cyld* deubiquitinase activity

Cyld DUB activity targets components of several signalling pathways in different cell types. Negative regulation of nuclear factor ' κ -light-chain-enhancer' of activated B-cells (NF- κ B) signalling pathway through *Cyld*-mediated deubiquitylation was initially reported in three simultaneous publications in 2003. The authors showed that *Cyld*-mediated deubiquitylation of NEMO, tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2) and to a lesser extent TRAF6, negatively affects the recruitment and activation of inhibitor of ' κ -light-chain-enhancer' of B-cells, kinase (I κ B kinase/IKK) complex and, subsequently, reduces translocation of NF- κ B complex to nucleus (Fig. 26A). A single mutation in Cys601 within the DUB domain is sufficient to abolish *Cyld* DUB activity (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003). *Cyld* DUB activity is also dependent on the interaction of *Cyld*'s third CAP-Gly domain with the proline-rich domain of NEMO (IKK γ) as well as the direct binding of *Cyld* with the TRAF interacting protein (TRIP) (Massoumi, 2010).

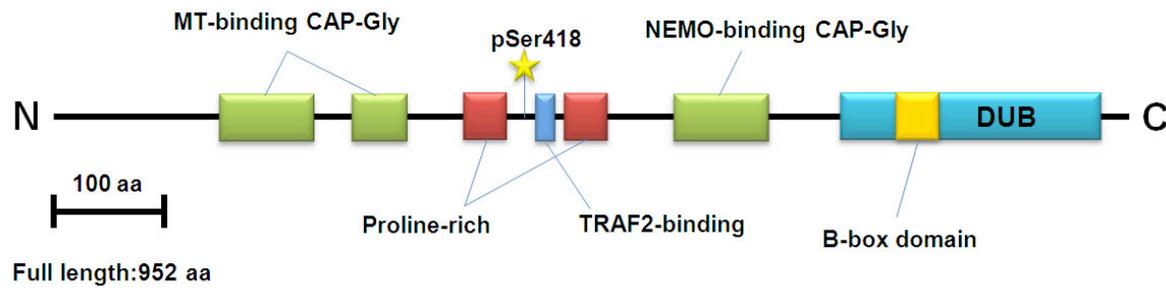


Figure 25. The structure of Cyld protein. Two N-terminal CAP-Gly domain bind to microtubules, while the third CAP-Gly binds to NEMO and plays a role in negative regulation of the NF- κ B signalling pathway. Two proline-rich motifs in Cyld potentially can bind to SH3-containing proteins. The C-terminus DUB domain is a Lys63-specific deubiquitinase. The zinc-finger-like B-box motif inside DUB domain is essential for Cyld intracellular localization.

X-ray crystallography of Cyld DUB domain revealed that Cyld specifically removes Lys63-linked polyubiquitin chains which mediate non-degradative signals. A catalytic triad containing Cys601 residue attacks the isopeptide bond between amine group of Lys and C-terminal of ubiquitin. In addition, a Zn-finger-like B-box motif identified inside the DUB domain is required for DUB activity. Its deletion impairs DUB capacity and leads to a sustained nuclear localization of Cyld (Komander et al., 2008).

Cyld deubiquitylation negatively controls detrimental effects of hyperactive immune responses. Cyld deubiquitinates TGF β -activated kinase (TAK1), which in turn, inhibits TAK1 autoactivation and its downstream signalling in T cells (Reiley et al., 2007). Cyld also was found as suppressor of T cell receptor signalling by erasing Lys48- and Lys63-linked polyubiquitin chain from lymphocyte-specific protein tyrosine kinase (Lck) (Reiley et al., 2006). B-cell activation, but not development, is dependent on Cyld deubiquitylation. Deletion of NEMO and TRAF2 binding motifs of Cyld in mice increases mature B lymphocyte population in lymphatic organs (Hovelmeyer et al., 2007). Cyld also negatively controls pathogen-induced inflammation which is driven by the NF- κ B signalling pathway. Lim et al. (2007) showed that Cyld-mediated TRAF6 and TRAF7 deubiquitination protects mice from nontypeable haemophilus influenzae (NTHi)-induced inflammatory response.

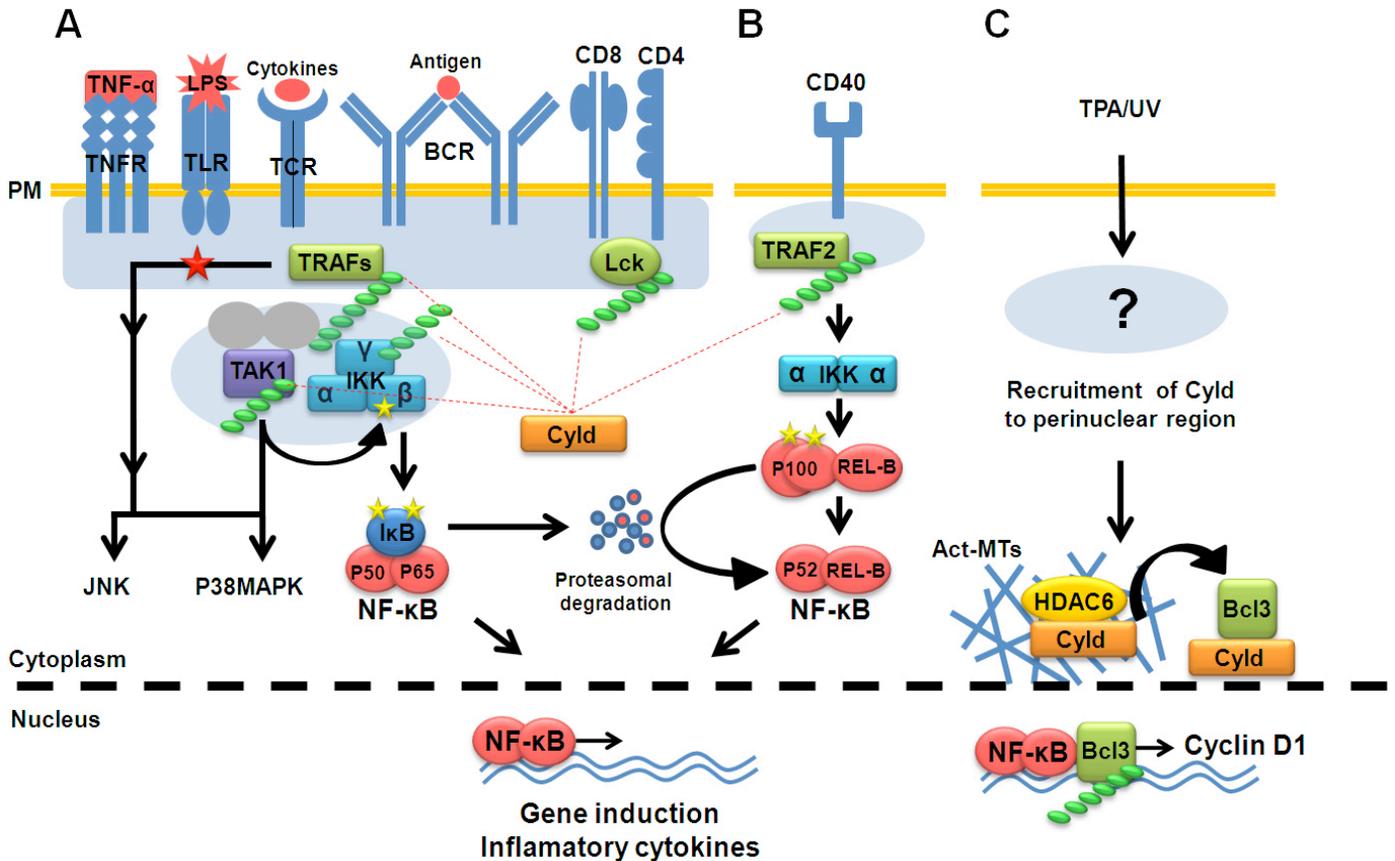


Figure 26. Cyld negatively controls NF- κ B signalling pathway. (A) Cyld affects canonical NF- κ B signalling pathway by deubiquitinating TRAFs (TRAF2, 6 and 7), NEMO (IKK γ), TAK1 and Lck. The recruitment of IKK complex and TAK1 is dependent on Lys63 polyubiquitin chain of TRAFs. It has been shown that TRAF2 deubiquitylation (red asterisk) by Cyld negatively affects JNK and P38 signalling pathways. TAK1 is also upstream of JNK and P38 pathways and triggers phosphorylation (yellow asterisk) of IKK β in canonical NF- κ B signalling pathway. Phosphorylation of I κ B by IKK triad complex induces its proteasomal degradation and releases NF- κ B dimer into the nucleus. (B) Cyld can affect noncanonical NF- κ B signalling pathway by TRAF2 deubiquitination in B lymphocytes, which in turn, downregulates formation of IKK α homodimer and reduces phosphorylation of P100 proteasomal degradation to P52. (C) In keratinocytes, TPA/UV-induced Cyld-Bcl3 complex formation in perinuclear region, promotes Bcl3 deubiquitination by Cyld, inhibits Bcl3 translocation to nucleus and downregulates Cyclin D1 expression. Moreover, TPA/UV-induced inhibitory interaction of Cyld with HDAC6 in perinuclear region of keratinocytes elevates acetylation of MTs (Act-MT) and facilitates Cyld-Bcl3 complex formation. Abbreviations are TNF- α : tumor necrosis factor α ; TNFR: TNF receptor; LPS: lipopolysaccharide; TLR: toll-like receptor; TCR: T cell receptor; BCR: B cell receptor; CD4/8/40: cluster of differentiation glycoprotein 4/8/40.

In keratinocytes, B-cell CLL/lymphoma 3 (Bcl3)-dependent *cyclin D1* gene expression and cell cycle progress is regulated by the Cyld DUB activity. Upon TPA or UV treatment, Cyld is recruited from cytoplasm to the perinuclear region, where it binds to Bcl3, removes the Lys-63-linked polyubiquitin chain and thereby prevents Bcl3 nuclear accumulation and cyclin D1 expression (Massoumi et al., 2006).

The effects of Cyld DUB activity is not restricted to NF- κ B pathway and can influence also other signalling systems. TAK1 deubiquitylation by Cyld is responsible for the resistance against bacterial infections through the crosstalk with the p38 MAPK pathway. Another example is Cyld-mediated TRAF2 deubiquitylation that suppresses the TNF- α -induced JNK signalling pathway and cell survival (Massoumi, 2010; Reiley et al., 2004; Reiley et al., 2007).

7.2. Cyld regulates microtubule stability

Evolutionary conserved CAP-Gly domains are known to bind to MTs and regulate MT dynamic, stability and MT-mediated trafficking of vesicles (Steinmetz and Akhmanova, 2008). The first and second CAP-Gly domains of Cyld directly bind to tubulin and/or MTs. Gao et al. (2008) showed that Cyld binding to tubulin through the first CAP-Gly domain promotes tubulin polymerization into MTs and facilitates cell migration. In keratinocytes, Cyld binds to MTs via the first and second CAP-Gly domains and negatively controls cell cycle progression. Mechanistically, TPA-activated Cyld is transferred to the perinuclear region, where it binds to the catalytic domain of histone deacetylase 6 (HDAC6), inhibits HDAC6 activity and increases acetylation and stability of MTs (Wickstrom et al., 2009). This negatively affects cell cycle progression at least by two different mechanisms: firstly, the inhibition of HDAC6 by Cyld and hyperacetylation of MTs facilitates TPA-induced Cyld-Bcl3 complex formation, blocks Bcl3 translocation to nucleus and downregulates NF- κ B signalling pathway. Secondly, Cyld binding to MTs and inhibition of HDAC6 activity in the midbody reduces the rate of cytokinesis (Wickstrom et al., 2009). HDAC6 is a multifunctional signalling molecule that controls many biological processes. Negative regulation of HDAC6 activity by Cyld could potentially extend the influence of Cyld to all HDAC6-induced processes. It remains open to further investigate the potential consequences of Cyld-HDAC6 interaction on signalling pathways in different cell types.

7.3. Cyld phosphorylation

Cyld undergoes transient and rapid Ser phosphorylation by members of the I κ B kinases (IKKs). TNF- α -induced Ser phosphorylation of Cyld by IKK α and IKK β in Jurkat T cell

transiently inactivates Cyld DUB activity, elevates TRAF2 ubiquitylation and activates its downstream signalling. The phosphorylation occurs mainly at Ser418 five minutes after TNF- α induction and requires IKK γ , the regulatory subunit of IKK complex (Reiley et al., 2005). The phosphorylation of Cyld Ser418 was much more efficient by IKK ϵ breast cancer oncoprotein. The phosphorylation reduced Cyld deubiquitylation activity and promoted IKK ϵ -driven transformation (Hutti et al., 2009). Similarly, constitutive Ser phosphorylation of Cyld inhibits Cyld-mediated deubiquitylation of the viral oncoprotein Tax in transformed T cells (Wu et al., 2011). Transient upregulation of Cyld was identified in SILAC-based proteomics of multiprotein complexes that were affinity-purified with anti-phosphotyrosine antibodies after EGF stimulation (Blagoev et al., 2004). As non-phosphorylated proteins complexed with phosphoproteins may also be immunoprecipitated, authors did not clarify whether Cyld directly undergoes tyrosine phosphorylation. Moreover, the consequence of such Tyr phosphorylation has been not investigated so far.

Publication summaries

Publication I

The induction of membrane circular dorsal ruffles requires integrin/ILK and EGF receptor co-signalling

Azimifar S. Babak, Böttcher RT, Zanivan S, Grashoff C, Krugel M, Legate K, Mann M and Fässler R.

Here we showed that the formation of transient actin-based circular dorsal ruffles (DRs) is the consequence of a signalling crosstalk between a fibronectin/ $\alpha 5\beta 1$ integrin/ILK signalling module and epidermal growth factor receptor (EGFR). ILK is essential for the precise spatial activation of Src at focal adhesions by integrin and EGFR signals, which in turn, mediates DR formation.

To identify novel molecular players linking integrin and EGFR signalling pathways, we established a screening strategy using the combination of SILAC-based quantitative phosphoproteomics followed by sh/siRNA-mediated depletion of candidate proteins. DRs served as readout to identify positive hits during screening. Using SILAC-based proteomic, we identified and quantified 2000 proteins and 140 specific phosphorylation sites. To test the involvement of these proteins in DR formation and integrin/EGFR signalling crosstalk, we monitored DR dynamics in sh/siRNA-mediated knockdowns of candidate genes in ILKf/f cells to obtain a shortlist of candidate proteins with high SILAC ratios in 4 independent SILAC experiments. DR frequencies decreased significantly in tumor suppressor Cyld- and Asap2- depleted cells. We decided to further analyse the involvement of Cyld in integrin/ILK and EGFR co-signalling pathway.

We show that Cyld tyrosine phosphorylation controlled by integrin/ILK and c-Src is a prerequisite for DR formation. Taken together we found that (i) DR formation is the result of fibronectin/ $\alpha 5\beta 1$ integrin/ILK and EGFR signalling crosstalk, (ii) DRs can be used as readout to screen for novel molecular players that involve in integrin/EGFR signalling crosstalk, (iii) integrin/ILK and EGFR signalling pathways crosstalk to mediate fast actin-based cytoskeletal rearrangements through Cyld tyrosine phosphorylation.

Publication II**Integrin-linked kinase at a glance**

Moritz Widmaier, Emanuel Rognoni, Korana Radovana, **S. Babak Azimifar**, and Reinhard Fässler.

Here we review the biological function of integrin linked kinase (ILK) and highlight the recent evidences indicating that ILK is an adaptor molecule rather than a kinase. ILK forms a ternary complex with Pinch and Parvin (IPP complex) before being recruited to cell adhesion sites. The assembly of distinct IPP complexes, which composed of tissue- or developmental stage-specific isoforms of Pinch and Parvin, can mediate multiple alternative signalling outputs. ILK has been found predominantly in focal adhesions, while it also reported to be in cell-cell adhesion sites, centrosomes and the nucleus. Genetic studies in flies, worms and mice demonstrated that ILK is a scaffolding molecule mainly regulating F-actin cytoskeletal rearrangement. Recently, it has been shown that ILK locally stabilizes microtubule tips in the cell cortex and promotes insertion of caveolin into the plasma membrane. Moreover, we discussed why ILK kinase function cannot be executed by comparing the crystal structure of the ILK pseudo-kinase domain with Protein Kinase A (PKA) kinase domain. Taken together, ILK is an important scaffolding and signalling molecule that regulates F-actin cytoskeletal rearrangement and stabilizes microtubule tips at the cell cortex.

Publication III**Babak-1 and Babak-2 are novel focal adhesion proteins that bind paxillin and regulate the assembly of membrane circular dorsal ruffles**

Azimifar S. Babak, Mandoph Wang and Faessler R.

Here, we identified a novel family of focal adhesion (FA) molecules called Babak-1 and -2 (Plekhh-1, -2) that localized to integrin adhesion sites as well as punctate structures in the cytoplasm of mouse fibroblasts and Hela cells. Babak-2 was identified as an intersector of integrin and growth factor signalling pathways in the SILAC-based phosphoproteomics screen (**publication I**). Murine Babak-2 and its homolog, Babak-1 are multidomain proteins that are highly conserved in vertebrates.

SILAC-based analysis of Babak-2 immunoprecipitates revealed that Babak-2 undergoes Ser/Thr phosphorylation and interacts with Paxillin, a FA-associated adaptor molecule. Immunoprecipitation experiments and mutational analysis of Babaks revealed that their recruitment to focal adhesions was dependent on their interaction with Paxillin, which was mediated through a paxillin binding sequence (PBS) present in the N-terminal half of the protein. Quantitative RT-PCR and northern assays revealed that Babak-1 and -2 transcripts are expressed at all embryonic stages after E7.5 as well as in most of adult murine tissues. To test the functional properties of Babak-2 *in vivo*, we generated a conditional Babak-2-null mouse strain. Babak-2-null mice were fertile and did not show an obvious phenotype suggesting that Babak-2 function can be compensated by its homologous protein, Babak-1.

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Curriculum Vitae

PERSONAL INFORMATION

First Name: Seyed Babak **Last Name:** Azimifar

Date and place of Birth: 1978, Ahvaz, Iran.

EDUCATION

(2006-present) **Ph.D. student:** Ludwig-Maximilians-University, Munich, Germany.

Project: Integrin/ILK and EGFR cosignalling

Mentor: Dr. Reinhard Fässler

(2001 - 2003) **M.Sc. in Human Genetics:** S. Beheshti University, Tehran, Iran.

Project: Carrier detection and prenatal diagnosis of Hemophilia A

Mentor: Dr. S. Yoosef Seyedna

(1997 - 2001) **B. in See Biology:** S. Chamran University, Ahvaz, Iran.

HONORS & AWARDS

(2001-2003) First Class Honors among graduate students (S. Beheshti Univ.)

(May 2001) National Iranian Scientific Olympiad on Biology (Ranked third)

(1997-2001) First Class Honors among bachelors of biology (S. Chamran Univ.)

EXPERIENCE

(2003-2006) **Lab supervisor:** Research Center for diagnosis of hereditary disease.

(2003-2006) **Teaching:** workshops (software skills and statistics).

PUBLICATIONS

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I) Prof. Dr. Reinhard Fässler (Dept. of Molecular Medicine, Max Planck Institute of Biochemistry, D-82152 Martinsried, Germany).

II) Dr. Hans-Joerg Schäffer (IMPRS for Molecular and Cellular Life Sciences, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany; www.imprs-ls.de).

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Appendix

In the following, publications I to III are reprinted.

The induction of membrane circular dorsal ruffles requires integrin/ILK and EGF receptor co-signalling

S. Babak Azimifar¹, Ralph T. Böttcher¹, Sara Zanivan^{2,#}, Carsten Grashoff¹, Marcus Krüger^{2,§}, Kyle R. Legate¹, Matthias Mann² and Reinhard Fässler^{1,*}

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, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, United Kingdom

§present address: Biomolecular Mass Spectrometry, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany

*Corresponding author: faessler@biochem.mpg.de

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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/ $\alpha 5\beta 1$ integrin/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 tumor-suppressor Cyld to be required for integrin/EGF-induced DR formation. Furthermore, EGF-induced Cyld tyrosine phosphorylation is controlled by integrin/ILK and c-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.

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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 α/β subunit combinations which confer substrate and signalling specificity. As 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 was shown to directly bind the $\beta 1$ and $\beta 3$ 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, *D. melanogaster* and *C. 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 c-Src is one of the kinases that localizes to FAs. Src activity is regulated by both integrin and RTK signalling (Huveneers 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). c-Src also regulates F-actin cytoskeleton remodeling through activation of various effector proteins, including small GTPases (Huveneers and Danen, 2009; Timpson et al., 2001), kinases such as c-Abl as well as p120-Catenin and cortactin (Castano et al., 2007; Chang et al., 1995; Plattner et al., 1999).

Circular dorsal ruffles/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 c-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 $\beta 3$ 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 FN/ $\alpha 5\beta 1$ 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 dorsal ruffle formation

We generated ILK-floxed ($ILK^{f/f}$) and ILK-deficient ($ILK^{-/-}$) fibroblasts to investigate the consequence of ILK deletion *in vitro* (Sakai et al., 2003). During our experiments we realized that stimulation of starved $ILK^{f/f}$ cells with media containing 10% fetal calf serum induced DRs in approximately 30% of $ILK^{f/f}$ cells, while $ILK^{-/-}$ 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 $ILK^{f/f}$ and $ILK^{-/-}$ fibroblasts that were seeded on FN-coated surfaces. Consistent with our observation using 10% fetal calf serum, about 25% of $ILK^{f/f}$ cells formed DRs after EGF stimulation whereas $ILK^{-/-}$ cells showed very few ruffles (Fig. 1B, C). Similarly, $ILK^{-/-}$ 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 since we consistently found a significant reduction of DRs in all $ILK^{-/-}$ clones compared to their $ILK^{f/f}$ 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 $ILK^{-/-}$ cells was not due to reduced EGF receptor (EGFR) phosphorylation or ERK1/2 activation as their relative levels were similar in $ILK^{f/f}$ and $ILK^{-/-}$ cells with the exception of phosphorylation of EGFR Tyr992, which was increased in $ILK^{-/-}$ cells (Fig. 1D, supplementary material Fig. S1D). Similarly, EGF-induced Rac1 activation was similar in $ILK^{-/-}$ and $ILK^{f/f}$ cells, although the activation was prolonged in $ILK^{-/-}$ cells but the differences were not statistically significant (Supplementary material Fig. S1E, F). $ILK^{-/-}$ cells show spreading defect, raising the possibility that impaired DR formation is a consequence of the reduced spread area of these cells. However, when $ILK^{-/-}$ cells were allowed to spread for longer time periods, up to two 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 $ILK^{-/-}$ cells.

ILK has been suggested to interconnect integrins with growth factor pathways through Pinch1. Additionally, ILK and Pinch1 are components of the ILK-Pinch-Parvin (IPP)

complex, whose members depend on complex formation for maintaining their stability (Legate et al., 2006). Western blot analysis showed that the Pinch1 expression level is strongly reduced in ILK^{-/-} cells (Fig. 1E). To test whether the DR formation defect in ILK^{-/-} 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^{-/-} cells. The presence of 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 ILK^{-/-} cells was not due to reduced Pinch1 protein levels. Expression of ANK-FLAG had no effect on DR formation in ILK^{fl/fl} cells (Supplementary material Fig. S1H, I). Conversely, Pinch1^{-/-} cells showed strongly reduced ILK protein (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.

ILK^{-/-} 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 ILK^{-/-} cells migrate toward a source of EGF or PDGF less efficiently than ILK^{fl/fl} cells or ILK-FLAG rescued ILK^{-/-} cells (Fig. 2A). Furthermore, the internalization of activated EGFR was significantly reduced in ILK^{-/-} cells whereas the internalization of transferrin receptor remained unchanged (Fig. 2B, Supplementary material Fig. S2). Reduced internalization of EGFR is expected to result in prolonged signaling, and indeed the relative level of phosphorylation of EGFR in ILK^{-/-} cells is increased, perhaps as a result of impaired downregulation through internalization (Supplementary material, Fig S1D). Therefore, ILK^{-/-} cells displayed phenotypic differences that are consistent with a reduction in the formation and number of DRs.

DRs are the result of $\alpha 5\beta 1$ integrin and EGFR signalling co-signalling

The finding that ILK plays a critical 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 ILK^{fl/fl} cells seeded on fibronectin (FN) or poly-L-lysine (PLL). While FN can be recognized by many integrin

receptors, most notably $\alpha 5\beta 1$ and $\alpha v\beta 3$, PLL-mediated adhesion is integrin-independent. Only FN-seeded ILK^{ff} cells formed DRs (Fig. 3A), and the rate of DR formation in ILK^{ff} 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 substratum and formed paxillin-rich focal adhesions on FN, VN and Col1 but not on PLL (Fig. 3E). While 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 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 $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins while VN is bound by $\alpha v\beta 3$ but not $\alpha 5\beta 1$ integrin (Hynes, 2002). Therefore, our results suggest that only $\alpha 5\beta 1$ integrin signals trigger DRs. To confirm this, we examined EGF-induced DRs in serum-starved FN-seeded integrin $\beta 1^{ff}$ and $\beta 1^{-/-}$ fibroblasts (which lack $\alpha 5\beta 1$ but express $\alpha v\beta 3$ integrin). In agreement with the previous experiments, about 30% of $\beta 1^{ff}$ cells formed DRs whereas $\beta 1^{-/-}$ cells showed significantly reduced DR frequency (Fig. 3G). Re-expression of $\beta 1$ integrin in $\beta 1^{-/-}$ cells rescued DR formation in these cells (Supplementary material Fig. S3E, F). These results suggest that DRs are the consequence of FN/ $\alpha 5\beta 1$ integrin/ILK and EGFR co-signalling.

ILK affects active c-Src localization to FAs

Both integrin and RTK signalling stimulate c-Src tyrosine kinase activity, which is known to play a central role in DR formation (Chang et al., 1995; Huvneers and Danen, 2009). In line with these previous reports, ILK^{ff} cells pre-treated with a Src inhibitor (PP1) failed to form DRs (Supplementary material Fig. S4A). Therefore we decided to investigate the role of c-Src in more detail. First, we investigated whether c-Src activation is impaired in ILK^{-/-}

cells. Immunostaining with a pY416 c-Src antibody showed that active c-Src levels were dramatically reduced in FAs of FN-seeded ILK^{-/-} cells before EGF stimulation and remained reduced after EGF stimulation (Fig. 4A, B). Re-expression of ILK-EGFP in ILK^{-/-} cells rescued the level of active c-Src in FAs (Supplementary material Fig. S4B-D). Western blot analysis showed that total c-Src levels were similar in ILK^{fl/fl} and ILK^{-/-} cells (Fig. 4C) and that non-adherent ILK^{fl/fl} and ILK^{-/-} cells showed a similar (2-fold) increase in c-Src activity after EGF treatment (Fig. 4C, D). Plating cells on FN caused a basal increase in c-Src phosphorylation in ILK^{fl/fl} cells that did not manifest in ILK^{-/-} cells, but EGF-treatment induced a similar activation of c-Src in both cell lines, resulting in a net decrease in active c-Src in ILK^{-/-} cells of about 20% (Fig. 4C, D). Co-immunoprecipitation of ILK with anti-c-Src antibody in ILK-FLAG-rescued ILK^{-/-} cells (Fig. 4E), and c-Src with anti-GFP antibody in ILK-GFP-rescued ILK^{-/-} cells (Fig. 4F) indicated that c-Src and ILK form a complex in our fibroblast cell lines. However, a complex between c-Src and endogenous ILK was not easily detectable in our cells (data not shown).

Importantly, transient expression of constitutively active c-Src^{Y527A}-EGFP localized to FAs and rescued DR formation in ILK^{-/-} cells (Supplementary material Fig. S4E-G). Furthermore, a decreased level of active c-Src at FAs significantly correlated with decreased DR frequency, whereas c-Src activity and the number of DRs concomitantly increased in ILK^{fl/fl} 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 controlling c-Src activity at FAs.

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 c-Src in FAs. To identify potential ILK-dependent substrates for EGFR/c-Src that are involved in DR formation, we compared the phosphoproteome of 30 sec and 2 min EGF treated ILK^{fl/fl} and ILK^{-/-} cells by combining phosphotyrosine immunoprecipitation and SILAC-based mass spectrometry (Fig. 5A). Candidate proteins involved in β1 integrin/EGFR induced DR formation were defined as those which displayed increased phosphorylation upon EGF stimulation in ILK^{fl/fl} cells, but not in ILK^{-/-} cells. Our analyses identified and quantified more than 2000 proteins and 140 specific

phosphorylation sites (Supplementary material datasets. S1, 2) after excluding proteins that are expressed at different levels in ILK^{fl/fl} and ILK^{-/-} 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^{fl/fl} and ILK^{-/-} cells, while certain proteins were upregulated in an ILK-dependent manner (Fig. 5B).

To test the involvement of these proteins in DR formation we performed sh/siRNA-mediated knockdown in ILK^{fl/fl} cells, of candidate proteins that were consistently represented in 4 independent SILAC screens (Fig. 5C). Knock down 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 β 1 integrin/ILK and EGFR co-signalling pathway.

Cyld tyrosine phosphorylation is essential for DR formation

The tumor suppressor protein Cyld tunes several signal transduction pathways including NF- κ B, JNK and Wnt/ β -catenin through its deubiquitinating (DUB) activity (Massoumi, 2010). We prepared Cyld^{-/-} 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^{-/-} fibroblasts (Fig. 6A; Supplementary material Fig. S6A), while re-expression of a catalytically inactive Cyld (Cyld C>S) (Brummelkamp et al., 2003; Trompouki et al., 2003) failed to restore DR formation in Cyld^{-/-} cells (Fig. 6A). Immunostaining of endogenous Cyld in wild type cells (Fig. 6B) and over expression of GFP-Cyld in Cyld^{-/-} cells revealed that Cyld was recruited to DRs (Supplementary material Fig. S6B-D). Like in ILK^{-/-} cells, Rac1 activation was not impaired in Cyld^{-/-} cells (Supplementary material Fig. S6E, F).

As our phospho-proteomics 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 signaling (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 Col1 (Fig. 6E, F). Consistent with our phosphoproteomics data, Cyld phosphorylation was reduced in FLAG-Cyld rescued *Cyld*^{-/-} cells in which ILK was depleted (Fig. 7A). Similarly, Cyld phosphorylation was diminished in *ILK*^{-/-} cells expressing FLAG-Cyld when compared with control *ILK*^{fl/fl} cells (Fig. 7B), whereas localization of endogenous Cyld was not changed in *ILK*^{-/-} cells upon EGF stimulation (Supplementary material Fig. S7A). Finally, c-Src inhibition with PP1 abolished EGF-induced Cyld tyrosine phosphorylation in FLAG-Cyld rescued *Cyld*^{-/-} cells indicating that EGF-induced Cyld tyrosine phosphorylation occurs downstream of c-Src (Fig. 7C). Immunostaining of active Src in mCherry-Cyld-expressing *Cyld*^{-/-} cells suggested that cytoplasmic Cyld was not localized to FAs (Fig. 7D). Although phosphorylation of Cyld on serine has recently been reported (Hatti 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 4 tyrosines (FLAG-Cyld-4X) identified in other phosphoproteomics experiments (data not shown, for the locations of these tyrosines see Supplementary material datasets. S3) reduced neither EGF-induced tyrosine phosphorylation of Cyld nor DR formation (Fig. 6A; Supplementary material Fig. S7B). The substitutions of additional tyrosines with alanines (FLAG-Cyld-9X and FLAG-Cyld-18X mutants, Supplementary material datasets S3) led to a significant decrease in EGF-induced tyrosine phosphorylation and DR formation (Fig. 6A, Fig. 7E).

Altogether these data demonstrate that Cyld lies downstream of an integrin/ILK-EGFR co-signalling pathway leading to the formation of DRs in fibroblasts. While 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 c-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, c-Src and Cyld.

The specificity of the involvement of $\alpha 5\beta 1$ 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 α/β integrin subunit combinations (Humphries et al., 2009). For example, $\alpha 5\beta 1$ and $\alpha V\beta 3$ have distinct effects on actin cytoskeletal regulation through different modulation of Rho GTPases (Danen et al., 2005; Huvneers et al., 2008). While adhesion to FN by $\alpha 5\beta 1$ causes high levels of RhoA activity and low levels of Rac activity, adhesion via $\alpha V\beta 3$ induces low levels of RhoA activity (Danen et al., 2002; Huvneers et al., 2008). Additional actin modulators, such as VASP, are also regulated differently by $\beta 1$ and $\beta 3$ integrins, leading to changes in actin-dependent processes such as migratory behavior (Worth et al., 2010). By plating cells on different substrates and making use of $\beta 1^{-/-}$ cells we show that the specific signalling complex that assembles on $\alpha 5\beta 1$ integrin tails supports DR formation, whereas the signals emanating from $\beta 3$ integrins or collagen-binding integrins do not. This is consistent with a recent study demonstrating that $\beta 1$ integrin is essential for both PDGF-induced DRs and chemotaxis in fibroblasts (King et al., 2011). We also show that ILK is a key component of the DR signalling complex downstream of $\alpha 5\beta 1$ integrin. $ILK^{-/-}$ cells formed only a few DRs, while ILK^{ff} cells as well as ILK-EGFP- or ILK-FLAG-rescued $ILK^{-/-}$ cells formed a full complement of DRs. This effect of ILK is downstream of $\beta 1$ integrin as $\beta 1^{-/-}$ cells are also unable to support DRs despite ample levels of ILK expression.

ILK has been intensively studied as a FA adaptor molecule that is involved in integrin-mediated actin cytoskeletal rearrangements (Bottcher et al., 2009; Legate et al., 2006; Legate et al., 2009). We found that ILK affects DR formation by regulating the levels of active c-Src in FAs in the absence of growth factors. c-Src is a tyrosine kinase that mediates signalling pathways involved in actin reorganization and DR formation, and is activated

downstream of RTK and integrin signalling (Huveneers and Danen, 2009). There is increasing evidence that c-Src activation and its biological functions are tightly controlled by its subcellular localization. Whereas c-Src at FAs inhibits Rho GTPase by inducing p190GAP activation, it activates Rho GTPase when localized to podosomes (Arthur et al., 2000; Bass et al., 2008; Berdeaux et al., 2004). In addition, PDGF receptor uses different pools of c-Src to initiate distinct pathways; while PDGF activates caveolae-associated c-Src for mitogenesis, PDGF-activated c-Src outside of caveolae affects F-actin assembly leading to DR formation (Veracini et al., 2006). The absence of ILK strongly reduces the levels of active c-Src in FAs, but rescuing ILK^{-/-} cells with ILK-FLAG or ILK-GFP restores its presence in FAs, and induces an ILK/c-Src complex. It has been suggested that an ILK/c-Src complex regulates actin polymerization by phosphorylating cofilin (Kim et al., 2008). The ILK/c-Src interaction seems to be important for c-Src activation in FAs, but does not appear to play a role in c-Src phosphorylation in response to EGF stimulation. Therefore the localization of c-Src to FAs via associating with ILK is necessary for DR formation, whereas activation of c-Src in other subcellular compartments is not sufficient for this process. However, the precise mechanism by which ILK affects active c-Src levels in FAs is still unclear.

The mechanism by which c-Src, and in particular FA-associated c-Src, induces DR formation is largely unknown. c-Src at FAs activates Rac1 locally through phosphorylation of specific GEFs, and thereby induces lamellipodia (Huveneers and Danen, 2009). In concert with RTK signals active Rac could participate in DR induction (Buccione et al., 2004). In addition, several c-Src substrates have been implicated in DR formation, such as Cortactin (Lai et al., 2009), c-Abl (Plattner R et al., 1999) and c-Cbl (Sirvent et al., 2008). In this study we used a SILAC-based phosphoproteomics screen to detect proteins differentially phosphorylated by EGF in the presence or absence of functional integrin/ILK signalling. We confirmed the reliability of our SILAC list by monitoring DR formation in cell lines depleted of selected candidate proteins by sh/siRNA. In this way we identified Cyld as a new player in DR formation downstream of c-Src. This screen also identified additional candidates that could potentially serve key roles in integrin/RTK crosstalk.

The function of Cyld in the regulation of signalling pathways has previously been linked to its Lysine-63 deubiquitinase activity (Brummelkamp et al., 2003; Kovalenko et al., 2003; Massoumi et al., 2006; Reiley et al., 2004) which can be controlled by phosphorylation on

serine 418 by IKK ϵ (Hutti 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, $\alpha 5\beta 1$ integrin and ILK. As ILK does not localize to DRs and Cyld does not localize 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 may be required for the interaction of Cyld with 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 the histone deacetylase-6 (HDAC6) (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-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 signaling 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 Signalling Technology. Gefitinib (Iressa) was supplied by Selleck. The following antibodies were used: ILK, Rac1 and paxillin (BD Bioscience), EGF receptor, pY1173-, pY1068-, pY992- and pY845-EGF receptor and pY416-Src (Cell Signalling Technology); pY-4G10 and Lasp-1 (Millipore); β 1 integrin and Cyld antibodies were homemade antibodies raised in rabbit; anti-FLAG, SHC2, and vinculin (Sigma); CDC42BPB, Dock4 (ABNOVA); anti-mouse-HRP, anti-rabbit-HRP, anti-Rat-HRP (BioRad); phalloidin-Alexa-488 (Invitrogen); anti-rabbit Cy3, anti-mouse Cy3 (Jackson Immunoresearch); Pinch1 (Li et al., 2007).

Immunofluorescence

Cells were cultured on glass cover slips coated with 10 μ g/ml FN (Calbiochem). For staining, cells were fixed in 2-4% paraformaldehyde in PBS for 15 min and permeabilized for 10 min with 0.2% Triton X-100 in PBS. The cells were blocked with 3% BSA in PBS for 1 h and incubated with the primary antibody for 1 h at room temperature or overnight at 4°C. Secondary antibodies were incubated for 1 h at room temperature. Images were collected using a confocal microscope (DMIRE2; Leica, Bensheim, Germany) equipped with 63x/1.4 or 100x/1.4 oil objectives and the Leica Confocal Software (version 2.5, build 1227), or collected with a AxioImager Z1 microscope (Zeiss, Germany) with the 63x/1.4 oil objective, using Metamorph software.

Immunoprecipitation and western blotting

Cell lysate was prepared by quickly washing cells in ice-cold PBS prior to addition of lysis buffer (50mM Tris-HCl, pH7.6, 150mM 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 (Sigma, Cat.No. A2220). For other IPs, 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, washed three times with lysis buffer, eluted with SDS loading buffer and analyzed by SDS-PAGE. GTP-Rac1 pulled down by PAK-CRIB peptide from EGF-triggered cell lysates and blotted with Rac1 antibody.

Constructs and siRNAs

cDNA constructs of FLAG-Cyld, FLAG-ILK, FLAG-ANK ILK, pEGFP-ILK were amplified by PCR. DUB-dead FLAG-Cyld C>S was kindly donated by Dr. Rene Bernards, NKI Amsterdam. FLAG-Cyld Y>A mutant constructs were generated using the QuikChange Site-Directed Mutagenesis kit (#200518 Stratagene). Mutations in 9x and 19X Y>A FLAG Cyld mutants are summarized in Supplementary material datasets. S3. Mission siRNAs were ordered from Sigma. shRNAs were supplied from a shRNAmir30 library (Thermo Scientific). For sh/siRNAs sequences, see Supplementary material datasets S3.

Cell cultivation and transfection/transduction

Cells were transiently transfected with Lipofectamine 2000 (Invitrogen). To generate stable cell lines cDNAs were cloned into pCLMFG retroviral vectors and transiently transfected into human embryonic kidney (HEK293T) cells; viral particles were used for infection of ILK^{-/-} fibroblast as previously described (Pfeifer et al., 2000). For stable knockdowns Phoenix viral packaging cells were used to generate virus that was subsequently used to infect ILK^{fl/fl} fibroblasts (Pear et al., 1993). Integrin $\beta 1^{fl/fl}$ fibroblasts were isolated from murine kidney and immortalized by SV40 Large T-antigen. $\beta 1^{-/-}$ cells were generated by adenoviral infection of $\beta 1^{fl/fl}$ cells with GFP-Cre recombinase, followed by flow cytometry cell sorting of GFP-positive cells. $\beta 1^{-/-}$ cells were rescued by retroviral infection with $\beta 1$ integrin cDNA. For DR experiments, fibroblasts were serum starved overnight, trypsinized, seeded on FN, VN, Col1 or PLL coated dishes, stimulated with 50ng/ml EGF and monitored for DR formation using a Zeiss Axiovert 200 M microscope (Zeiss, Germany) at 37°C. Time lapse images were captured for 15 minutes with interval time of 90 seconds. We isolated Cyld^{-/-} fibroblasts from kidney of Cyld^{-/-} mice (Massoumi et al., 2006).

EGFR internalization assay

ILK^{fl/fl} and ILK^{-/-} fibroblasts were serum starved for four hours at 37°C. Medium was exchanged for ice cold medium containing 25 ng/ml Alexa488-EGF (Invitrogen) and cells

were incubated on ice for 30 minutes to allow for full ligand binding. Plates were washed twice with cold PBS, and prewarmed DMEM was added to induce internalization of EGFR. Plates were incubated at 37°C for the indicated times, and placed on ice to stop internalization. Cells were washed for 5 minutes with 0.2 M acetic acid/500 mM NaCl, pH 2.8, washed 3 times with cold PBS and scraped into tubes for counting by FACS. Negative and 100% binding controls were kept on ice and either acid washed or directly scraped into tubes, respectively. Fluorescence intensity was measured by FACS, and the mean values of the peaks were normalized against 100% binding controls to obtain percent internalization.

Chemotaxis assay

Chemotactic migration assay was performed as previously described (Legate et al., 2011). Briefly, 30000 cells were resuspended in serum-free conditions in Cell culture inserts (cat#353097; BD Biosciences), that were pre-coated on the underside with 5 µg/ml FN, and were allowed to migrate for 4 hours at 37°C toward the lower chamber containing 50ng/ml PDGF-BB, 50ng/ml EGF or BSA control. Cells were fixed and stained with 0.1% crystal violet in 20% methanol for 5 minutes in room temperature and non-migratory cells were manually removed with a cotton swab. Four random fields were captured by a Zeiss Axioskop microscope equipped with a LEICA DC 500 digital camera, and cells were counted manually.

SILAC-based proteomics

SILAC-based proteomics was performed as described before (Mann, 2006). Briefly, cells were metabolically labelled with SILAC medium, serum starved overnight, trypsinized, seeded on FN for 90 minutes and EGF (50ng/ml) stimulated for 30 seconds or 2 minutes. Cells lysates were prepared, mixed 1:1 and subjected to immunoprecipitation using phosphotyrosine antibodies (4G10 from Millipore and anti-pY100 from Cell Signalling). The immunoprecipitated proteins were subjected to in gel digestion; peptides were concentrated and desalted using the micropurification system StageTips, separated by online reverse phase nanoscale capillary LC and analyzed by ES MS/MS on a linear trap quadrupole (LTQ)-Orbitrap mass spectrometer (Thermo Fisher Scientific). Mass spectra were processed with the software MaxQuant in combination with the Mascot search engine.

Live cell imaging and image analysis

Images of live cells were acquired at 37°C and 5% CO₂ on a Zeiss Axiovert 200 M microscope (Zeiss, Germany) with a 10x1.6 objective; the microscope was equipped with a motorized stage (Märzhäuser, Germany), an environment chamber (EMBL Precision Engineering, Germany) and a cooled CCD camera (Roper Scientific, Princeton, NJ). Microscope control, image acquisition and post-acquisition analysis were carried out using MetaMorph software (Molecular Devices, Downington, PA). To monitor dynamics of DR formation in different cell types, serum starved cells were seeded on FN for 90 minutes, stimulated with EGF (50 ng/ml) or PDGF-BB (50 ng/ml) and time-lapse movies were captured for 17 minutes in the case of EGF stimulation (40 and 60 minutes for PDGF and serum stimulation respectively) with 90 seconds interval time. DRs were classified as transient actin-rich structures that appeared after growth factor stimulation. To measure active Src levels at FAs, serum starved cells were seeded on FN for 90 min, stimulated with EGF, fixed, immunostained with pY416-Src and vinculin antibodies and images were captured with a confocal microscope (DMIRE2; Leica, Bensheim, Germany) equipped with 63x/1.4 oil objectives. Images were then processed by MetaMorph software to calculate the intensity of active Src and vinculin at FAs. The averages of active Src intensities normalized to vinculin intensities are presented in histograms (means±s.d.). The intensity of western blot bands was quantified by MultiGauge software (Fujifilm).

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 t-test was used for comparisons between different data sets. Asterisks indicate significant differences (*P<0.05, **P<0.01 and ***P<0.005).

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Figure legends

Fig. 1. ILK is required for DR formation. (A) Kinetics of DR formation in serum starved ILK^{ff} and ILK^{-/-} cells when treated with serum or (B) when seeded on FN and stimulated with EGF. DR formation is significantly decreased in ILK^{-/-} cells. (C) Actin (green) and Cortactin (red) staining of FN-seeded ILK^{ff} and ILK^{-/-} cells after three minutes of EGF stimulation. Arrows indicate DRs in ILK^{ff} cells. Scale bars: 20µm (D) Western blot analysis of EGF-triggered EGFR and ERK phosphorylation in ILK^{ff} and ILK^{-/-} cells. ILK^{-/-} cells expressed higher levels of EGFR, however, downstream ERK signaling was not changed (E) Western blot analysis of protein lysates from ILK^{-/-} cells 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 the mean±s.d. Stars indicate *P*-value for *t*-test (****P*<0.005).

Fig. 2. Functional consequence of aberrant DR formation in ILK^{-/-} cells. (A) Chemotaxis migration of ILK^{ff}, ILK^{-/-} and ILK-FLAG rescued ILK^{-/-} cells toward EGF or PDGF. Chemotaxis migration to EGF/PDGF was significantly decreased in ILK^{-/-} cells. n=4 independent experiments. (B) EGFR internalization after EGF stimulation is diminished in ILK^{-/-} cells. n=3 independent experiments. Data expressed as the mean±s.d. Stars indicate *P*-value for *t*-test (**P*<0.05 and ****P*<0.005).

Fig. 3. Integrin dependency of DRs. (A) Quantification of DR formation in FN- or PLL-seeded ILK^{ff} cells. DR formation decreased significantly in cells plated on PLL. (B) DR frequency is increased in ILK^{ff} cells by increasing the FN concentration in the coating solution. (C) Differential DR formation in serum starved FN^{-/-} cells seeded onto FN, VN, PLL or Col1 coated dishes (phase contrast images; scale bar: 50µm) or (D, E) coverslips (immunofluorescence; scale bar: 20µm) and stimulated with EGF for three minutes. Arrows indicate DRs in FN-seeded FN^{-/-} cells. (D) Overlay of F-actin (green) and Paxillin (red) immunostaining. (E) FAs as visualized by Paxillin immunostaining. Note the absence of FAs in PLL-seeded FN^{-/-} cells while FN-, VN- and Col1-seeded FN^{-/-} cells form FAs with a distinct morphology. (F) Kinetics of EGF-induced DR formation when cells are

seeded on FN, VN, PLL or Col1. **(G)** The frequency of DRs is reduced significantly in integrin $\beta 1^{-/-}$ fibroblasts in comparison with control integrin $\beta 1^{+/+}$ cells. $n=4$ independent experiments. Data expressed as the mean \pm s.d. Stars indicate P -value for t -test (** $P<0.005$).

Fig. 4. ILK affects active Src levels at FAs during spreading on FN. **(A)** pY416-Src (green) and vinculin (red) immunostaining of serum starved FN-seeded ILK^{+/+} and ILK^{-/-} cells. Note the reduced pY416-Src staining in ILK^{-/-} cells. Scale bars: 10 μ m. **(B)** The quantification of pY416-Src/vinculin intensity at FAs in cell stainings showed reduced active Src intensity at FAs of ILK^{-/-} cells. Data expressed as the mean \pm s.d. $n=3$ independent experiments. **(C)** pY416-Src immuno-blotting of serum starved ILK^{+/+} and ILK^{-/-} cells when seeded on FN (FN) or kept on suspension (S) and stimulated with EGF. **(D)** The measurement of pY416-Src band intensity in immunoblots indicated that FN-induced Src activation is impaired in ILK^{-/-} cells. Data expressed as the mean \pm 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. Stars indicate P -value for t -test (* $P<0.05$, ** $P<0.01$ and *** $P<0.005$).

Fig. 5. Analysis of the ILK-dependent phosphoproteome. **(A)** Schematic representation of the SILAC-based phosphoproteomics screening strategy (see material and methods). Non-stimulated ILK^{+/+}, EGF-triggered ILK^{-/-} and EGF-triggered ILK^{+/+} cells were labeled 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)** The panel of identified anti-phosphotyrosine immunoprecipitated proteins after 2 min EGF stimulation in ILK^{+/+} cells (x axis), and ILK^{-/-} cells (y axis). The 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 sh/siRNAi mediated knock down. Quantification of DR formation in Cyld **(D)** or Asap2 **(E)** knock down cells demonstrates their involvement in the DR pathway downstream of EGF stimulation. $n=4$ independent experiments. Data expressed as the mean \pm s.d. Stars indicate P -value for t -test (** $P<0.01$ and *** $P<0.005$).

Fig. 6. DR formation requires EGF-triggered tyrosine phosphorylation of Cyld. (A) DR frequency three minutes after EGF induction for $Cyld^{-/-}$ cells, $Cyld^{+/+}$ cells and $Cyld^{-/-}$ cells expressing wild type HA-Cyld, DUB-dead HA-Cyld C>S, wild type FLAG-Cyld, FLAG-Cyld-4X and FLAG-Cyld-9X or FLAG-Cyld-18X tyrosine to alanine point mutant cDNAs. (B) Immunostaining of endogenous Cyld (green) and α -actinin (red) in FN-seeded wild type cells after EGF stimulation. Arrows indicate DR. Scale bar: 10 μ m. (C) FLAG immunoprecipitation from FLAG-Cyld rescued $Cyld^{-/-}$ cells followed by phosphotyrosine blotting revealed transient EGF-triggered Cyld tyrosine phosphorylation. (D) Inhibition of EGFR signaling by Gefitinib (20nM/ml) abolished Cyld tyrosine phosphorylation. The inhibitory effect of Gefitinib on EGFR phosphorylation was verified by immunoblotting for EGFR phospho-tyrosine 992 and 1173. (E) EGF-triggered Cyld phosphorylation in cells seeded on FN, but not on PLL (E), VN or Coll 1 (F). Data expressed as the mean \pm s.d. Stars indicate *P*-value for *t*-test (***P*<0.005).

Fig. 7. ILK and Src are essential for EGF-triggered tyrosine phosphorylation of Cyld. (A) SiRNA-mediated depletion of ILK in FLAG-Cyld rescued $Cyld^{-/-}$ cells decreased Cyld tyrosine phosphorylation. (B) Western blot analysis of FLAG immunoprecipitates from FN-seeded $ILK^{f/f}$ and $ILK^{-/-}$ cells that expressed FLAG-Cyld before and after EGF stimulation. Note the reduced Cyld tyrosine phosphorylation and active Src levels in $ILK^{-/-}$ cells. (C) Pharmacological inhibition of Src activity by PP1 (5 μ M/ml) abolished Cyld tyrosine phosphorylation. (D) Immunostaining of pY416-Src (green) in $Cyld^{-/-}$ cells that expressed mCherry-Cyld, before and after EGF stimulation. mCherry-Cyld was distributed throughout the cytoplasm with partial enrichment at the cell periphery and perinuclear regions before EGF stimulation, and additionally to DRs after EGF stimulation. Scale bars: 10 μ m. (E) Cyld tyrosine phosphorylation is reduced in FLAG-Cyld-9X or FLAG-Cyld-18X tyrosine to alanine point mutants.

Supplementary Fig. S1. Impaired DR formation in $ILK^{-/-}$ cells. (A) Kinetics of DR formation in starved, FN-seeded $ILK^{f/f}$ and $ILK^{-/-}$ cells treated with PDGF-BB. $ILK^{-/-}$ cells formed a few DRs in comparison with control $ILK^{f/f}$ cells. Note the differential kinetics of PDGF- and EGF-induced (see Fig. 1B) DR formation. n=5 independent experiments (B) DR formation monitored in two different $ILK^{f/f}$ cell lines and their corresponding $ILK^{-/-}$

clones three minutes after EGF stimulation. The frequency of DRs decreased significantly in all ILK^{-/-} clones. n=3 independent experiments **(C)** Immunostaining of serum starved, FN-seeded ILK^{f/f} cells after 4 min EGF-stimulation against F-actin (green) and ILK (red). ILK immunostaining was absent at DRs. Scale bar: 10 μm. **(D)** Quantification of EGFR phospho-tyrosine levels upon EGF stimulation in ILK^{f/f} and ILK^{-/-} cells. The intensities measured from western blots of n=3 independent experiments and normalized to total EGFR levels. EGFR phosphorylation was not reduced in ILK^{-/-} cells. **(E)** Analysis of Rac1 activation after EGF stimulation in ILK^{f/f} and ILK^{-/-} cells. **(F)** Quantification of Rac1 activation upon EGF stimulation in ILK^{f/f} and ILK^{-/-} cells. n=3 independent experiments **(G)** Frequency of DRs in ILK^{f/f}, ILK^{-/-} and ILK-GFP rescued ILK^{-/-} cells after three minutes of EGF stimulation. n=3 independent experiments. **(H)** Expression of ANK-FLAG did not affect DR formation in ILK^{f/f} cells. n=4 independent experiments **(I)** Western blot analysis of protein lysates from ILK^{f/f} cells that expressed ANK-FLAG. **(J)** EGF-induced DR formation decreased significantly in PINCH (-/-) cells. n=4 independent experiments. Data expressed as the mean±s.d. Stars indicate *P*-value for *t*-test (**P*<0.05, ***P*<0.01 and ****P*<0.005). n.s.= not significant.

Supplementary Fig. S2. Transferrin internalization was not affected in ILK^{-/-} cells. Internalized transferrin was measured by flow cytometry internalization assay in ILK^{f/f} and ILK^{-/-} cells. Transferrin internalization was normal in ILK^{-/-} cells. One example from 3 independent experiments is shown.

Supplementary Fig. S3. Integrins are crucial for DR formation. **(A)** Cell spreading area is significantly reduced only when FN^{-/-} cells are seeded on PLL. FN-seeded FN^{-/-} cells that spread for 30 minutes had same reduced spreading area. n=3 independent experiments. **(B)** Restricting the spreading time to restrict the spread area did not impair DR formation 3 minutes after EGF stimulation. n=4 independent experiments. **(C)** Quantification of DR formation in ILK-depleted FN^{-/-} cells 3 min after EGF-stimulation. n=3 independent experiments. **(D)** Western blot analysis of ILK levels in ILK-depleted FN^{-/-} cells. **(E)** Rescue of DR formation in integrin β1^{-/-} after re-expression of β1 integrin cDNA. n=4 independent experiments. **(F)** Western blot analysis of β1 integrin levels in β1^{f/f}, β1^{-/-} and

$\beta 1$ integrin-rescued $\beta 1^{-/-}$ cell lysates. Data expressed as the mean \pm s.d. Stars indicate *P*-value for ANOVA in panel **A** (**P*<0.05) and *t*-test in panel **B**, **C** and **E** (**P*<0.05).

Supplementary Fig. S4. DR formation is rescued in ILK^{-/-} cells that expressed GFP-ILK or constitutively active c-Src. **(A)** DR formation in ILK^{fl/fl} cells is blocked when treated with Src selective inhibitor PP1. n=4 independent experiments. **(B)** The quantification of pY416-Src/vinculin immunofluorescence intensity at FAs showed increased active Src level in FAs of ILK-GFP rescued ILK^{-/-} cells. n=3 independent experiments. **(C and D)** Serum starved ILK^{-/-} cells and ILK-GFP rescued ILK^{-/-} cells were seeded on FN, stimulated with EGF and immunostained for pY416-Src (red) and **(C)** vinculin or **(D)** actin (white). Scale bars: 10 μ m. Arrows indicate DRs. **(E)** Starved ILK^{-/-} cells that transiently express Y527A Src-GFP were stimulated with EGF for three minutes and immunostained for actin (red) and vinculin. Scale bars: 10 μ m. Arrows indicate DR. **(F)** Western blot analysis and **(G)** the frequency of DRs for ILK^{-/-} cells that transiently express pEGFP or Y527A-Src-pEGFP constructs. n=5 independent experiments. **(H)** The levels of active Src at FAs correlate with (compare to Fig. 2B) DR frequencies and FN coating concentrations. n=3 independent experiments. Data expressed as the mean \pm s.d. Stars indicate *P*-value for *t*-test (**P*<0.05 and ****P*<0.005).

Supplementary Fig. S5. Evaluation of knock down efficiency for selected candidate proteins from the SILAC list, using (A) quantitative RT-PCR or (B) western blotting.

Supplementary Fig. S6. Cyld is essential for DR formation. **(A)** Dynamics of DR formation in Cyld^{+/+}, Cyld^{-/-} and FLAG-Cyld rescued Cyld^{-/-} fibroblasts. n=3 independent experiments. **(B)** Localization of GFP-Cyld to DRs. Staining of F-actin (white) in Cyld^{-/-} cells that co-expressed GFP-Cyld (green) and mCherry (red). **(C)** Fluorescence intensity measured for three different regions (red line) at DR rings for actin (gray), GFP-Cyld (green) and mCherry (red). Cells with same background intensities of GFP-Cyld and mCherry were selected for further analysis. **(D)** Graph showing GFP-Cyld and mCherry intensities normalized to actin levels for 10 different cells with DR. **(E)** Rac1 activation in Cyld^{-/-} and Cyld^{+/+} cells after EGF stimulation. **(F)** Quantification of western blot intensities from Rac1 activation assay for Cyld^{-/-} and Cyld^{+/+} cells. n=3 independent experiments. Data

expressed as the mean±s.d. in panel A and D and mean±s.e.m in panel F. Stars indicate *P*-value for *t*-test (***P*<0.01 and ****P*<0.005). n.s.= not significant.

Supplementary Fig. S7. Cyld is essential for DR formation. (A) Immunostaining of Cyld (green) and Vinculin (red) in FN-seeded ILK (-/-) and ILK (f/f) cells before and after EGF-stimulation. Cyld was distributed in the cytoplasm and partially enriched at perinuclear regions in Ilk (-/-) and ILK (f/f) cells. Scale bars: are 10 μm. (B) The EGF-triggered Cyld tyrosine phosphorylation was not affected in quadruple tyrosine to alanine FLAG-Cyld mutants (4X) or in a triple mutant Cyld (3X) that contains the three N-terminal mutations of the FLAG-Cyld-9X mutant. See Supplementary material dataset S3 for mutated amino acid positions.

Supplementary dataset. S1. The list of proteins that were identified and quantified in our SILAC-based (phospho)-proteomics experiments. Peptides and proteins are listed in the sheets. The list of all detected proteins from SILAC experiments (protein groups are presented in Excel sheet). The list of identified (phospho)-proteins that showed up-regulation (>2.5 fold) or down-regulation (<0.4 fold) in ILK^{f/f} cells (_HL>2.5 or <0.4 Excel sheets) or ILK^{-/-} cells (_ML>2.5 or <0.4 Excel sheets) when cells were stimulated with EGF for 30 seconds or 2 minutes. L, M and H in tables indicate non-stimulated ILK^{f/f}, EGF-triggered ILK^{-/-} and EGF-triggered ILK^{f/f} cells respectively. H/L and M/L indicates the normalized SILAC up-regulation ratio in EGF-triggered ILK^{f/f} and ILK^{-/-} cells respectively as compared with non-stimulated ILK^{f/f} cells.

Supplementary dataset. S2. The Excel list of phospho-(Ser/Thr/Tyr)-peptides that were identified and quantified by our SILAC-based (phospho)-proteomics experiments. The identified phospho-peptides that were upregulated in ILK^{f/f} or ILK^{-/-} cells upon 30 seconds/2 minutes EGF stimulation were shown separately.

Supplementary dataset. S3. The sequence of sh/siRNAs used in this study and the position of tyrosine to alanine mutations in FLAG-Cyld mutants.

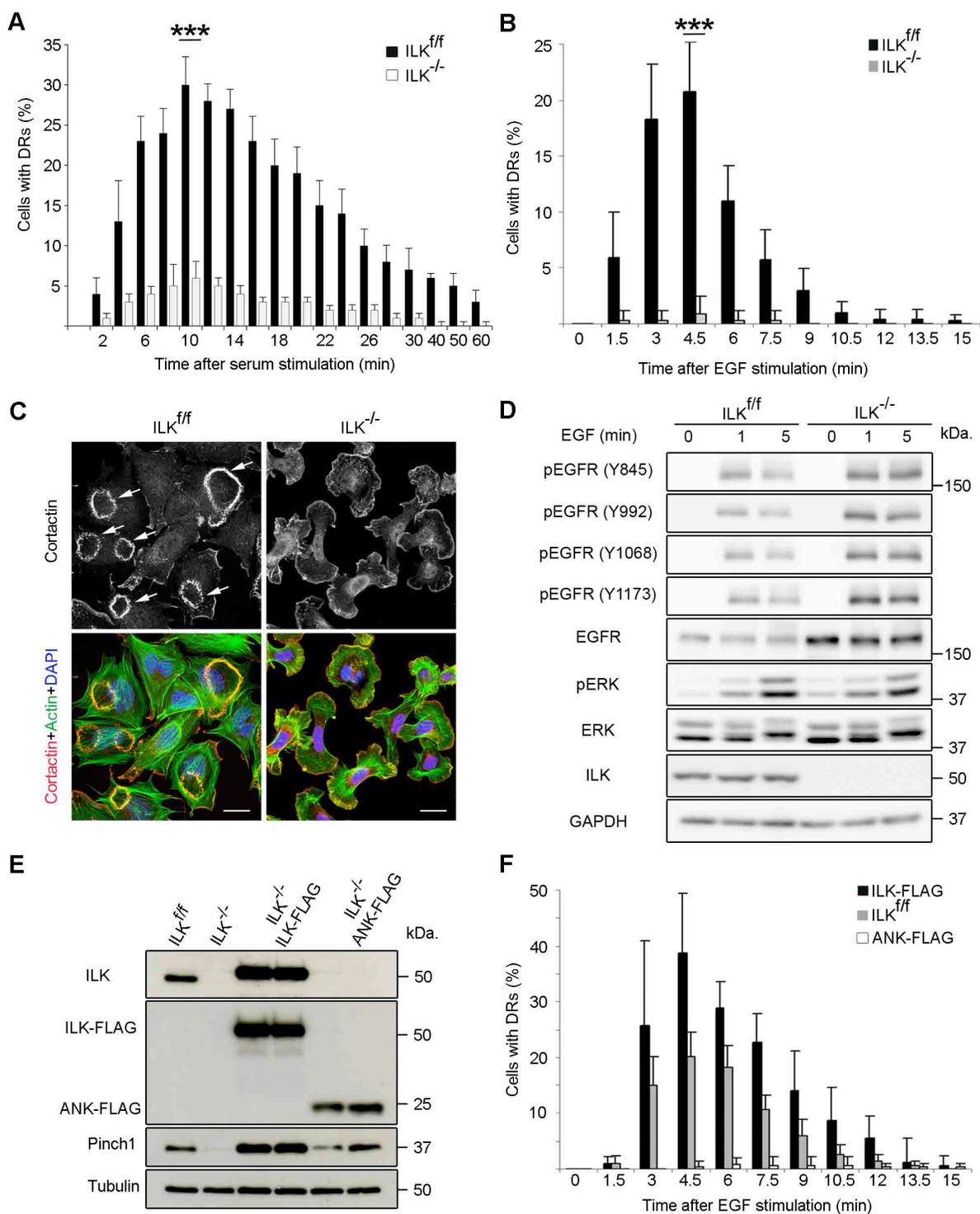


Figure 1

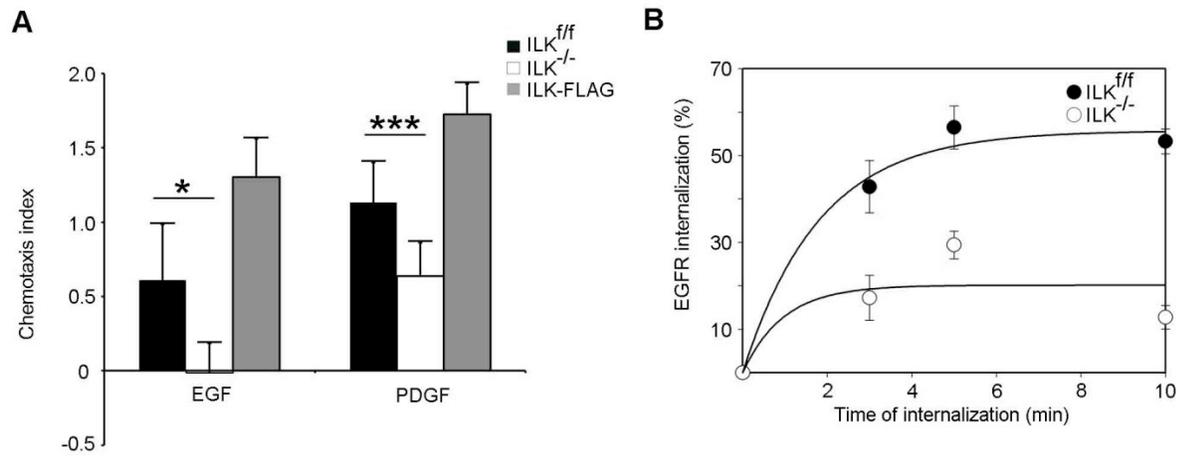


Figure 2

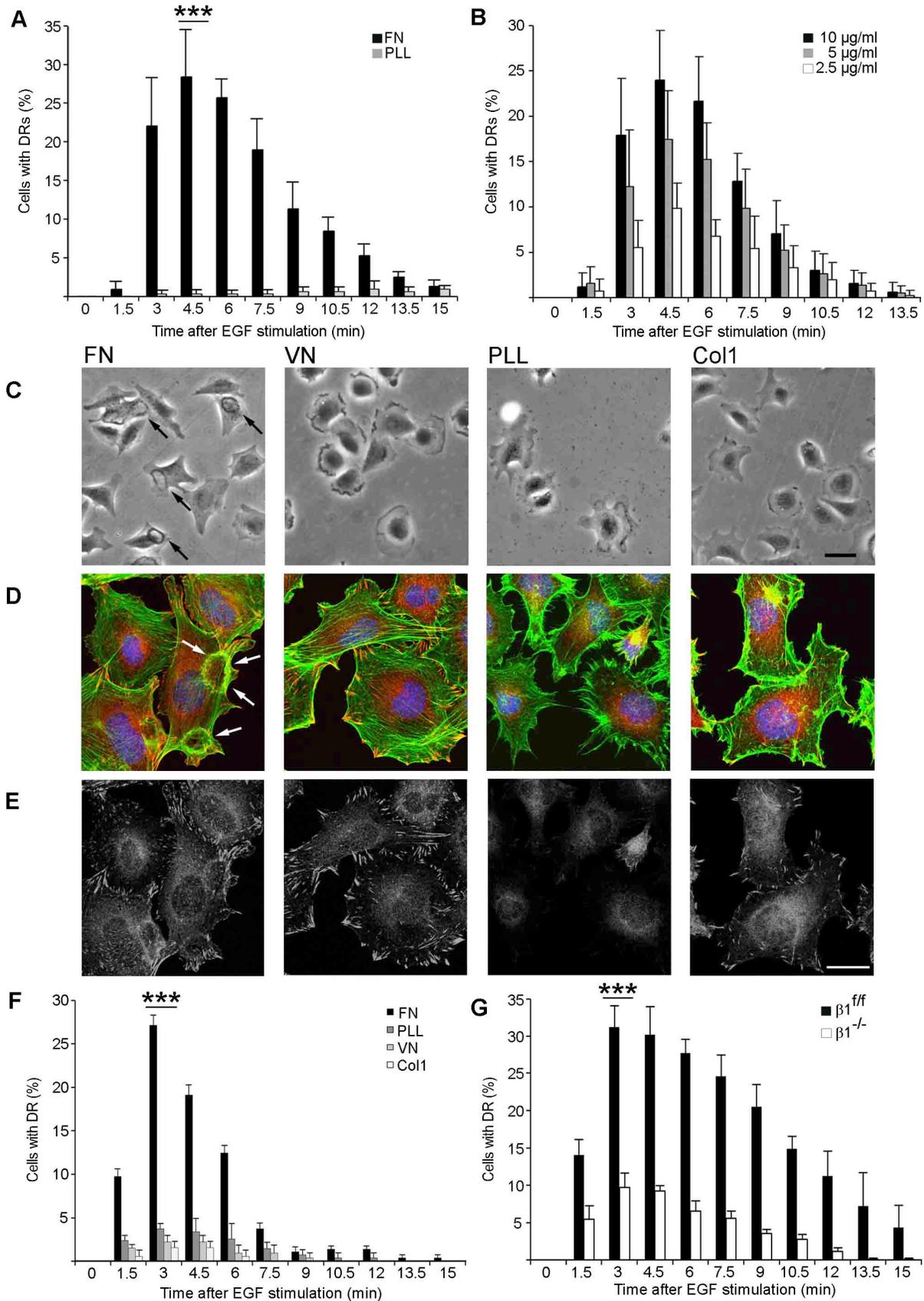


Figure 3

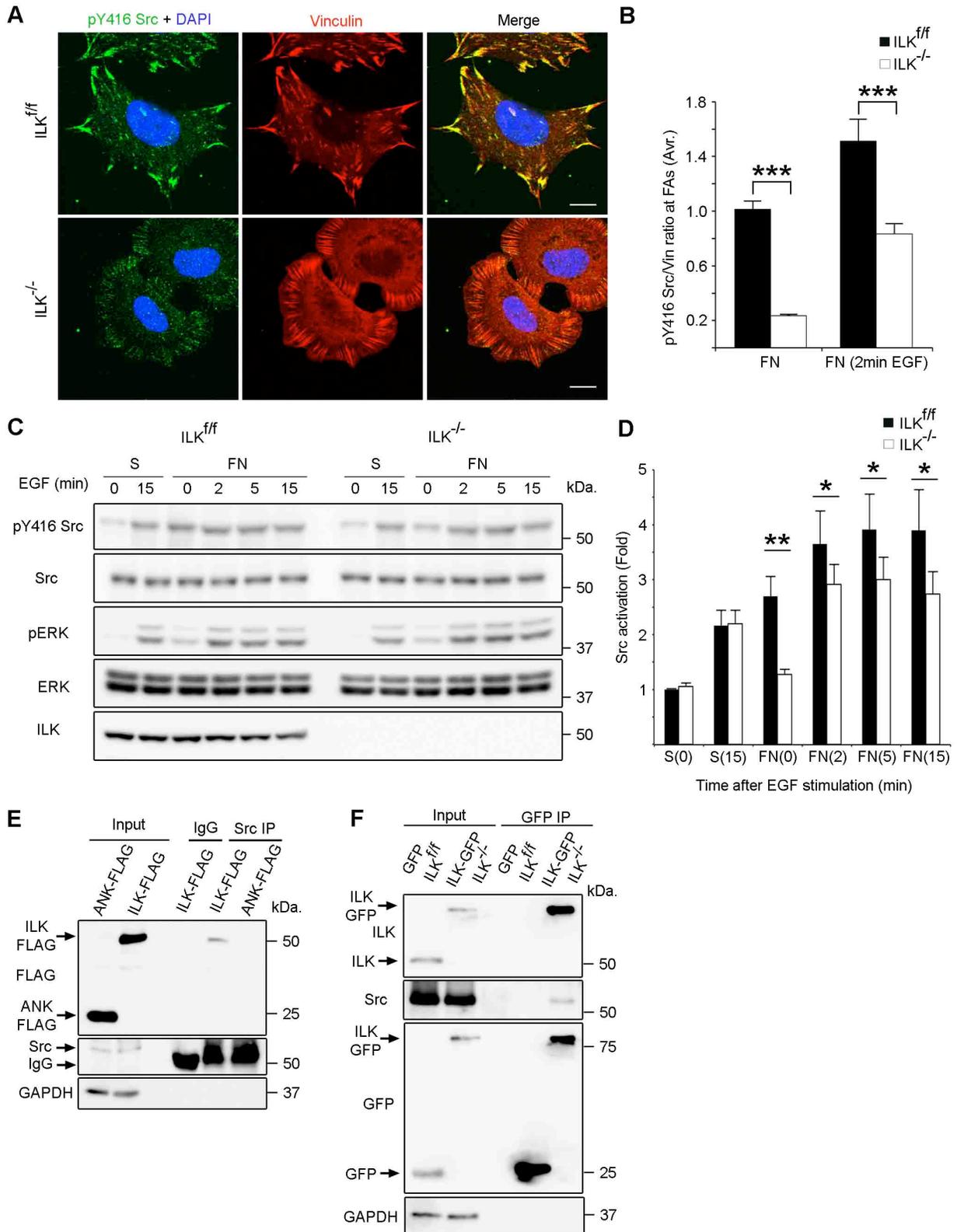


Figure 4

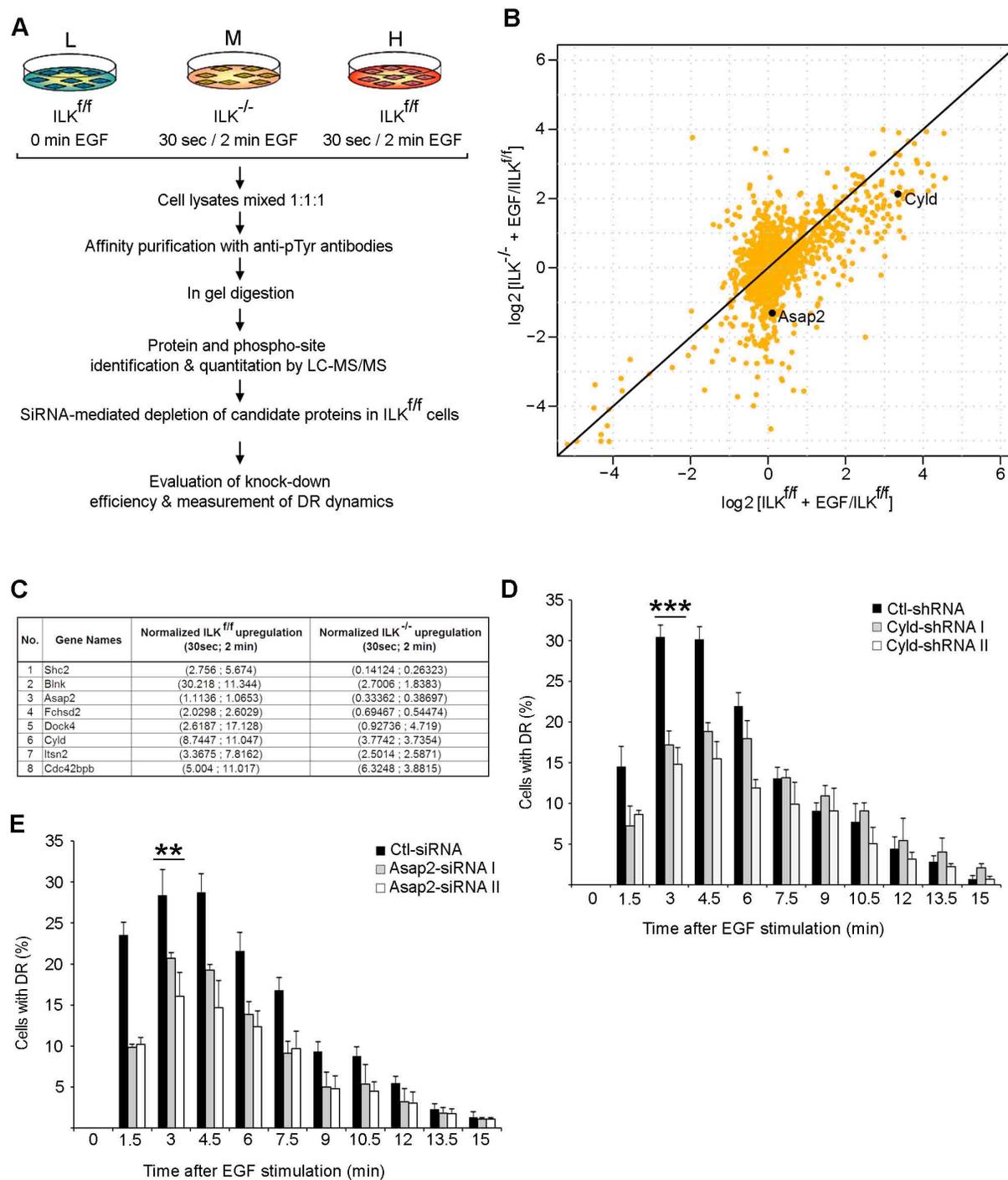


Figure 5

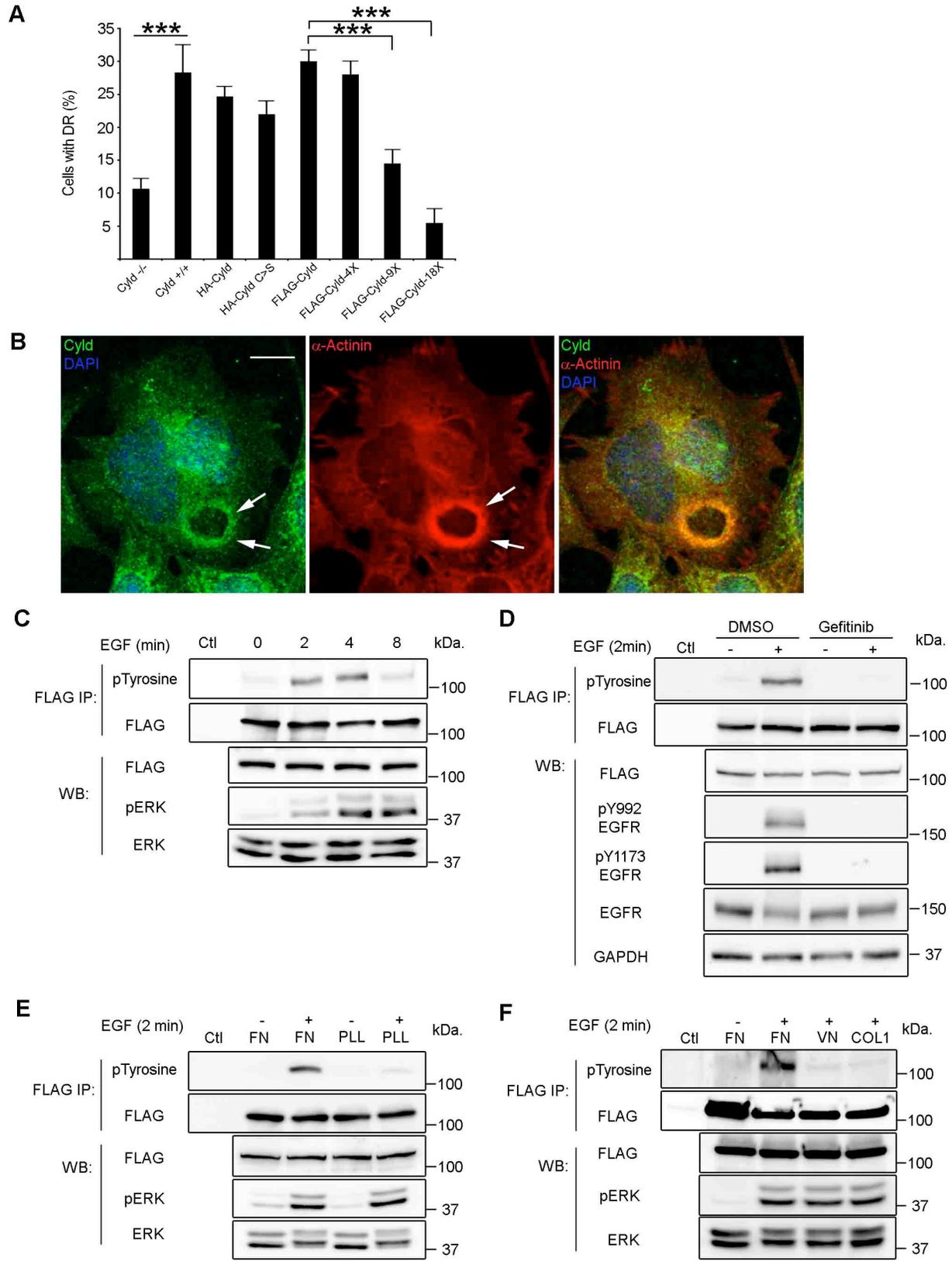


Figure 6

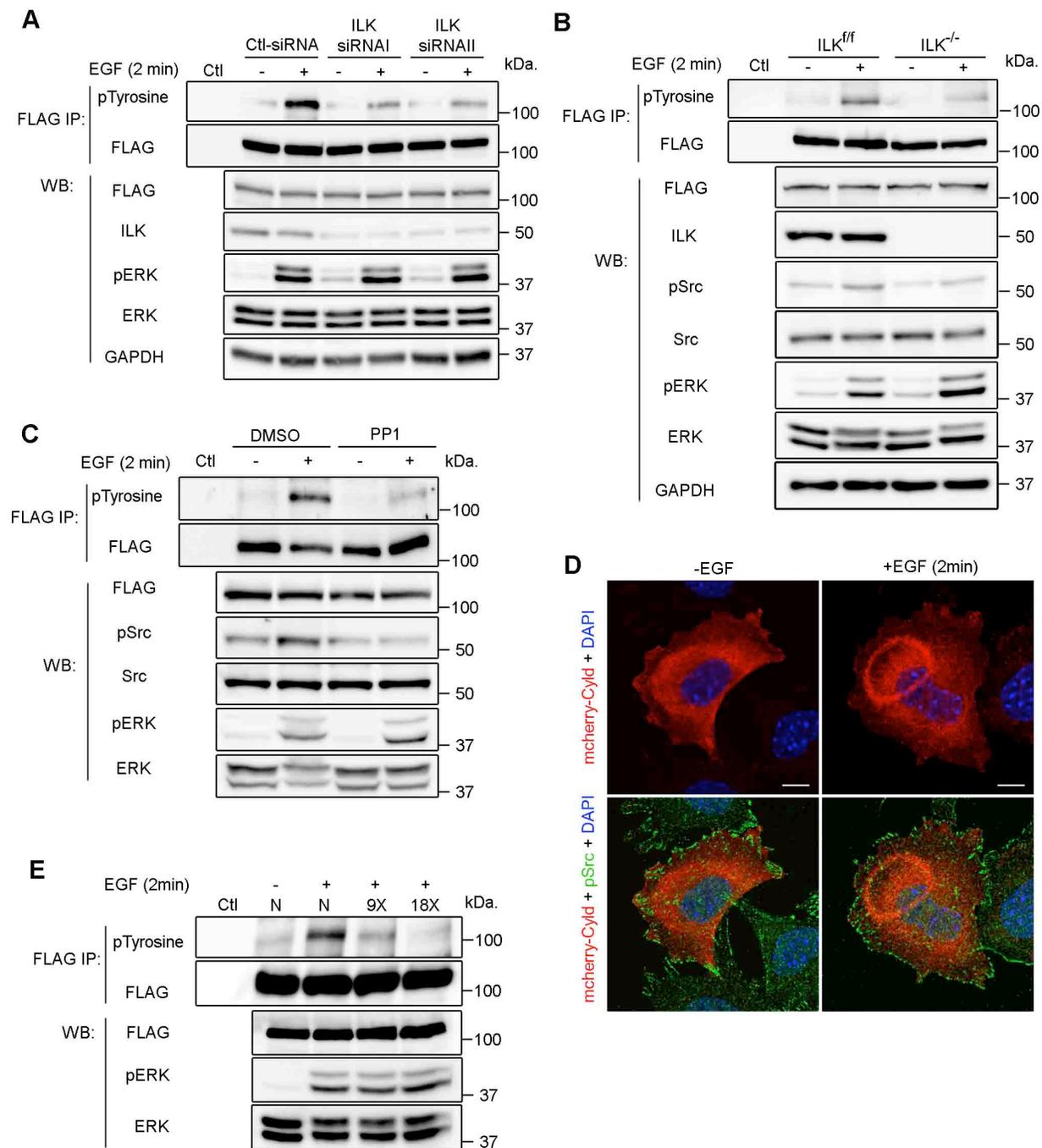
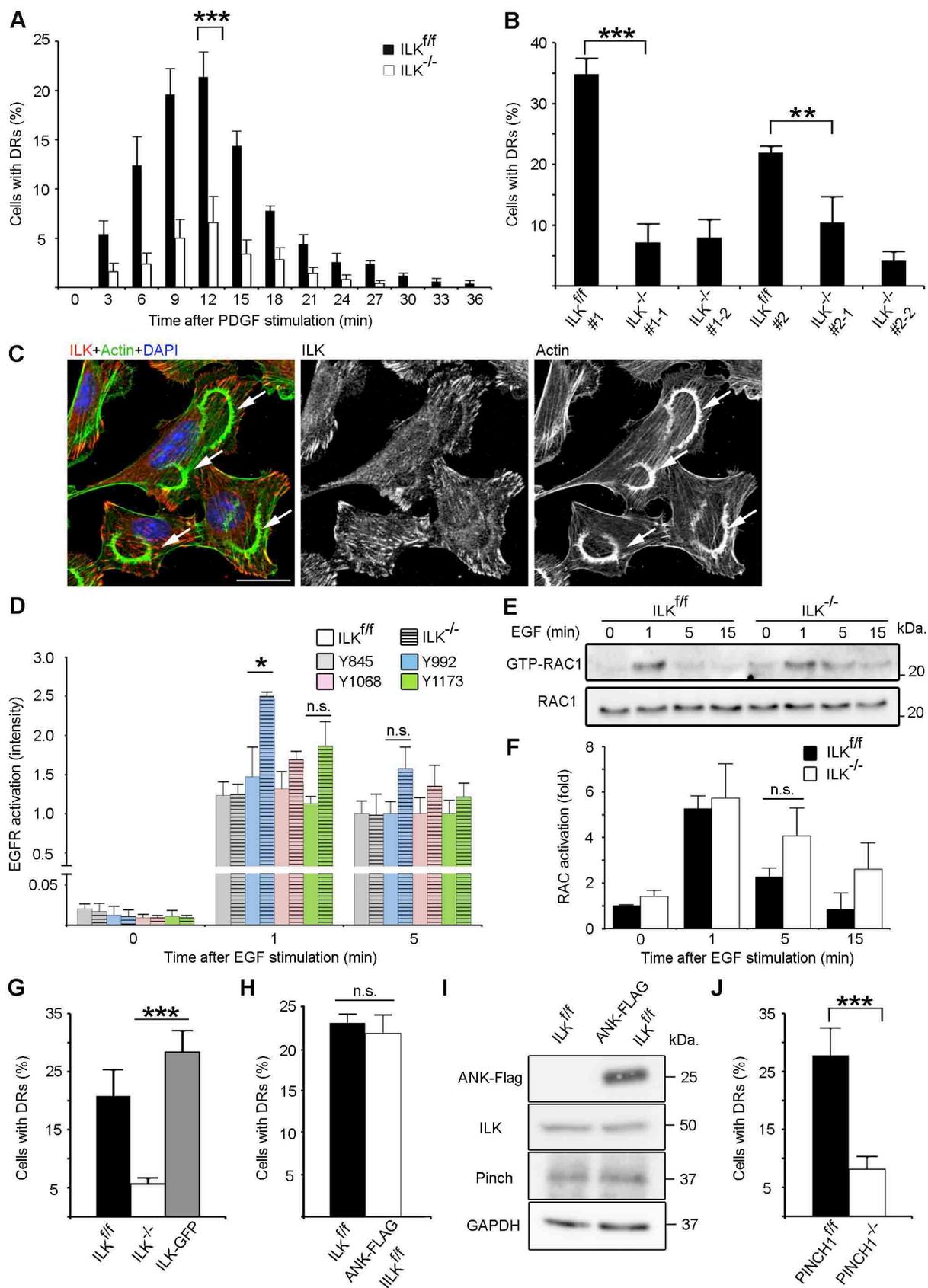
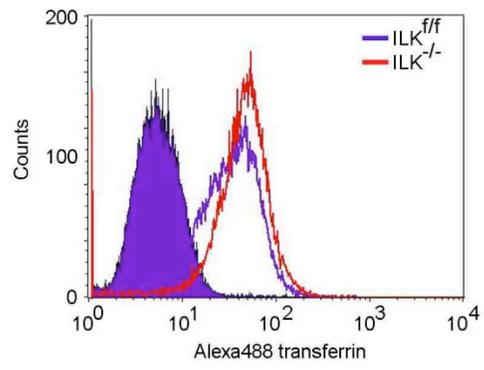


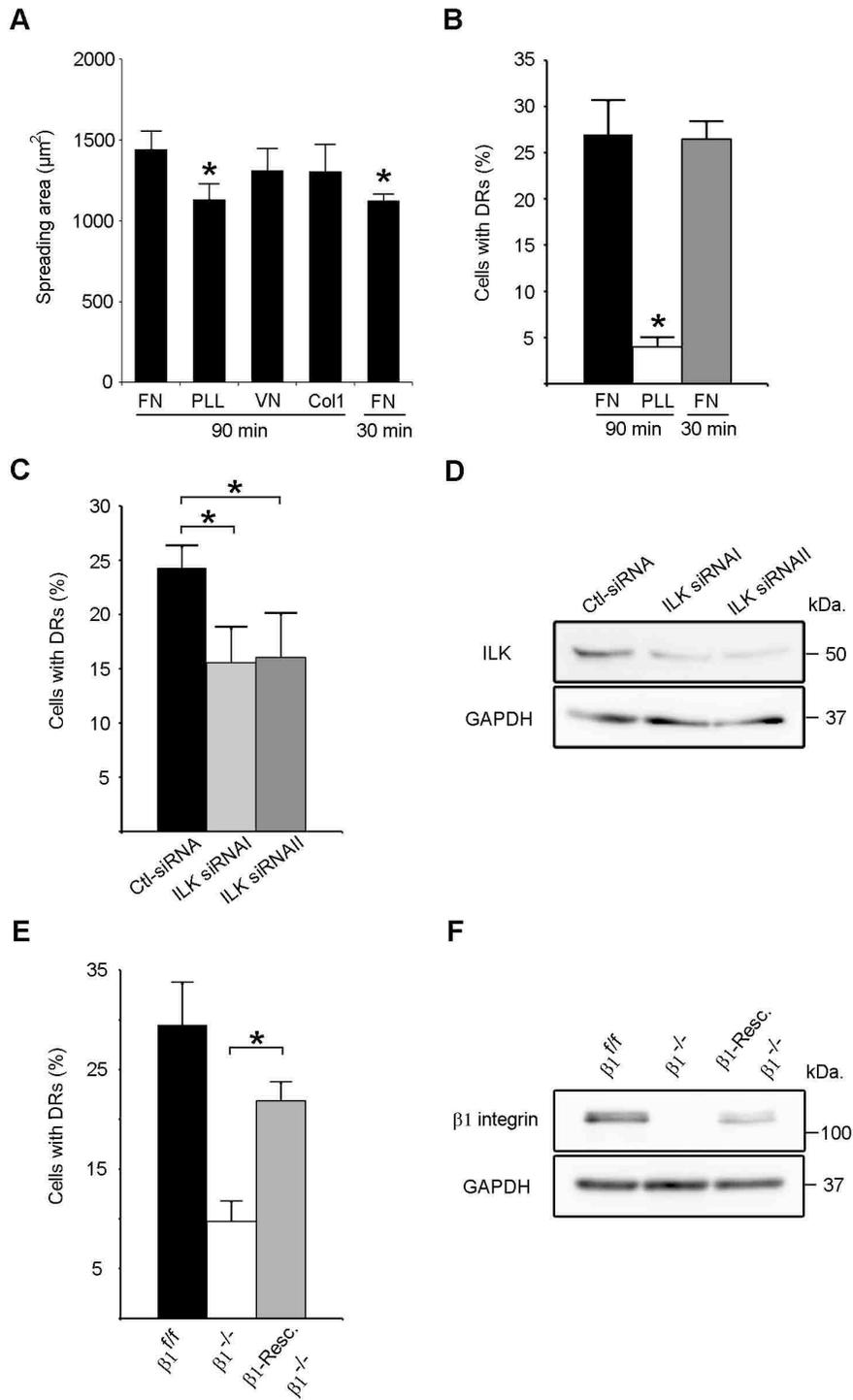
Figure 7

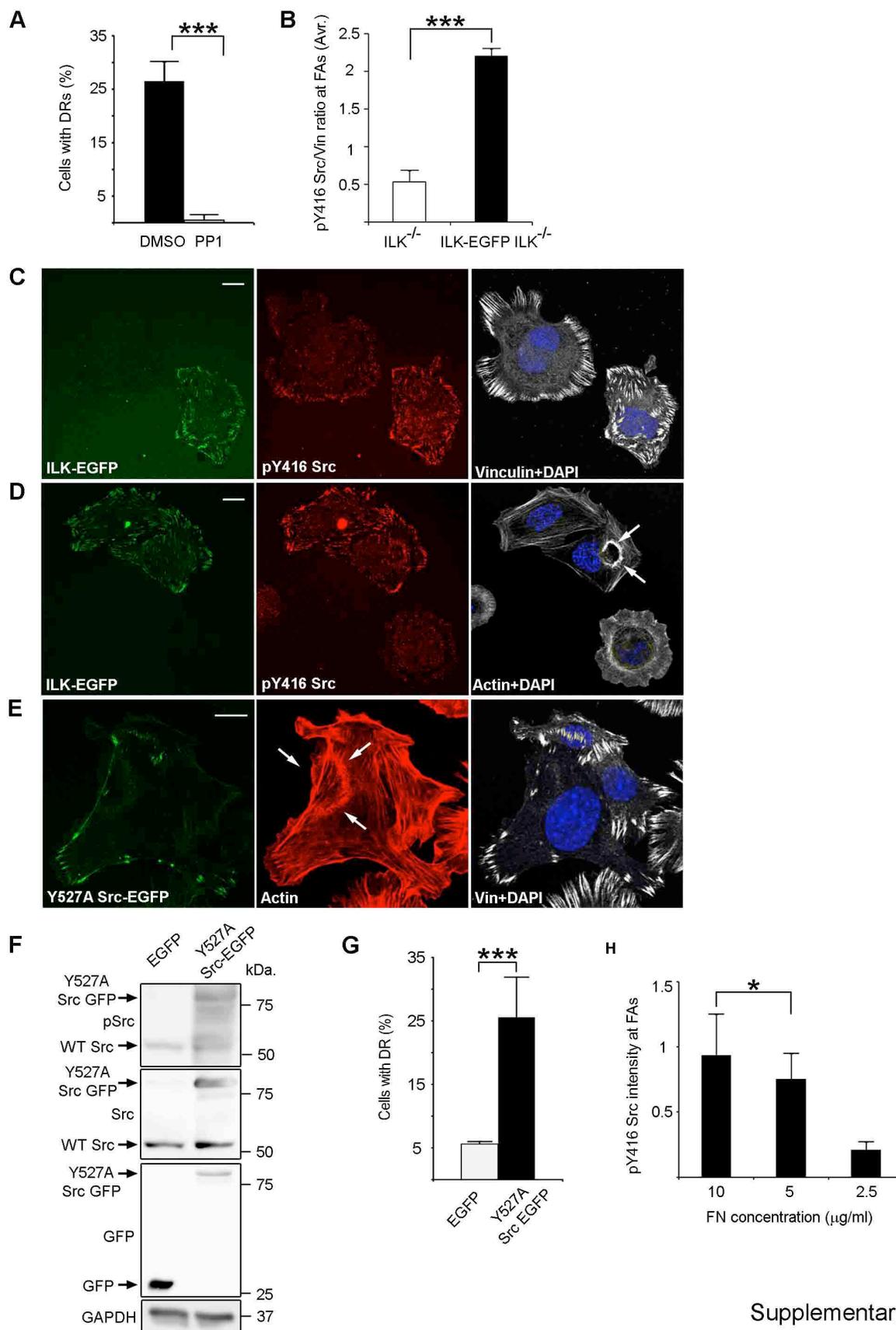


Supplementary1

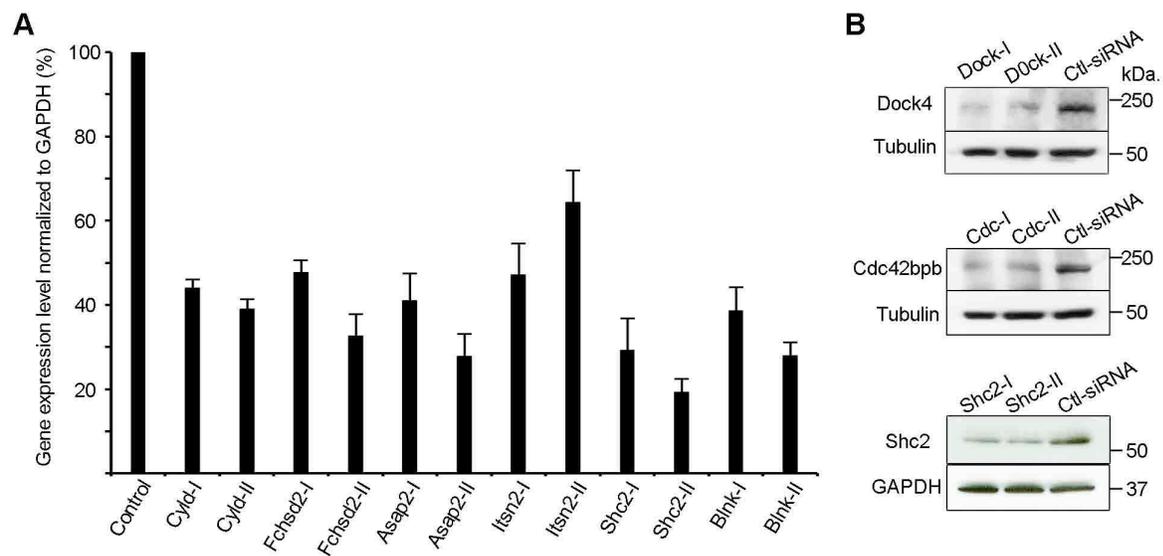
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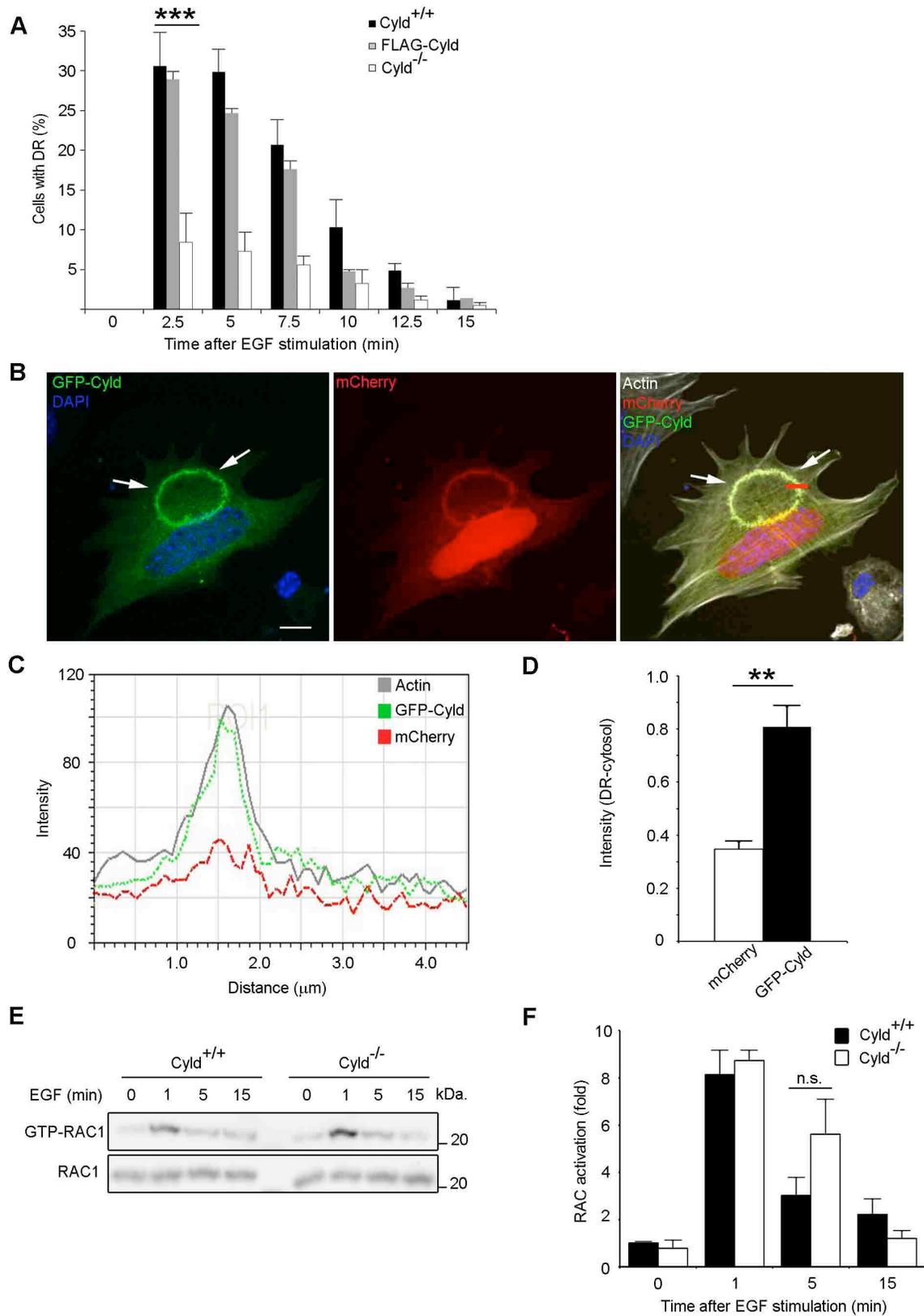


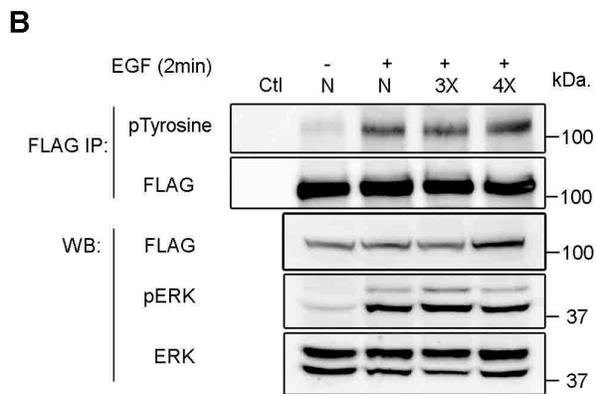
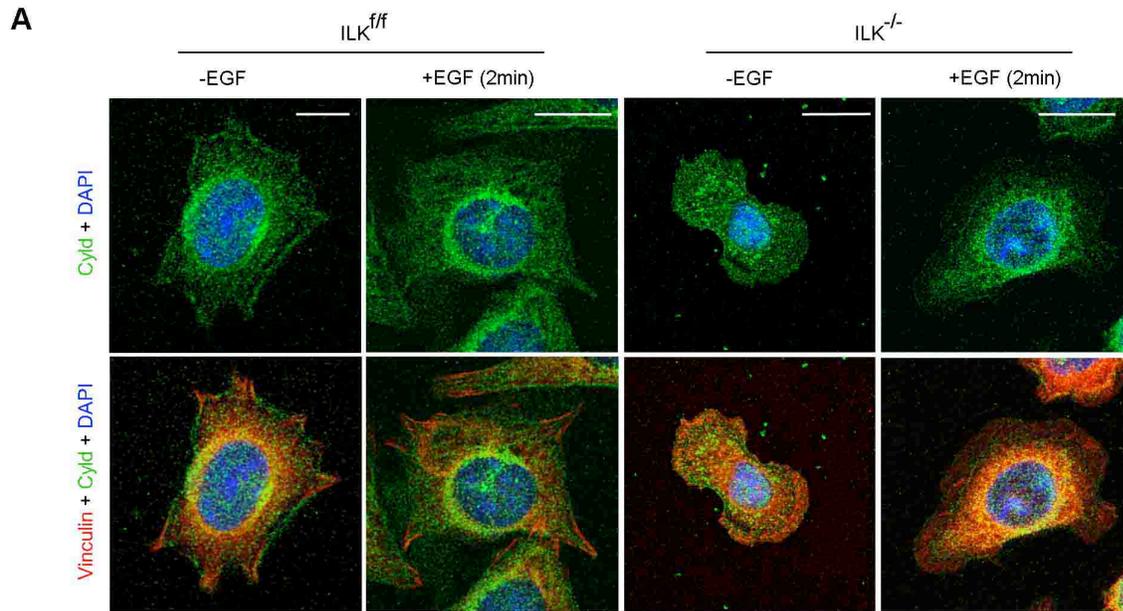




Supplementary 4







Integrin-linked kinase at a glance

Moritz Widmaier, Emanuel Rognoni, Korana Radovanac, S. Babak Azimifar, Reinhard Fässler.

Max Planck Institute of Biochemistry, Department of Molecular Medicine, Am Klopferspitz 18,
D-82152 Martinsried, Germany.

Correspondence to faessler@biochem.mpg.de

Integrins constitute a large family of cell-extracellular matrix (ECM) and cell-cell adhesion molecules that regulate development and tissue homeostasis by controlling cell migration, survival, proliferation and differentiation (Hynes, 2002). Integrins are non-covalently associated heterodimers consisting of α and β subunits. On the cell surface integrins exist in a conformation with high (active) or low (inactive) ligand affinity. Upon activation (inside-out signalling) (Moser et al., 2009), integrins cluster and form nascent focal adhesions, which eventually mature into focal adhesions (FAs) (outside-in signalling) (Legate et al., 2009; Geiger and Yamada, 2011). Major functions of integrins in FAs include their ability to link the ECM to the actin cytoskeleton and to fine tune growth factor receptor signalling (Legate et al., 2009).

Since integrins lack intrinsic enzymatic activity, their signalling critically depends on recruiting adaptor and signalling proteins (Schiller et al., 2011). One of the best described of these proteins is integrin-linked kinase (ILK), which is directly recruited to activated $\beta 1$ and $\beta 3$ integrin cytoplasmic domains. Since the discovery of ILK 15 years ago (Hannigan et al., 1996), ILK has been shown to play crucial roles in actin rearrangement, cell polarisation, spreading, migration, proliferation and survival (Legate et al., 2006). Despite its predominant localisation in FAs, ILK was also shown to reside in cell-cell adhesion sites, in centrosomes and in the nucleus. Here we summarise the functional properties of ILK and highlight the recent evidence demonstrating that ILK serves as a scaffold protein rather than a kinase.

ILK and the Pinch-Parvin complex

In vivo studies revealed that ILK is a ubiquitously expressed protein, whose predominant function is to organise the actin cytoskeleton during invertebrate and vertebrate development and homeostasis. In *Caenorhabditis elegans*, the ILK ortholog PAT4 localises to integrins at muscle attachment sites. Deletion of the PAT4 gene causes a 'paralysed at two-fold-stage' (PAT) phenotype that is characterised by muscle detachment through defective integrin-actin linkage and very early lethality (Mackinnon et al., 2002). In *Drosophila melanogaster*, a germline deletion of ILK leads to muscle detachment and lethality (Zervas et al., 2001). Mice lacking ILK die during the peri-implantation stage due to a failure to organise the F-actin cytoskeleton in epiblast cells (Sakai et al., 2003). In addition to the constitutive deletion, the *ILK* gene has been deleted in several organs and cell types using the Cre/loxP system. The outcome of these studies has been extensively reviewed elsewhere (Rooney and Streuli, 2011; Ho and Bendeck, 2009; Hannigan et al., 2007; Wickström et al., 2010b).

Structurally, ILK is comprised of three different domains: five ankyrin repeats at the N-terminus followed by a pleckstrin homology (PH)-like domain and a kinase-like domain at the C-terminus (Chiswell et al., 2008; Yang et al., 2009) (see poster). Although ILK was shown to directly interact with integrin cytoplasmic tails, it appears that the recruitment of ILK to integrins may depend, at least in some cells, on Kindlin-2 (Montanez et al., 2008; Chen et al., 2008), α -Parvin (Fukuda et al., 2009) or Paxillin (Nikopoulos et al., 2001). Structural studies of ILK revealed, however, that the proposed Paxillin interacting residues are buried within a polypeptide fold and thus are not directly accessible (Fukuda et al., 2009), arguing that these residues indirectly contribute to Paxillin binding. Prior to the recruitment of ILK to FAs, ILK forms a ternary complex with the two adaptor proteins Pinch and Parvin (termed the IPP complex). Although it is not understood how the IPP complex forms, its formation ensures stability of the individual components and faithful targeting to the adhesion site (Zhang et al., 2002; Fukuda et al., 2003a). Mammals have two Pinch genes (Pinch-1 and Pinch-2), which encode proteins consisting of five cysteine-rich, zinc binding LIM domains followed by a nuclear export signal. The first LIM domain of Pinch-1 and -2 binds to a concave surface that extends from the second to the fifth ankyrin repeat of ILK (Chiswell et al., 2008; Yang et al., 2009) (see poster). The three mammalian Parvin isoforms (α -, β - and γ -Parvin) are comprised of an N-terminal polypeptide followed by two calponin homology (CH) domains, of which the second one binds to the kinase-like domain of ILK (Tu et al., 2001; Fukuda et al., 2009) (see poster). As ILK can only bind one Pinch and one Parvin isoform at the same time (Chiswell et al., 2008; Montanez et al., 2009), ILK is capable of forming several distinct IPP complexes, each resulting in different signalling outputs (see poster and below).

The Parvins interact directly with F-actin (Legate et al., 2006) or recruit actin binding proteins such as α -Actinin [shown for β -Parvin (Yamaji et al., 2004)] or Vinculin via Paxillin (Turner, 2000) [shown for α - and γ -Parvin (Yoshimi et al., 2006)]. In addition, they control actin regulatory proteins such as testicular protein kinase 1 (TESK-1), which can bind α -Parvin and promote F-actin polymerisation through phosphorylation of Cofilin (LaLonde et al., 2005). In contrast, β -Parvin regulates actin dynamics through PAK-interactive exchange factor (α -PIX), a guanidine exchange factor (GEF) for Ras-related C3 botulinum toxin substrate 1 (Rac1) and cell division cycle 42 (Cdc42) (Mishima et al., 2004). Finally, α -Parvin was shown to inhibit G-proteins by recruiting Cdc42 GTPase-activating protein (CdGAP) to FAs (LaLonde et al., 2006) and to negatively regulate RhoA- and Rho-associated protein kinase (ROCK)-driven contractility in vascular smooth muscle cells (Montanez et al., 2009).

Pinch-1 binds the Ras suppressor protein 1 (RSU1), which is important for integrin-mediated cell adhesion and spreading (Kadmas et al., 2004; Ito et al., 2010). RSU1 is a negative regulator of growth factor-induced Jun N-terminal kinase 1 (JNK1) (Kadmas et al., 2004). Together these findings suggest that the assembly of distinct IPP complexes in a given cell together with the differential expression patterns of Pinch and Parvin isoforms provides a means for multiple alternative signalling outputs (see poster).

Emerging functions of ILK

The most prominent subcellular localisation of ILK is in integrin adhesion sites. In the past years it has been reported that ILK is also present in additional subcellular regions and compartments where it may exert integrin-independent functions.

MT trafficking networks

Keratinocytes and likely also other cells employ ILK to capture microtubule (MT) tips to connect them to the cortical actin network (see poster). ILK-mediated MT capture occurs exclusively in nascent FAs and is mediated by recruitment of the large scaffold protein IQ motif containing GTPase activating protein 1 (IQGAP-1) (Wickström et al., 2010a). The capture of MT tips can be achieved either directly via binding of IQGAP-1 to the MT tip protein cytoplasmic linker protein CLIP170, or indirectly via IQGAP-1-mediated recruitment of murine diaphanous homolog 1 (mDia1; Diaphanous-related formin-1 in human) which is also able to stabilise MTs. As both IQGAP-1 and mDia1 are also able to bind F-actin, the ILK-IQGAP-1-mDia1 complex connects MTs with actin tracks at β 1 integrin-containing nascent adhesion sites (Wickström et al., 2010a) (see poster). Exocytotic carriers that are transported via MT tracks require a switch from MT-based to actin-based motility at the plasma membrane to overcome the cortical F-actin network. Thus, the connection of both networks by the ILK-IQGAP-1-mDia1 complex at nascent adhesion sites is essential for the exocytosis of caveolar carriers (Wickström et al., 2010a). Consequently, ILK not only contributes to epithelial cell polarisation through actin remodeling, but also through vesicular trafficking and MT organisation.

Nuclear functions

Despite its prominent localisation in different integrin adhesion sites, ILK has also been observed in the nucleus of several cell lines, including COS-1 cells (Chun et al., 2005), MCF-7 cells (Acconcia et al., 2007), HeLa cells and keratinocytes (Nakrieko et al., 2008a) (see poster). The nuclear

function of ILK, however, is still not well understood. In keratinocytes, nuclear ILK was shown to induce DNA synthesis (Nakrieko et al., 2008a) and in MCF-7 cells it was found to control the expression of the connector enhancer of kinase suppressor of Ras 3 (*CNKSR3*) gene (Acconcia et al., 2007). *CNKSR3* was shown to regulate the epithelial Na⁺ (ENaC) channel through inhibition of MAP and Erk Kinase 1 (Mek1) (Ziera et al., 2009). However, the significance of ILK regulated *CNKSR3* expression is not understood.

It is also not well understood how ILK translocates into the nucleus. It is not known whether the nuclear import of ILK depends on its N-terminus (Acconcia et al., 2007) or on a C-terminal nuclear localisation signal (Chun et al., 2005). The nuclear export of ILK requires the kinase-like domain (Acconcia et al., 2007; Nakrieko et al., 2008a) and is apparently controlled by the nuclear export factor chromosome region maintenance 1 protein homolog (CRM1), the integrin-linked kinase-associated serine- and threonine phosphatase 2C (ILKAP) and p21 protein (Cdc42/Rac)-activated kinase 1 (PAK1) (Acconcia et al., 2007; Nakrieko et al., 2008a).

Cell-cell contacts organisation

ILK has been shown to serve as a scaffold for promoting the formation of cell-cell contacts (see poster) and the recruitment of tight junction proteins (Vespa et al., 2003; Vespa et al., 2005). Following treatment of cultured keratinocytes with Ca²⁺, they undergo differentiation. This process is accompanied by the translocation of ILK from FAs to cell-cell adhesion sites (Vespa et al., 2003). This translocation requires the N-terminal ankyrin repeats (Vespa et al., 2003), however it is unclear whether Pinch-1 or -2 translocate together with ILK. In contrast to these *in vitro* findings, deletion of the ILK gene in keratinocytes of mice neither affects cell-cell adhesion nor barrier function in the epidermis, but severely impairs their migration on and adhesion to the epidermal-dermal BM, resulting in skin blistering, epidermal hyperthickening and hair loss. (Lorenz et al., 2007; Nakrieko et al., 2008b).

Centrosome functions

A proteomic search for novel ILK interacting proteins identified a number of proteins including several centrosome- and mitotic spindle-associated proteins, such as α -, β -tubulin and the tubulin-binding proteins RUVB like protein 1 (RUVBL1) and colonic and hepatic tumor over-expressed gene protein (ch-TOG) (Dobrev et al., 2008) (see poster). Although ILK probably binds these proteins in an indirect manner (Fielding et al., 2008), it co-localises with them in centrosomes from interphase and mitotic cells where it plays an essential role in controlling centrosome function during mitotic

spindle organisation and centrosome clustering (Fielding et al., 2008; Fielding et al., 2011). The organisation of the mitotic spindle requires Aurora A kinase and the association of ch-TOG with the centrosomal protein TACC3 (transforming acidic coiled-coil-containing protein 3), which in turn promotes the polymerisation and stabilisation of centrosomal microtubules (Barr et al., 2007). In ILK-depleted cells, Aurora A kinase, although active, is unable to phosphorylate and thus activate TACC3 resulting in disrupted mitotic spindles. Similarly, the clustering of supernumerary centrosomes in cancer cells is also achieved by the TACC3-ch-TOG complex in an ILK- and Aurora A-dependent manner (Fielding et al., 2011). ILK associates with ch-TOG, but neither with TACC3 nor with Aurora A. Therefore, it is not clear how ILK supports phosphorylation of TACC3 by Aurora A kinase. Similarly, it is also unclear how ILK is recruited to centrosomes. The centrosomal localisation of ILK requires RUVBL1 expression and occurs without the known ILK binding partners, α -Parvin and Pinch (Fielding et al., 2008). Finally, it is also not known why the treatment of cells with QLT-0267, a small chemical compound that binds to the ATP binding site of ILK, is as effective as siRNA-mediated depletion of ILK in blocking the association of TACC3 with Aurora A (Fielding et al., 2008). The mechanistic interpretation of this work is based on the assumption that ILK acts as a kinase, which has been disproved by genetic and structural studies (see below). A potential explanation for the inhibitory effect of QLT-0267 could be an impairment of the stability of ILK (see end of next paragraph).

The kinase controversy

The experimental evidence that the ILK kinase-like domain lacks catalytic activity is overwhelming (Wickström et al., 2010b). Although ILK was initially identified by Dedhar and colleagues as a serine- and threonine-kinase (Hannigan et al., 1996), it lacks several important motifs that are conserved in most kinases (Hanks et al., 1988) (see poster). Furthermore, genetic studies in flies, worms and mice demonstrated that the putative kinase activity is not required for development and homeostasis (Zervas et al., 2001; Mackinnon et al., 2002; Lange et al., 2009). Despite this compelling evidence, many papers have been and are still published claiming that ILK is a *bone fide* kinase, with only marginal evidence at best.

The crystallisation of the ILK kinase-like domain in complex with the CH domain of α -Parvin and its comparison to the kinase domain of protein kinase A (PKA) provided a mechanistic explanation for why its kinase function is not executed (Fukuda et al., 2009). The catalytic activity of a kinase depends on a coordinated interplay of the N- and C-lobes and the catalytic loop of the kinase domain with the substrate and ATP. The N- and C-lobes and the catalytic loop of ILK show major

differences to *bona fide* kinases that render the 'kinase' of ILK non functional. The catalytic loop lacks important acidic and positively charged residues. The acidic residue (D166 in PKA), which polarises the hydroxyl group of the substrate and accepts its proton, is substituted in ILK with the uncharged alanine residue (A319) (Fukuda et al., 2009). The positively charged residue in catalytic loops (K168 in PKA), which stabilises the intermediate state of the phospho-transfer reaction by neutralizing the negative charge of the γ -ATP phosphoryl-group, is replaced in ILK by N321 resulting in a misrouting of ATP to the C-lobe. In the N-lobe, the ATP binding p-loop captures ATP at a too great distance from the active center (10 Å), which precludes its movement towards the catalytic loop; and the lysine residue K220 contacts the α - and γ -ATP phosphoryl-groups instead of the α - and β -phosphoryl-groups resulting in an aberrant ATP orientation. Furthermore, the C-lobe of ILK chelates ATP with only one instead of the expected two metal ions. The metal ion is bound by the aspartate (D339) of the DVK motif of ILK (DFG-motif in PKA), whereas the second potential metal binding residue (S324) remains unoccupied. Another divergence from *bona fide* kinases is the coordination of the γ -ATP phosphoryl-group by the lysine (K341) of the DVK motif, which usually occurs by the catalytic loop (Fukuda et al., 2009). Nevertheless, despite this structural evidence, dissenting views are still expressed and the controversy rages on (Hannigan et al., 2011). Thermodynamic and structural analysis of ILK mutants revealed that K220A or K220M mutations, previously described to affect kinase function, destabilise the global ILK structure (Fukuda et al., 2011), thus reducing ILK stability and binding of interaction partners such as α -Parvin. This observation provides an explanation for the severe kidney defects observed in mice which either lack α -Parvin expression or carry K220A or K220M mutations in ILK (Lange et al., 2009).

Redefining the role of ILK in cancer

ILK is overexpressed in many types of cancer, and its depletion or inhibition with the small molecule inhibitor QLT-0267 was reported to inhibit anchorage-independent growth, cell cycle progression and invasion (Hannigan et al., 2005). The oncogenic effects of ILK have been attributed for the most part to the catalytic activity of the kinase domain resulting in the activation of protein kinase B (PKB), also known as proto-oncogene c-Akt (Akt), and glycogen synthase kinase-3 beta (GSK3 β) which in turn regulates the stability of proto-oncogenic β -Catenin (Hannigan et al., 2005). The recent findings that mammalian ILK lacks catalytic activity and serves as a scaffold protein in FAs of mammalian cells (Lange et al., 2009; Fukuda et al., 2009) raise the question of how ILK mediates its oncogenic potential despite this functional twist.

One possibility is that ILK controls the activity of oncogenes, such as PKB/Akt by controlling their subcellular localisation. For example, the ILK binding partners α - and β -Parvin promote the recruitment of PKB/Akt to the plasma membrane where the kinase mediates its oncogenic activity (Fukuda et al., 2003b; Kimura et al., 2010). An alternative possibility is that ILK and ILK-interacting protein(s) regulate oncogenic kinases by controlling the activity of phosphatases. This has been shown for Pinch-1, which binds to and inhibits the protein phosphatase 1 α (PP1 α) resulting in sustained PKB/Akt phosphorylation and activity (Eke et al., 2010). Consequently, reducing the amounts of the IPP complex in FAs will concomitantly result in an increased PP1 α activity and decreased PKB/Akt function. It is also conceivable that ILK exerts its oncogenic function by its ability to cluster supernumerary centrosomes in cancer cells, which prevents their genomic instability and death (Fielding et al., 2011). Finally, ILK may also promote oncogenesis by regulating gene expression in the nucleus, or by modulating the assembly of ECM proteins, as shown for fibronectin (Wu et al., 1998), which has been reported to affect cancer development and invasion (Akiyama et al., 1995).

Outlook and perspectives

ILK research has significantly advanced in the past years by settling the long-lasting debate regarding the catalytic activity of ILK and by identifying novel functions for ILK, many of which occur outside of FAs. Most of the emerging functions of ILK (e.g. in the nucleus, at cell-cell adhesion sites, in the centrosome) have only been studied in cultured cells thus far and still await confirmation *in vivo*.

In addition, several basic functions of ILK in FAs are still open, including the mechanism(s) of the recruitment of ILK to FAs, ILK's role in FA maturation and as a potential stretch sensor (Bendig et al., 2006), the turn-over and modifications of ILK, to name a few. It is currently debated whether the recruitment of ILK to FAs occurs through a direct association with the integrin cytoplasmic domains or indirectly, e.g. through binding to Kindlins (Montanez et al., 2008) or Paxillin (Nikopoulos et al., 2001). In this regard it is also not known whether ILK binds or associates with all β integrin tails, or is more selective. A co-crystallisation of the kinase-like domain of ILK with β integrin tails should help to answer some of these questions. Similarly, a structural analysis of the predicted PH domain of ILK would clarify whether it adopts a classical PH fold as predicted in the original publication (Hannigan et al., 1996), or a different motif whose function would then have to be determined. Zebrafish studies point to a stretch sensing function of ILK in cardiomyocytes (Bendig et al., 2006). This observation raises the question of whether mechanical stress sensing by

ILK is restricted to cardiomyocytes or also occurs in other cells, and how ILK is executing this function at the molecular level. Finally, it will be important to re-evaluate the role of ILK in cancer. Ideally, these experiments are performed in an unbiased manner with tumor models in mice (e.g. colon cancer and mammary cancer models) that lack ILK expression, and are complemented by sophisticated *in vitro* studies with cells derived from the tumors.

It is obvious that despite the rapid progress in ILK research many questions are still unanswered. Recent advances in imaging and proteomics combined with genetics, cell biology and biochemistry will make the years to come exciting for all ILK aficionados.

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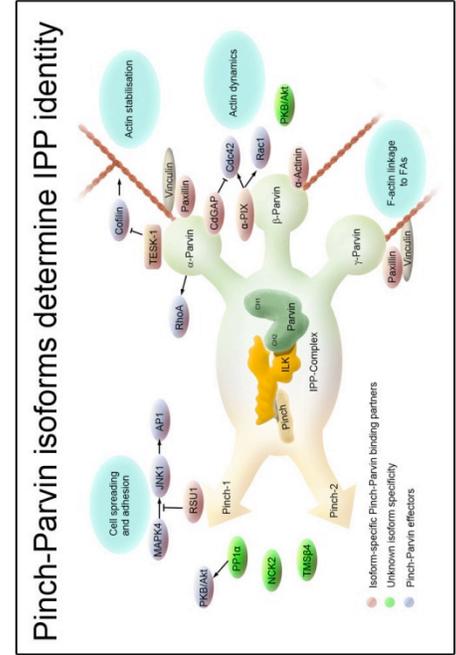
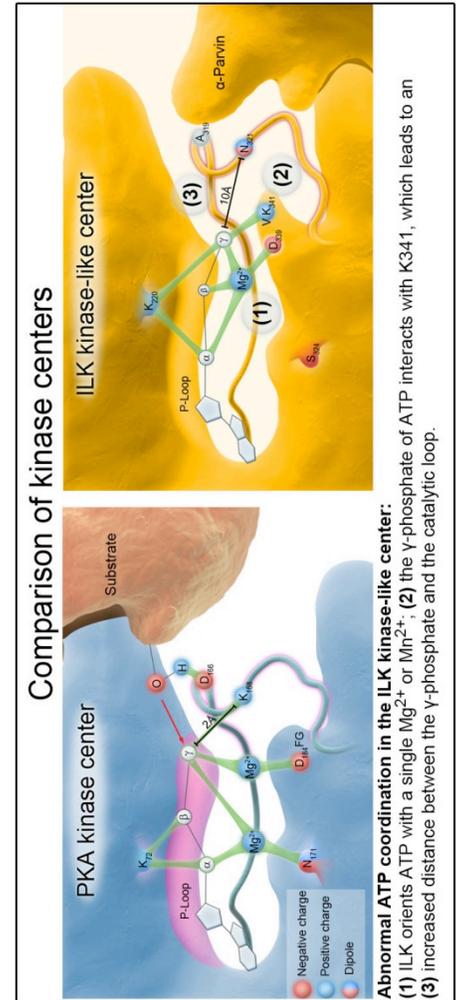
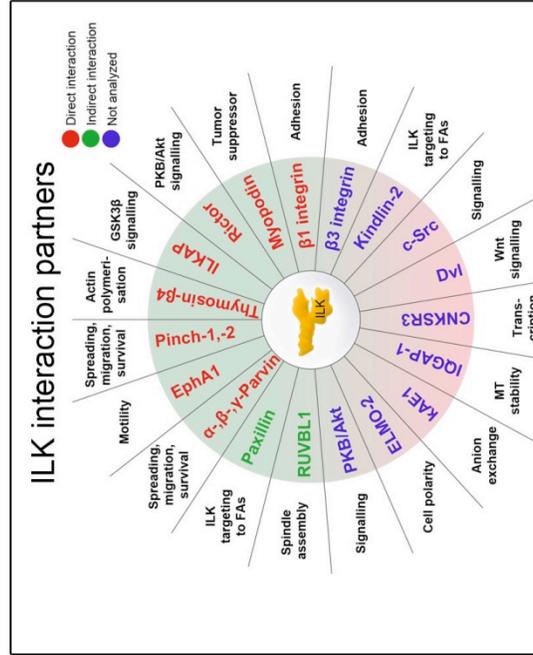
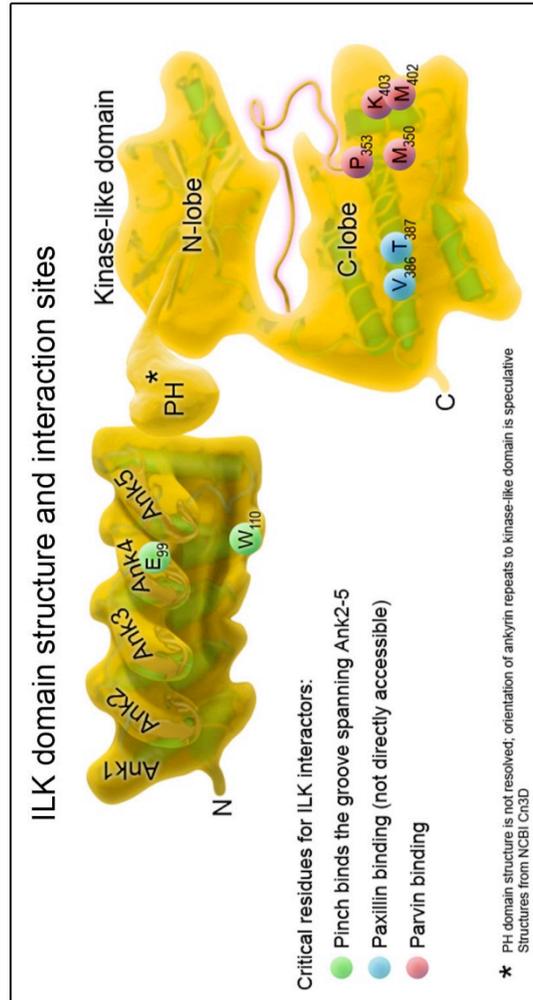
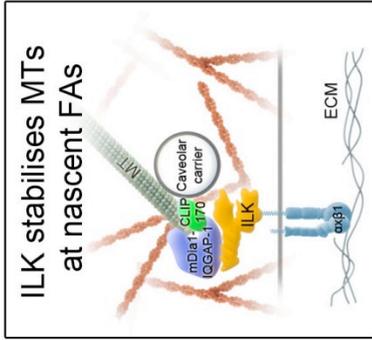
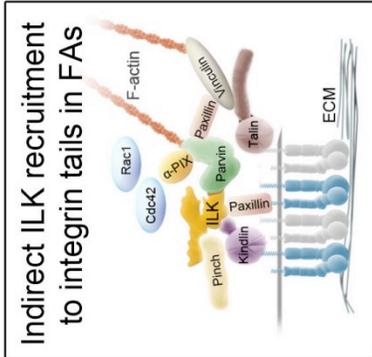
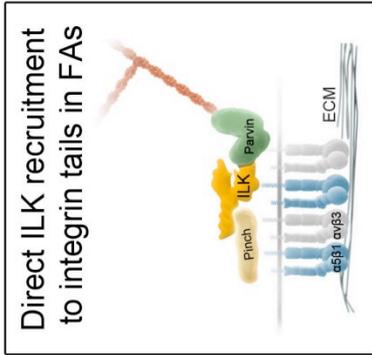
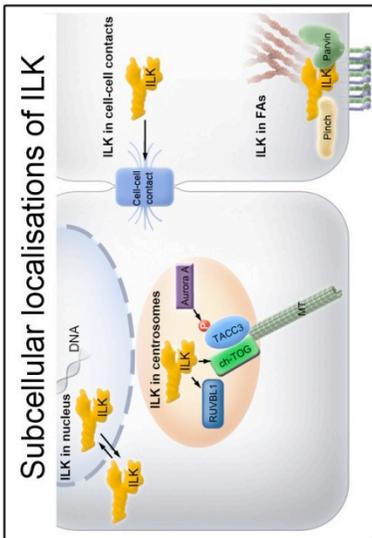
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ILK at a glance



Babak-1 and Babak-2 are novel focal adhesion proteins that bind paxillin and regulate the assembly of membrane circular dorsal ruffles.

S. Babak Azimifar¹, Manddoph Wang¹ and Reinhard Fässler^{1,*}

¹Department of Molecular Medicine, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany

*Corresponding author: faessler@biochem.mpg.de

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Summary

Integrin-based adhesion sites are multiprotein complexes that link the actin cytoskeleton to the extracellular matrix (ECM) and trigger intracellular signalling pathways. Here, we identified a novel family of focal adhesion (FA) molecules called Babak-1 and -2 (Plekhh-1, -2) that localized to integrin adhesion sites as well as punctate structures in the cytoplasm of mouse fibroblasts and HeLa cells. SILAC-based proteomics of Babak-2 immunoprecipitates identified paxillin, a FA-associated signalling adaptor, as interaction partner. Immunoprecipitation experiments with GFP-tagged Babak-1 showed that also Babak-1 associates with paxillin. Indeed, the recruitment of both Babaks to FAs was dependent on their interaction with paxillin, which was mediated through a paxillin binding sequence (PBS) present in the N-terminal half of the protein. Quantitative RT-PCR and northern assays revealed that Babak-1 and -2 are expressed in most adult murine tissues. To test the functional properties of Babak-2 *in vivo*, we generated a conditional Babak-2-null mouse strain. Babak-2-deficient mice were fertile and did not show an obvious phenotype suggesting that Babak-2 function can be compensated by Babak-1.

Word count: 169 words.

Introduction

Cell adhesion to extracellular matrix (ECM) molecules controls a broad range of biological functions. Integrins are prominent mediators of cell-ECM adhesions. They link the actin cytoskeleton to the ECM and signal into as well as out of cells. Integrin cytoplasmic tails are short and lack enzymatic activity. Therefore, integrins recruit signalling and adaptor molecules to their cytoplasmic tails resulting in the formation of a large signalling hub called focal adhesions (FAs) that mediate the signalling task of integrins. About 200 FAs molecules have been identified so far that control the numerous functions of integrins including actin dynamics, biochemical signalling and the crosstalk with growth factors (1, 2).

Integrin signals converge with growth factor receptor (GFR) cues to collaboratively control a cellular functions (3). As integrins and GFRs diversify in different tissues and developmental stages, the consequence of integrin-GFR co-signalling is vast and complex and ranges from influencing cell proliferation to cell survival, migration, angiogenesis and immune response (4). We showed recently that transient actin-based, circular dorsal ruffles (DRs) are the consequence of a collaborative signalling effort between the fibronectin/ $\alpha 5\beta 1$ integrin/ILK complex and epidermal GFR (EGFR) in mouse fibroblasts (Azimifar et al., 2011). We used DRs to establish a screening strategy that combined stable isotope labeling by amino acids in cell culture (SILAC)-based phosphoproteomics and siRNA-mediated depletion of candidate proteins to identify new molecular players in the integrin-EGFR signalling crosstalk. Among the identified proteins that were specifically phosphorylated upon integrin adhesion and EGF treatment was a FERM domain-containing protein that we termed Babak-2. It belongs to a new family of proteins that consist of Babak1 (Plekhh1 or Max-1) and -2 (Kia2028 or Plekhh2). They are members of the H family of Pleckstrin homology (PH) domain-containing proteins. Loss-of-function mutations of the Babak-1 orthologue in *C. elegance* cause axon guidance defects (5), and depletion of Babak-1 in Zebrafish leads to patterning abnormalities of intersegmental blood vessels (6). Murine Babak-2 was reported to be expressed in kidney podocytes (7) and its locus was linked to diabetic nephropathy (8).

The number of signalling and scaffolding proteins in FAs is vast (1, 9). Paxillin is such a FA-associated, phosphotyrosine-modifiable adaptor protein that associates integrin and growth factor receptor signaling pathways. Paxillin consists of five N-terminal leucine- and aspartate-rich (LD) motifs followed by a proline-rich motif and four C-terminal Lin11, Isl-1 and Mec-3 (LIM) domains. In addition, it contains several tyrosine, serine and threonine residues scattered throughout the molecule that can become phosphorylated (10). Paxillin LD motifs form amphipathic α -helices that, despite their small size and similar sequence, are able to mediate distinctive interaction with paxillin binding sequence (PBS)-containing proteins such as vinculin, focal adhesion kinase (FAK), α -parvin (actopaxillin) and ILK (11-13). Paxillin phosphorylation mainly mediated by kinases downstream of growth factor signaling pathways regulates the interaction with other proteins (10). As paxillin serves as a molecular nexus intersecting integrin and growth factor signaling pathways, it is important to ascertain novel paxillin interactors and their potential role in cell signaling networks.

In the present study, Babak-2 was identified in a SILAC-based phosphoproteomics screen to be required for growth factor-induced dorsal ruffle (DR) formation in fibroblasts. Expression of Babak-2 and its homologous Babak-1 in fibroblasts and HeLa cells showed that they localize to FAs and punctate structures in the cytoplasm. Furthermore, we found that Babak-2 is recruited to FAs through a N-terminal PBS motif that binds to paxillin.

Results

Identification and modular composition of the murine Babak-1 and -2 proteins

We identified Babak-2 in a SILAC-based phosphoproteomics analysis as a possible intersector of integrin and EGFR signaling pathways (Azimifar et al., 2011). To confirm the involvement of Babak-2 in the integrin-EGFR dependent induction of DRs, we depleted Babak-2 in fibroblasts using specific siRNA and indeed observed reduced EGF-triggered DR formation in fibroblasts (Supplementary material Fig. S1A-C).

Since the biological function of Babak-2 in mouse is unknown, we decided to further investigate the functional properties of Babak-2 *in vitro* and *in vivo*. Mining the National Center for Biology Information (NCBI) database revealed that Babak-2 together with a homologous protein that we termed Babak-1 (kiaa1200/Plekhh1) form a novel family of proteins that are highly conserved among vertebrates (Fig. 1A-C). Murine Babak-1 and -2 are highly homologous in their C-terminal segments which contain two PH domains, a Myosin tail homology 4 (Myth4) domain and a 4.1 protein, Ezrin, Radixin and Moesin (FERM) domain. The low homology N-terminal part of both Babak proteins revealed different hits of several low-scored, overlapping small domains including a structural maintenance of chromosomes (SMC) homology domain for Babak-2, a DNA polymerase III subunits gamma and tau (PRK05563) for Babak-1, and a coiled coil motif for both Babak-1 and -2 at the very N-terminus. Moreover, we found several conserved proline-rich motifs and phosphorylation sites throughout the protein sequence of Babak-1 and -2 (Fig. 1A-B). Altogether, these data show that Babak-1 and -2 are highly conserved multi-domain proteins and that they link integrin and growth factor signaling pathways.

Babak-1 and -2 localize to cell-matrix adhesion sites

Next we tested the subcellular localization of both Babak proteins using GFP-tagged cDNA constructs that were transiently expressed in mouse fibroblasts. Babak-1 and -2 co-localized with paxillin in nascent and mature FAs (Fig. 2A, B). In addition, Babak-1 and 2 signals were also observed in punctate structures that were present throughout the cytoplasm (Fig. 2A, B). EGFP-tagged Babak-1 fully overlapped with Cherry-tagged Babak-2 in the

cytoplasmic puncta and in FAs (Fig. 2C). However, Babak-positive puncta neither colocalized with Rab5 (endosomal marker), nor with EE1 (early endosomal marker), Rab7 (late endosomal marker), Rab21 (marker of β 1 integrin-containing recycling endosomes), Lamp1 (lysosomal marker), GM130 (Golgi marker), caveolin-1, flotillin-1 and transferrin receptor (recycling endosomes; Supplementary material Fig. S3A). Taken together, these data indicate that Babak-1 and -2 localize to FAs and cytosolic punctate structures whose identity is unknown.

SILAC-based analysis of the Babak-2 interactome

Next we used SILAC (14), followed by EGFP-Babak2 immunoprecipitation and mass spectrometry to identify Babak-2 interaction partners (Fig. 3A). To exclude nonspecific interactions, EGFP-expressing fibroblasts were labeled by light SILAC media and used as control experiment. We identified 360 proteins and ranked them based on SILAC ratios (Supplementary dataset 1). Babak-2 ranked first in the list of proteins with the highest SILAC ratio, indicating specificity and accuracy of the interactome list. We also identified several Babak-2 serine/threonine phosphopeptides (Fig. 3B). Interestingly, paxillin a FA-associated signaling and adaptor protein (10) was among the proteins potentially binding to Babak-2. Consistent with the proteomic data and our immunostaining (Fig. 2A), paxillin was immunoprecipitated together with EGFP-tagged Babak-2 in fibroblasts (Fig. 3C). These results indicate that Babak-2 undergoes phosphorylation and forms a complex with paxillin.

Babak-1 and -2 are recruited to FAs through a paxillin binding sequence

To examine the significance of the Babak-2 interaction with paxillin, we generated a series of EGFP-tagged Babak-2 expression plasmids (Fig. 4A). Paxillin was readily immunoprecipitated from HeLa cells that transiently expressed GFP-tagged full-length Babak-2 (GFP-Babak-2) or GFP-tagged C-terminally truncated Babak-2 (GFP-B2- Δ CT), but not from N-terminally truncated Babak-2 variants (GFP-B2- Δ NTP and GFP-B2- Δ NT), indicating that Babak-2 binds paxillin through its N-terminal part (Fig. 4B). Several

paxillin interacting proteins bind to paxillin via a conserved PBS motif (amino acids 377-396 in ILK, 919-938 in FAK and 951-970 in vinculin) (13). Homology search revealed a potential PBS motif in the N-terminal part of both, Babak-1 and -2 (residue 128 to 139) that is conserved in the sequence of other vertebrate Babaks (Fig. S2A). The interaction of paxillin with Babak-2 was dramatically reduced when conserved residues of PBS motif were mutated in the full length or N-terminal half of the Babak-2 polypeptide (GFP-Babak-2^{VT>GG} or GFP-B2- Δ CT^{VT>GG}) (Fig. 4C). Furthermore, the immunoprecipitation of paxillin with GFP-Babak-1 was also abolished when the PBS motif of Babak-1 was mutated (Fig. 4D).

Consistent with the immunoprecipitation experiments, GFP-Babak-2 and GFP-B2- Δ CT, but not GFP-B2- Δ NTP or GFP-B2- Δ NT co-localized with paxillin in FAs of Hela cells (Fig. 4E). N-terminally truncated GFP-B2- Δ NTP and GFP-B2- Δ NT were unable to localize to FAs and the punctate structures and distributed diffusely throughout the cytoplasm. Interestingly, GFP-B2- Δ CT was recruited to FAs but not to the cytoplasmic puncta, while GFP-B2- Δ CT^{VT>GG} or GFP-B1- Δ CT^{VT>GG} localized to the punctate structures but were absent from FAs (Fig. 4E, F). These results showed that N-terminal PBS motifs of Babak1 and 2 are mediating their interaction with Paxillin and recruitment to FAs and that both the C-terminal and N-terminal parts of the polypeptides are required for the recruitment to the punctuate structures.

***In vivo* analysis of Babak-2 in mice**

To evaluate the expression pattern of Babak-1 and -2 in mouse tissues, cDNAs of different tissues from 4-week old normal mice were subjected to quantitative RT-PCR and Northern blot analysis (Fig. 5A,B). Babak-1 and -2 were highly expressed in gonads, brain, lung, stomach, small intestine and kidney, and to a lesser extent in spleen, skin, liver, organs of immune system and muscle (Fig. 5A,B). Babak-1 expression was dominant in brain, stomach, small intestine and kidney, while Babak-2 showed high expression levels in testis, spleen and bone marrow (Fig. 5A). In addition, RT-PCR analysis of whole embryonic cDNAs indicated strong expression of Babak-1 and -2 throughout all embryonic stages tested, from E7.5 to E17.5 (Fig. 5C).

To examine the role of Babak-2 in mouse development and homeostasis, we generated a conditional floxed (f) allele of the *Babak2* gene by flanking exons 12 and 13 with loxP sequence (Fig. 6A). Out of 800 embryonic stem cell clones, we found only one clone with a correct integration of the targeting cassette (Fig. 6B). Cre recombinase-mediated deletion of floxed exons in Babak-2^{f/f} mice or in kidney-derived Babak-2^{f/f} fibroblasts led to a frame-shift mutation followed by premature stop codon in all splice variants of Babak-2 mRNAs (Fig. 6C-F). Offspring from Babak-2^{f/f} intercrosses yielded a normal Mendelian distribution of Babak-2^{-/-} mice (Fig. 6G). Moreover, Babak-2^{-/-} mice were fertile and did not develop any apparent defects. These results indicate that Babak-2 protein is not essential for mouse development and reproduction.

Discussion

FAs are integrin-nucleated multi-protein complexes that are involved in many cell signalling events. The composition and molecular interaction at FAs govern the nature of their signalling response and are diversified in a cell type-, tissue- and developmental stage-specific manner. A large number of proteins have been identified that are recruited to integrin-mediated adhesion sites (1). Here, we identified a novel family of FA proteins, that we intelligently termed Babak-1 and -2. Both Babak isoforms are expressed in different tissues and are conserved among vertebrates. Using SILAC-based quantitative proteomics, we identified paxillin as interaction partner of both Babak proteins. Paxillin serves as a scaffold that recruits many signaling molecules to FAs, where they can interact with and modulate downstream signaling pathways. Like many other paxillin-binding FA proteins (10, 13), the recruitment of Babaks to FAs was mediated through a conserved PBS motif, suggesting PBS as an evolutionary conserved versatile module that mediates recruitment of various FA molecules to a specific signaling compartment within FAs.

In addition to FAs, Babaks localized to the punctate structures that are scattered throughout the cytoplasm. The nature of these structures remains unclear, as they did not colocalize with the Golgi apparatus, endosomes, lysosomes, caveolin-1, flotilin-1, Rab21 and transferrin receptor. One possibility is that the Babak proteins are part of molecular machinery organizing a novel intracellular trafficking route. Since integrins do not localize to the Babak-positive puncta, these routes must serve integrin-independent functions. Membrane trafficking is essential to the functions of eukaryotic organisms through transporting cargoes to specific cellular sites as well as providing microenvironments for signalling events (15, 16). Therefore, it requires further efforts to characterize the potential role(s) of Babak-positive punctate structures in the cellular context.

Babak-2-null mice were viable, fertile and showed no obvious phenotype, indicating that Babak-2 alone is not essential for normal murine physiology. As both Babak-1 and Babak-2 share same molecular structure and subcellular distribution, it is possible that Babak-1 compensates for Babak-2 functions *in vivo*. This assumption can be further examined by constitutive or conditional deletion of Babak-1, or both Babak-1 and -2 in mice.

Materials and methods

Reagents and antibodies

The following antibodies were used: paxillin, caveolin1, flotillin1, GM130 and EEA1 (BD Bioscience); Rab5 and Rab7 (Cell Signalling Technology); Rab21 and phalloidin-TRITC (Sigma); anti-mouse-HRP and anti-rabbit-HRP (BioRad); transferrin receptor (Invitrogen); anti-rabbit Cy3 and anti-mouse Cy3 (Jackson ImmunoResearch).

Southern and northern blotting

For Southern blotting, 15 µg of DNA was digested by restriction enzyme(s), separated on a 0.8% agarose gel and transferred onto Hybond N+ membranes (GE Healthcare). For northern blotting, 10 µg of total RNA was extracted with TRIzol (Invitrogen) from four-week murine tissues, separated on a denaturing agarose gel and transferred onto Hybond N+ membranes (GE Healthcare). Probe preparation and membrane hybridization were described previously (17).

Immunofluorescence, immunoprecipitation and western blotting

Cell immunostainings and confocal microscopy were performed as described previously (Azimifar et al., 2011). Cell lysates were prepared by quickly washing cells in ice-cold PBS prior to addition of lysis buffer (50mM Tris-HCl, pH7.6, 150mM NaCl, 1% Triton-X-100, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma-Aldrich)). GFP-tagged protein immunoprecipitation was performed according to the manufacturer's instructions (MiltenyiBiotec). For western blotting, a total of 10-25 µg of protein per lane was separated on a 6% SDS-PAGE and transferred onto a PVDF membrane (Millipore). Membrane blocked with Tris-buffered saline (TBS) pH 7.6 containing 5% (w/v) skimmed milk powder (Fluka) and 0.1% Tween 20 (Serva). Membranes were incubated for 1 hour at room temperature or overnight at 4°C with primary antibodies and were incubated with appropriate HRP-coupled secondary antibodies (BioRad) for 30 minutes at room temperature. Subsequently, membranes were washed, developed with chemiluminescence detection kit (Western Lightning, PerkinElmer) and images were captured by ImageReader LAS-4000 (Fujifilm).

Constructs, siRNAs and primers

Full-length Babak-1 and Babak-2 cDNAs were amplified as a single amplicon from E15.5 embryonic brain and cloned into pEGFP-C1 plasmid (Clontech). Truncated Babaks constructs were PCR amplified from full-length pEGFP-Babak-1 and -2. Mission siRNAs for depletion of Babak-2 mRNA were ordered from Sigma. These primers were used: for Babak-1 quantitative RT-PCR: 5'-AGA CAT GAT GGC CAC CAA GTG-3' and 5'-AGT AGG GAT AAG TGA TGT TCA CCT G-3'; Babak-2 quantitative RT-PCR: 5'-ACT GTA TTT CTC AGT GCA AGC TC-3' and 5'-GCC TTG CAC TGG TTA GTC ACA G-3'; PCR genotyping of Babak-2 mice: 5'-ATG TCT TTT AAT ACG GCG CAC-3' and 5'-CCA CAT TGA ACT GAG TGC ATC C-3'; RT-PCR for Babak-2 mice: 5'-CCC TTT CCT GGA TGA CTC CTC-3' and 5'-TGG TGA GCA AGC CCT TTA CTG-3'.

Cell culture and transfection/transduction procedures

Cells were transiently transfected with Lipofectamine 2000 (Invitrogen). Babak-2^{f/f} fibroblasts were isolated from 4-week murine kidney and immortalized by retroviral transduction of the SV40 Large T-antigen. Babak-2^{-/-} cells were generated by adenoviral infection of Babak-2^{f/f} cells with Cre recombinase. Knock down and DR experiments were as described previously (Azimifar et al., 2011).

SILAC-based quantitative proteomics

SILAC-based proteomics was performed as described (Mann, 2006). Briefly, cells were metabolically labelled with SILAC culture media and transfected with EGFP or EGFP-Babak-2 constructs for 24 hours. Cells lysates were prepared and subjected to GFP-immunoprecipitation (MitrnyBiotech). The immunoprecipitated proteins were digested with trypsin in columns, eluted, mixed 1:1 and separated by online reverse phase nanoscale capillary LC and analyzed by MS/MS on a linear trap quadrupole (LTQ)-Orbitrap mass spectrometer (Thermo Fisher Scientific). Mass spectra were processed with MaxQuant and Mascot search engine.

Statistical Analysis

Results are expressed as means±sd. Statistical analysis was performed using Excel software. Student's *t*-test was used for comparisons between different data sets. Asterisks indicate significant differences (**P*<0.05, ***P*<0.01 and ****P*<0.005). The deviation from Mendelian ratios measured by χ^2 test for codominant inheritance.

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Figure legends

Fig. 1. Domain structure of murine Babak-1 and Babak-2 proteins. Conserved motifs of (A) Babak-1 and (B) Babak-2 were predicted by NCBI-CDD and Motif Scan servers. Babaks could potentially mediate interactions with other proteins/lipids through conserved phosphosites, proline-rich motifs and lipid-binding sequences. Abbreviations: Structural maintenance of chromosomes (SMC) protein homology domain; DNA polymerase III subunits gamma and tau (PRK05563); Pleckstrin homology (PH) domain; Myosin tail homology 4 (MyTH4) domain; 4.1 protein, ezrin, radixin and moesin homology (FERM) domain. (C) Pairwise alignment scores for Babak-1 and -2 proteins and DNAs sequences in human, mouse, dog and Zebrafish. Alignment scores are according to the National Center for Biotechnology Information (NCBI) database.

Fig. 2. Cellular localization of Babak-1 and Babak-2. (A) Immunostaining of paxillin (red) in fibroblasts that expressed EGFP-Babak-2 or EGFP-Babak-1. EGFP-Babak-2 and -1 colocalized with paxillin at FAs, and localize punctate structures that distributed throughout the cytoplasm. (B) Immunostaining of F-actin (red) in fibroblasts that expressed EGFP-Babak-2 or -1. EGFP-Babak-2 localized to focal complexes (arrow) at the leading edge of the cell. (C) EGFP-Babak-1 and mCherry-Babak-2 signals overlap in cytoplasmic puncta. Scale bars are 10 μ m.

Fig. 3. SILAC-based analysis of Babak-2 interactome. (A) Schematic representation of the SILAC-based proteomics experiment. EGFP (control) or EGFP-Babak-2 was expressed in fibroblasts that labeled with light (L) or heavy (H) SILAC media, respectively. Cell lysates were affinity purified by anti-EGFP antibody and digested with trypsin in columns. Tryptic peptides were mixed 1:1 and subjected for LC-MS/MS analysis. (B) Short list of Babak-2 phosphopeptides identified by LC-MS/MS analysis. The potential kinases triggering the phosphosites were predicted by PHOSIDA server (18). (C) Paxillin was detected in anti-GFP immunoprecipitates from fibroblasts that expressed EGFP-Babak-2.

Fig. 4. The interaction of Babak-2-PBS with paxillin is essential for its recruitment to FAs. (A) Schematic diagram of the various EGFP-Babak-2 constructs used. (B) Paxillin was detected in anti-GFP immunoprecipitates from cells that expressed full length EGFP-Babak-2 or EGFP-B2- Δ CT, but not EGFP-B2- Δ NT and EGFP-B2- Δ NTP. Paxillin immunoprecipitation with Babak-2 was dramatically reduced by mutating the PBS site (VT>GG) in (C) EGFP-Babak-2, EGFP-B2- Δ CT and (D) EGFP-Babak-1. (E) Paxillin (red) immunostainings colocalized with EGFP-Babak-2 or EGFP-B2- Δ CT (green) in FAs of Hela cells. EGFP-B2- Δ NT and EGFP-B2- Δ NTP were diffusely distributed through the cytoplasm and did not localize to FAs and the punctate structures. Mutating the PBS motif of Babak-2 (EGFP-B2^{VT>GG}) strongly reduced its recruitment to FAs, while it did not affect Babak-2 localization to the punctate structures. (F) Mutating PBS of Babak-1 (EGFP-B2^{VT>GG}) strongly disturbed its FA recruitment in Hela cells.

Fig. 5. Expression pattern of Babak-1 and Babak-2. (A) Quantitative RT-PCR of Babak-1 and Babak-2 in different murine tissues. (B) Northern blot of Babak-2 extracted from different murine tissues. (C) RT-PCR of Babak-1 and Babak-2 expression during different embryonic stages.

Fig. 6. Babak-2-deficient mice (A) Schematic representation of Babak-2 locus and targeting construct. (B) Southern blotting of Babak2-recombinant ES cell clones with the 3'-arm external probe. (C) Southern blotting of Babak-2 mice with the 5'-arm external probe. (D) Genotyping of Babak-2 mice with PCR. (E) cDNAs derived from mRNA extracted from Babak-2^{fl/fl} and Babak-2^{-/-} fibroblasts and brain, were analyzed by RT-PCR. A wild-type band of 614 bp (black arrow), an exon 11>14 frame shift mutant band of 330 bp (grey arrow) and an exon 11>15 frame shift mutant band of 270 bp (white arrow) were amplified by RT-PCR and (F) analyzed by DNA sequencing. (G) Offspring statistics of Babak-2^{fl/fl} intercrosses did not show significant deviation from normal codominant Mendelian ratios (2 degree of freedom, χ^2 : 3.62).

Supplementary Fig. S1. DR formation is reduced in Babak-2-depleted fibroblasts. (A) Babak-2 expression levels were evaluated by quantitative RT-PCR for two different Babak-2 knock down cell lines. (B) Phase contrast images of Babak-2 knock down (siRNA-II) and control cells (siRNA-ctl) when serum starved, seeded on fibronectin and stimulated with EGF (50ng/ml) for 3 minutes (scale bar: 50 μ m). Note absent of EGF-induced DRs (arrows) and morphological changes in the Babak-2 knock down. (C) DR formation was significantly reduced three minutes after EGF stimulation in Babak-2 knock down cells in comparison with their corresponding control cells, n=4 independent experiments. Data expressed as the mean \pm sd. Stars indicate *P*-value for *t*-test (***P*<0.01).

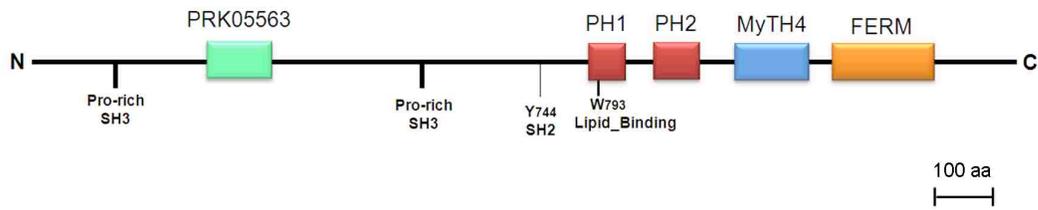
Supplementary Fig. S2. Conserved PBS motif in the N-terminus parts of Babak-1 and -2. (A) Alignment of N-terminal PBS (red boxes) of Babak-1 and -2 in different species. Red arrows indicate conserved K and E residues that are also present in the PBS motif of FAK, Vinculin and ILK (Nikolopoulos et al., 2001).

Supplementary Fig. S3. Babak-2 localizes to cytoplasmic puncta of unknown origin. (A) No colocalization of EGFP-Babak2 (green) with EEA1, Rab5, Rab7, Rab21, GM130, transferrin receptor (tfr), caveolin-1 (Cav1) and flotillin-1 (flot1) (all red) in Hela cells. Scale bar is 10 μ m.

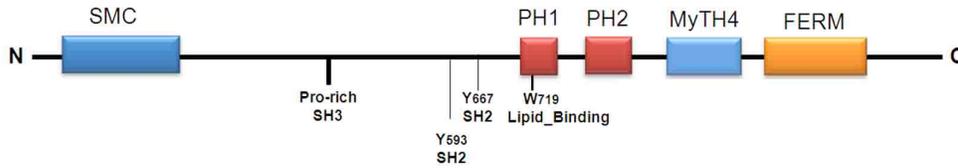
Supplementary dataset. S1. The list of Babak-2 interactome obtained by SILAC-based quantitative proteomics. All identified and quantified proteins were ranked based on their normalized SILAC ratio. L and H indicate SILAC labeling that represent EGFP (control) and EGFP-Babak-2 immunoprecipitates, respectively. H/L indicates the normalized SILAC ratio from EGFP-Babak-2 immunoprecipitates as compared with immunoprecipitates of control EGFP-expressing cells.

A

Babak-1 (1560 residues)

**B**

Babak-2 (1491 residues)

**C**

Species \ Gene	Babak-1		Babak-2	
	Protein	DNA	Protein	DNA
H.sapiens				
vs. C.lupus	87.3	88.2	95.2	91.6
vs. M.musculus	84.1	85.4	86.0	82.9
vs. D.ferio	59.8	62.1	58.0	58.2
C.lupus				
vs. H.sapiens	87.3	88.2	95.2	91.6
vs. M.musculus	80.4	81.9	85.1	82.3
vs. D.ferio	60.7	62.8	58.6	59.6
M.musculus				
vs. H.sapiens	84.1	85.4	86.0	82.9
vs. C.lupus	80.4	81.9	85.1	82.3
vs. D.ferio	59.0	61.5	56.6	59.2
D.ferio				
vs. H.sapiens	59.8	62.1	58.0	58.2
vs. C.lupus	60.7	62.8	58.6	59.6
vs. M.musculus	59.0	61.5	56.6	59.2

Fig. 1

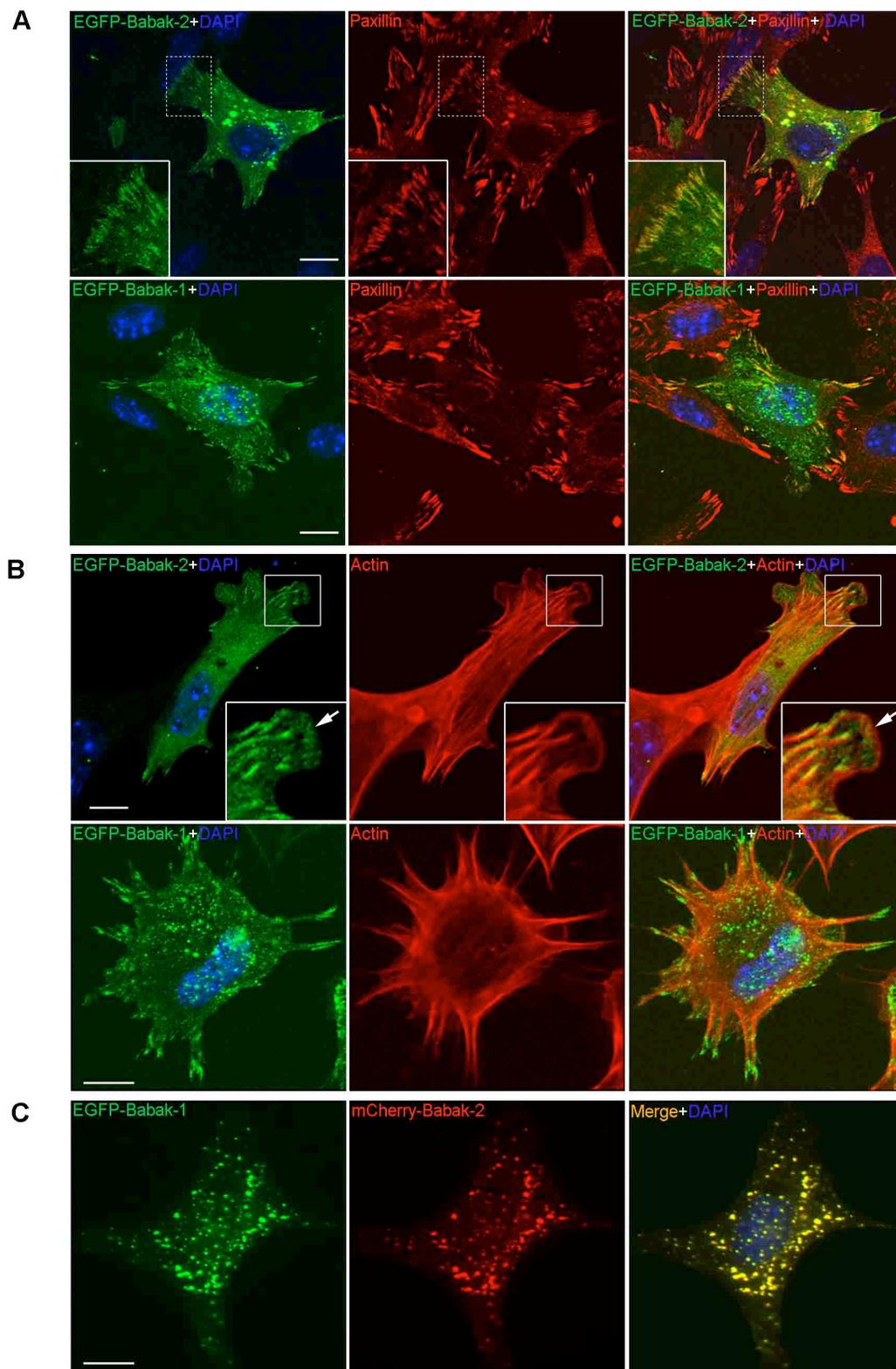


Fig. 2

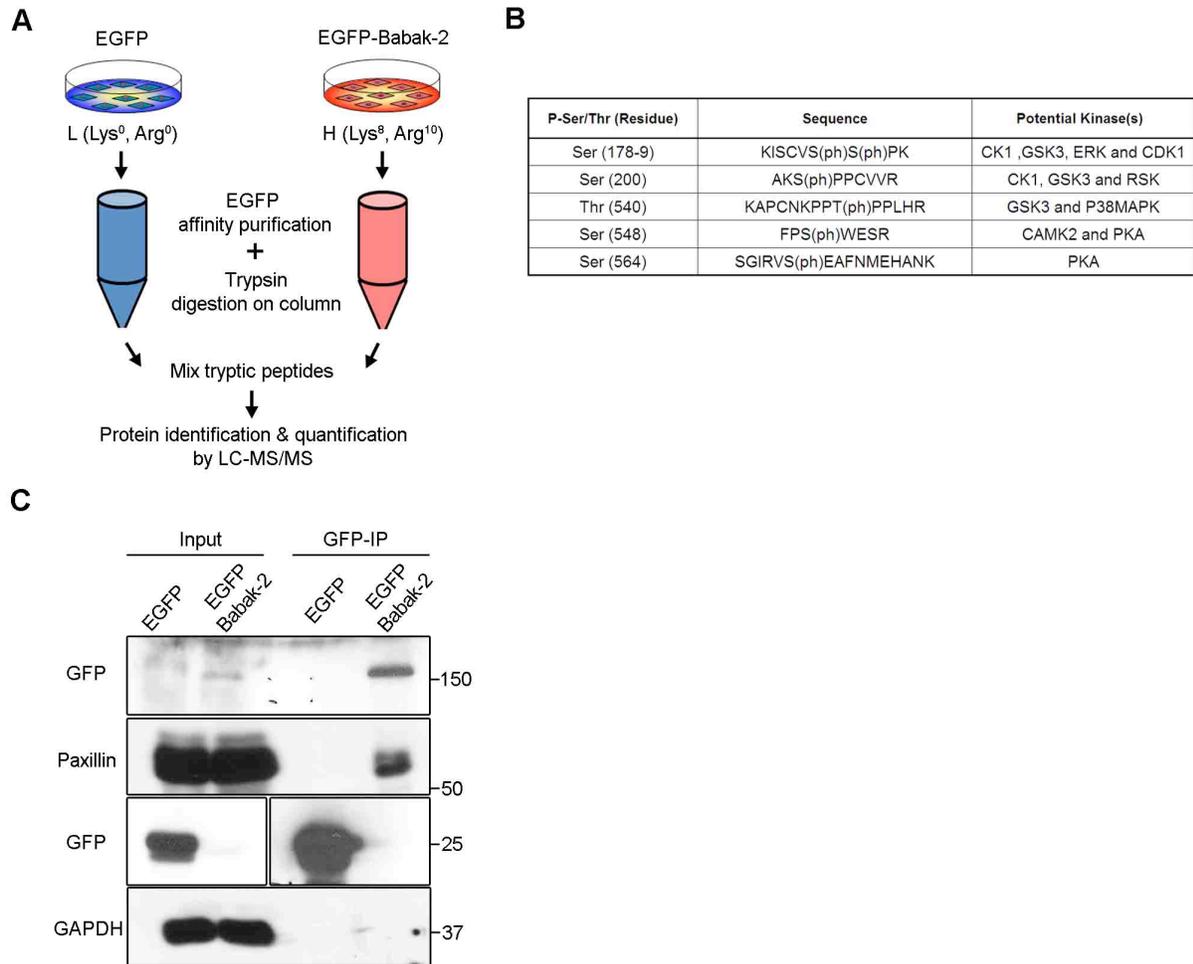


Fig. 3

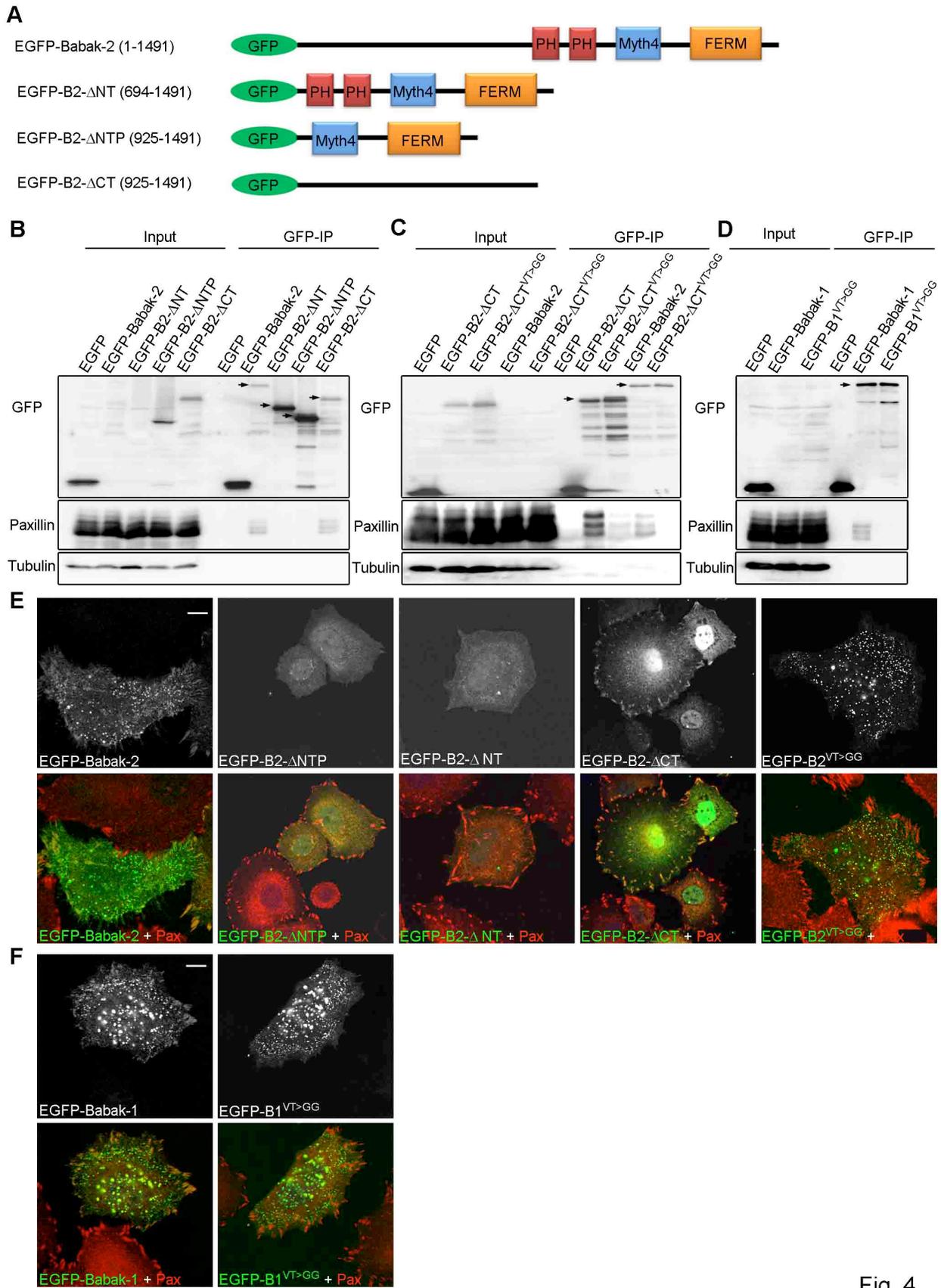


Fig. 4

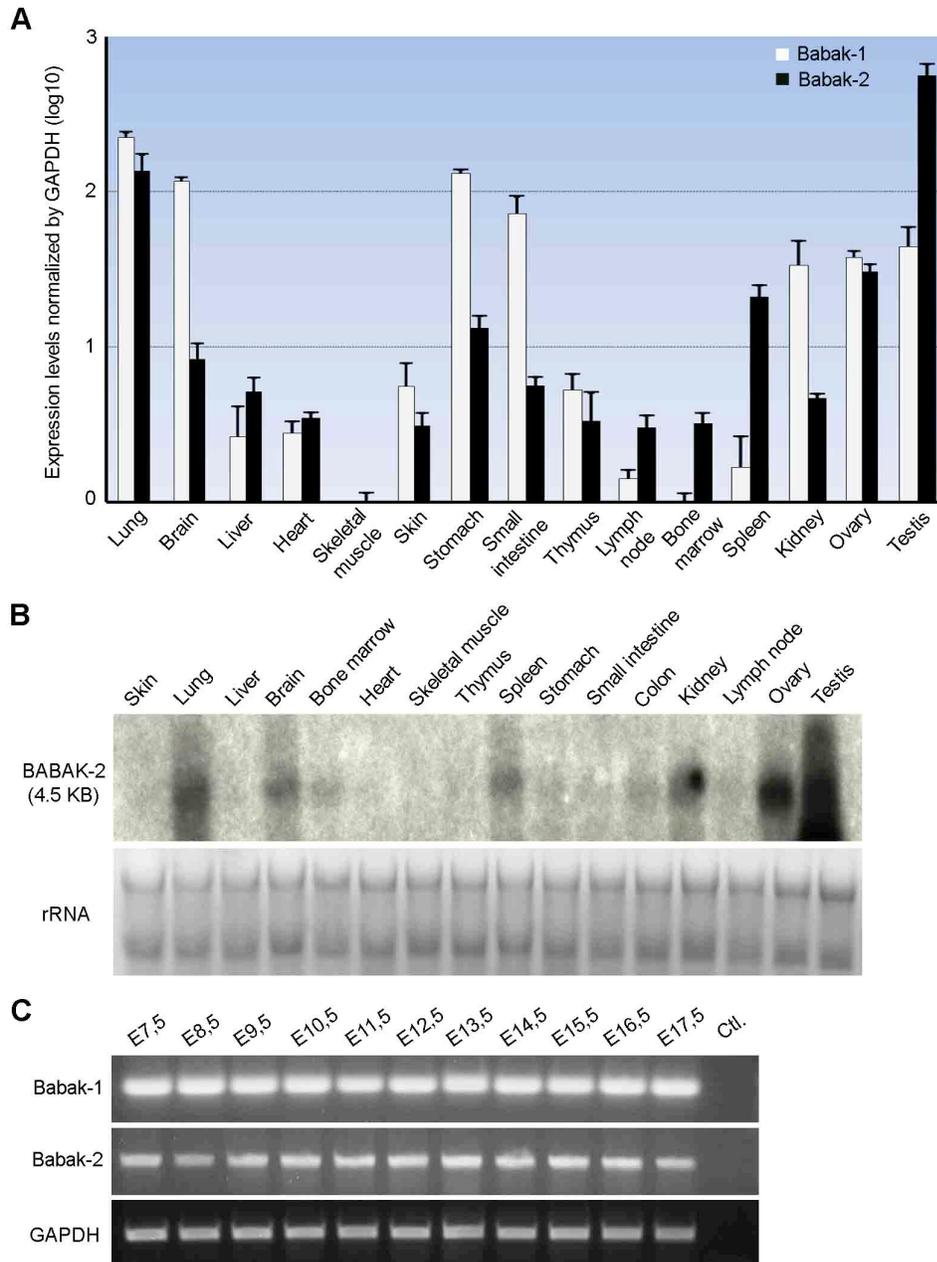


Fig. 5

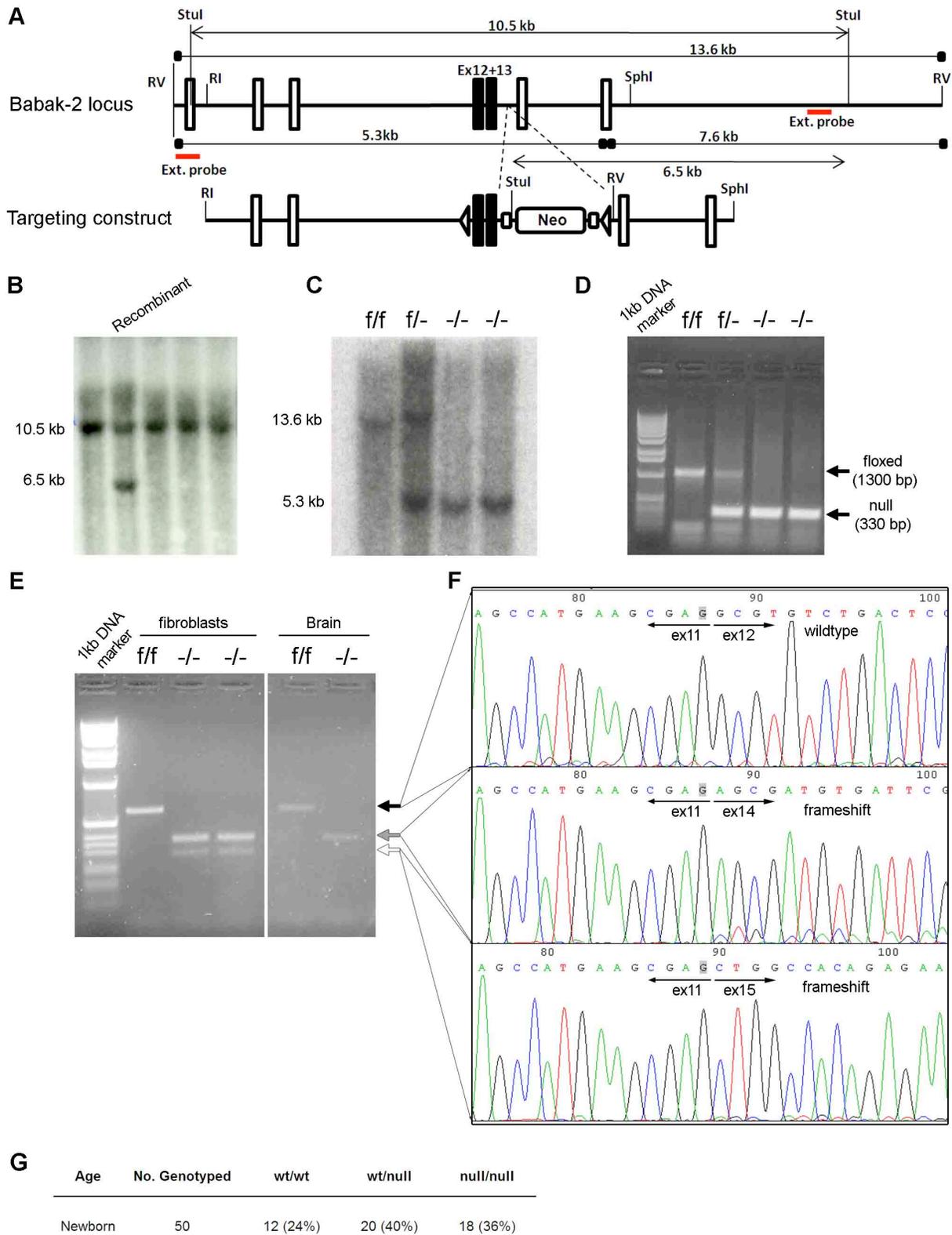
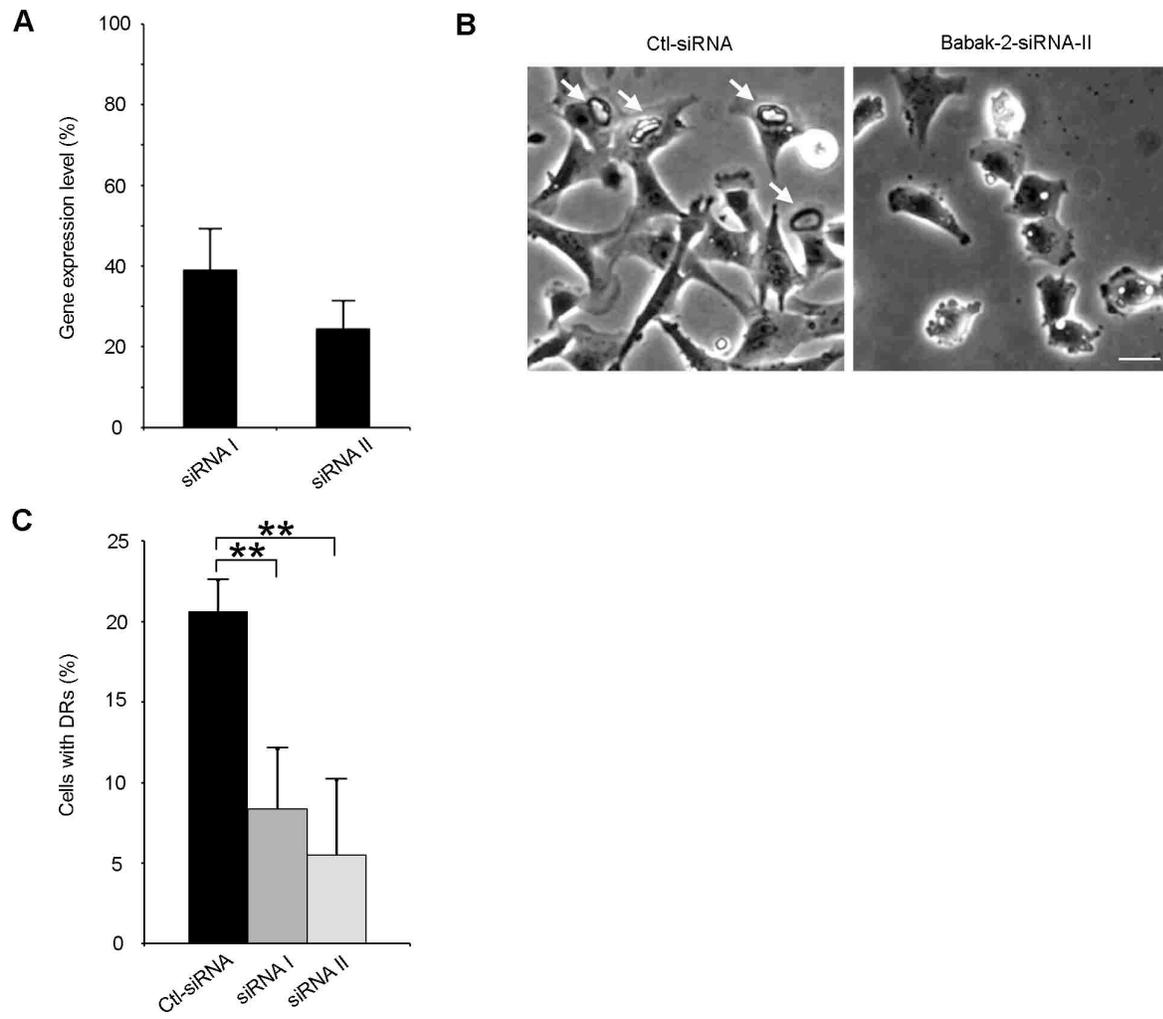


Fig. 6



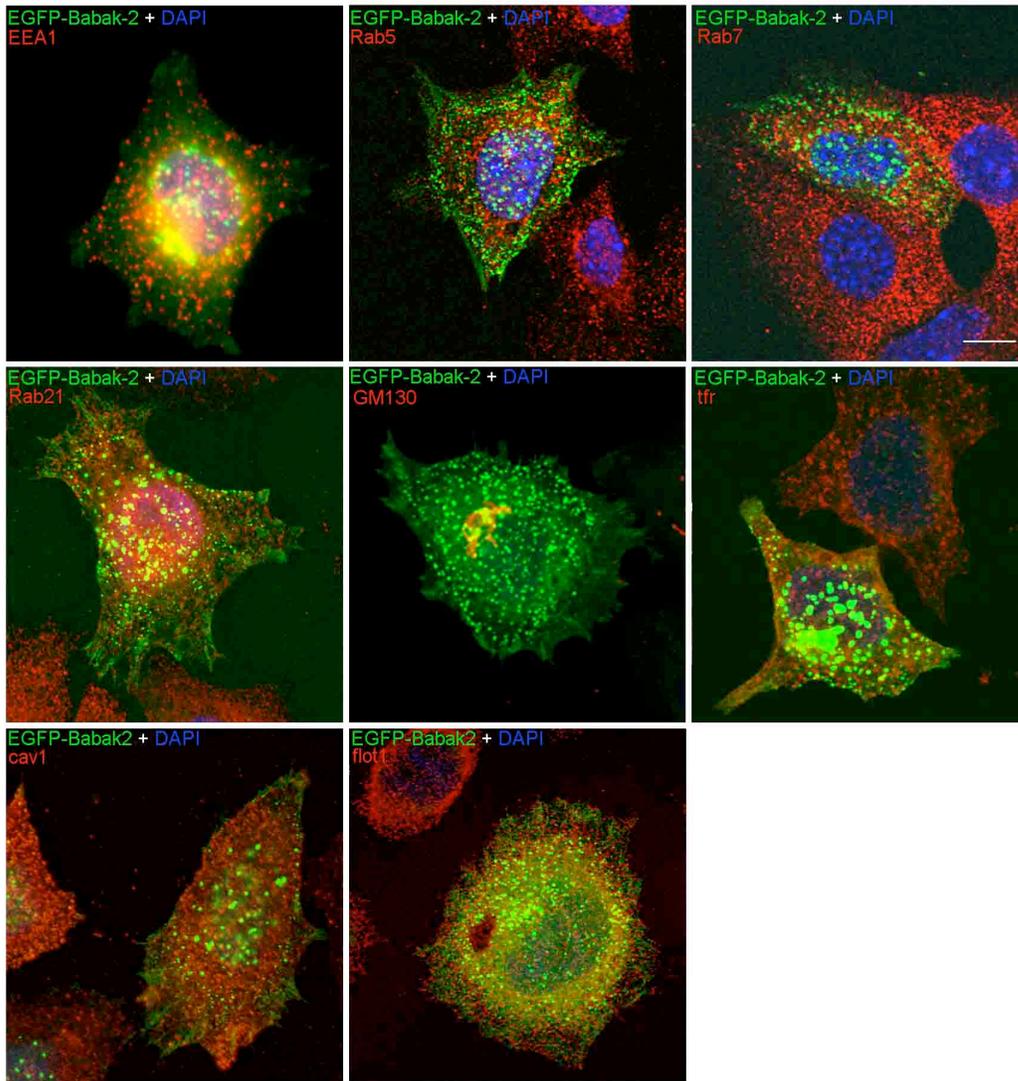
Supplementary Fig. 1

A

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Human_plekhh2 AKIIEEKAAKIKWVTVKLNLELEVENQNLRRLINQNTQEEIRTMOSKLEVOGKKSSTVST 179
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Chick_plekhh2 AKIIEEKAAKIKWVTVKLNLELEVENQNLRRLINQKQIKEMRTVQSKLQELQATGKK-SVTST 178
Anolis_plekhh2 AKIIEEKAAKIKWVTVKLNLELEVENQNLRMINQNTQTEEMRLLQNKLELQGGK-SVPST 178
Xenopus_plekhh2 AKMIEDKAAKIKDWTSLKRELELEVENQNLRRLTNKNYNEEITSLRSKLEIKDKKSSVGAT 179
Human_plekhh1 AKTVQEKAAKIKWVTVKLNLELEVENQHLKSHNQRLVEQVGSLODALEAIQIAPSRKLLV 180
Mouse_plekhh1 AKIVQEKAAKIKWVTVKLNLELEVENQQLKTCNQQLVEQVAALODALEDRMTPSELLV 179
Anolis_plekhh1 AKTVQEKAAKIKDWTSLKRELEVENHYLKDNCNRLTDQIKALQAAALQ----- 166
** :::*****:*** ** :***:**: * : * .:: :| *:
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Supplementary fig. 2

A



Supplementary fig. 3