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Review

How integrins control mammary epithelial differentiation: A possible role for the ILK–PINCH–Parvin complex

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ABSTRACT

Differentiation into tissue-specific cell types occurs in response to numerous external signals. Integrins impart signals from the extracellular matrix microenvironment that are required for cell differentiation. However, the precise cytoplasmic transducers of these signals are yet to be understood properly. In lactating mammary epithelial cells, integrin-linked kinase has been identified as an indispensable integrin-signalling adaptor that enables the activation of Rac1, which is necessary for prolactin-induced milk protein expression. Here we use examples from various tissues to summarise possible mechanisms by which ILK and its binding partners PINCH and Parvin (ILK–PINCH–Parvin complex) could be required for Rac activation and mammary epithelial differentiation.

1. Introduction

Cells in multicellular organisms require and receive signals from different sources, which cooperate to control cell fate decisions. Highly specific stimuli induce changes in gene expression and chromatin architecture, which leads to differentiation into tissue-specific cell types that behave in a particular manner. These stimuli can act globally (e.g. endocrine hormones) or locally, via paracrine and autocrine signals from secreted growth factors and cytokines. In addition, cells receive signals emanating directly from their specific microenvironment, imparted by adhesive interactions with other cells and the surrounding extracellular matrix (ECM) [1]. Cell adhesion to the ECM influences and controls the behaviour and organisation of groups of cells in tissues, and provides physical support for the maintenance of structures and shapes during development. Cues from cell-ECM interactions act as an underlying input affecting various aspects of cell fate decisions, and influencing processes as fundamental to biology as the progression of the cell cycle/mitosis, cell migration, cell survival and apoptosis and the establishment of cell polarity [2].

The ECM is a complex, tissue-specific network of fibrous proteins (e.g. collagens, fibronectins, etc.), sugars and glycoproteins that function to provide the structural framework around which tissues form. Foremost in mediating adhesive interactions between cells and the ECM are the integrin family of trans-membrane ECM receptors. Integrins are glycoprotein heterodimers of α and β subunits with a large extracellular domain (that interacts directly with the ECM) and a short intracellular cytoplasmic tail [3]. Outside-in signalling from integrins influences the internal behaviour of the cell and is mediated by a large and diverse complex of signalling, scaffolding and adaptor proteins that accumulate around integrin cytoplasmic tails [4]. Signals from integrins collaborate with cytokine/growth factor pathways, which enable the full transduction of these stimuli into the cell [5]. Integrin signals modulate the actin cytoskeleton and the activity of Rho family GTPases that coordinate cell migration and are important regulators of other processes such as differentiation [6]. Integrins also provide a physical linkage between the ECM and actin, whereby tensile forces can be transmitted [7].

In certain tissues and developmental contexts, significant advancements in our understanding of the molecular mechanisms of how integrins regulate cell fate have been made. In the mam-

Abbreviations: TGFβ1, transforming growth factor β1; LIM, Lin11, Isl-1 & Mec-3; EGFR, epidermal growth factor receptor; SOS, son of sevenless homologue 1; shRNA, short hairpin ribonucleic acid; LD motif, leucine and aspartic acid rich motif

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mary gland (which will be the focus of this review) the β1-integrin binding adaptor protein, integrin-linked kinase (ILK), has emerged as a key transducer of β1-integrin signalling required for prolactin-induced differentiation of pregnant mammary epithelial cells (MECs). ILK and the Rho family GTPase, Rac1, are both required for proper transduction of signals from the prolactin receptor (PrlR), which directs gene expression in pregnant and lactating MECs [8,9]. A better understanding of the signalling pathways emanating from integrins, including the exact protein–protein interactions that transduce them, is required to truly appreciate the role and requirement for integrin-mediated cell-ECM attachment.

2. The requirement for integrins in mammary gland function

The predominantly postnatal development of the mammary gland coupled with well established mouse primary mammary cell culture techniques makes the murine mammary gland a tractable tissue system in which to dissect the requirement for integrin-mediated adhesion during cell differentiation. Mammary glands undergo tremendous, regulated and defined morphological and functional changes during adulthood. In puberty, the immature mammary gland proliferates into an intricate network of branched ducts and tubules. During pregnancy, hormonal changes stimulate a surge in proliferation resulting in increased mammary epithelial cell (MEC) branching and invasion, and the formation of polarised lobuloalveolar units containing terminally differentiated MECs capable of milk production (Fig. 1). Following lactation, the unwanted milk-producing cells undergo regulated apoptosis [10,11]. While hormones temporally direct mammary gland development, there is also a fundamental requirement for spatial signals from the ECM to collaborate in these processes.

The mammary gland consists of two kinds of MECs, inner luminal epithelial and outer basal myoepithelial cells. Mammary ducts and acini are surrounded by a specific basement membrane (BM) ECM. The primary constituents of the BM in the mammary gland are four types of αβγ laminin trimers (111, 322, 511, 521), glycoproteins, proteoglycans, and type IV collagen [12]. MECs use a range of integrin αβ dimer combinations to interact with the BM. The BM separates MECs from the mammary stroma, which contains fibrous ECM components (such as type I and type III collagen, fibronectin and hyaluronan) and stromal cells including adipocytes, fibroblasts and endothelial cells [11,13].

MEC functional differentiation occurs during pregnancy leading to lactation, with the conversion of alveolar stem cells into highly polarised and differentiated milk producing cells. This process is driven by ovarian signals from oestrogen and progesterone and via pituitary prolactin [14]. When MECs extracted from pregnant mice are stimulated with the 22 kDa peptide hormone prolactin (Prl) in 3D laminin rich (LnR) culture, they differentiate and produce milk proteins, such as caseins. These MECs also form 3D polarised acini, which are highly reminiscent of in vivo mammary alveoli [15,16]. However, when MECs are grown in a non-physiological environment such as on a collagen rich stromal ECM, or on an artificial 2D plastic tissue culture dish, no such acini structures form and cells do not produce milk in response to Prl [16]. This identified a fundamental requirement for specific cell-ECM adhesion for correct acini formation, morphology and differentiation, representative of both specific ligand interactions and appropriate elasticity of the cellular environment.

The requirement for MECs to adhere to a laminin ECM is mediated through β1-integrin–laminin interactions [17]. Primary MECs that lack β1-integrin have several defects, including an inability to differentiate into milk producing cells when stimulated with Prl. They also have an inability to form hollow acini with clear lumens, and show incorrect polarisation of acini with displacement of basolateral and apical markers. β1-Integrin deficiency in vivo causes morphological defects; mice have similar expansion and branching of the ductal network in puberty but insufficient alveolar development during pregnancy [18,19]. β1-Integrin also regulates the orientation of the mitotic spindle in basal MECs, and many β1-null alveoli have cells growing into their luminal space, not attached to the basement membrane [19,20]. In β1-integrin deficient mice, lactation is either entirely prevented or severely decreased, and pups are malnourished compared to wild type. In addition to β1-integrins, MECs also express an additional laminin receptor, β-dystroglycan, which is required for differentiation, not as a signalling molecule, but as an organiser of the laminin ECM so that integrins can adhere and signal [21]. Laminin has specific elastic properties, which are 'mechanosensed' by MECs, and alterations in the mechanochemical properties of the LnR ECM can impinge on their capacity to differentiate [22].

3. Mammary epithelial cell differentiation: prolactin-JAK2/Stat5 signalling pathway

MECs in vivo, and in 3D LnR culture, express PrlR on their basolateral membranes. Prolactin induces dimerisation of PrlR and activation of the constitutively associated tyrosine kinase Janus kinase2 (Jak2) [23]. Jak2 phosphorylates the PrlR, which forms a phospho-tyrosine platform, which recruits the SH2 domain containing protein Signal Transducer and Activator of Transcription 5 (Stat5). Once recruited, Jak2 phosphorylates Stat5, inducing the formation of a pStat5 dimer via their respective SH2 domains. The pStat5 dimer translocates to the nucleus where it initiates a transcriptional response to the Prl signal, i.e. transcription of pro-differentiation genes and genes encoding milk proteins such as β-Casein [24–27]. Sustained activation of Stat5 requires β1-integrin-laminin interactions [28]. This canonical Jak/Stat signalling pathway was first characterised in MECs and is dependent on β1-integrins and laminin 111 (Fig. 2A).

4. How integrins regulate MEC differentiation

Although MECs do not differentiate unless stimulated to do so by hormones that circulate during pregnancy and lactation, research in our lab has focussed on understanding how β1-integrin signalling enables MEC differentiation and how it might crosstalk with cell surface receptor signalling pathways. Downstream of β1-integrins, the Rho family GTPase Rac1 is required for MEC differentiation and signalling through the PrlR/Jak2/Stat5 pathway [9]. MECs expressing a dominant-negative Rac1 have impaired Jak2-Stat5 signalling and thus do not produce β-casein or other milk products. It is not currently clear how Rac effects Prl/Jak/Stat5 phosphorylation events, but the mechanism may involve a Rac-dependent regulation of the tyrosine phosphatase SHP2, which can negatively regulate PrlR signalling [9]. Rac may also control Stat5 through a Pak1-mediated serine phosphorylation [29]. Mice with a mammary specific deletion of Rac1 have reduced milk production (unpublished data), and MECs that lack β1-integrin have reduced Rac1 activation [19]. Moreover, differentiation is rescued in β1-integrin-null cells that express a constitutively active Rac1. Together, this suggests that Rac1 functions as a vital node downstream of β1-integrin and is required for transducing the spatial signals provided by cell–matrix interactions through integrins, which influence the temporal signals for lactation that are provided by the endocrine hormone prolactin (Fig. 2B).

Integrins cannot directly associate with Rac1, however, they recruit signalling and adaptor proteins, which directly modulate Rac (and other GTPases) activity. However, very little is currently

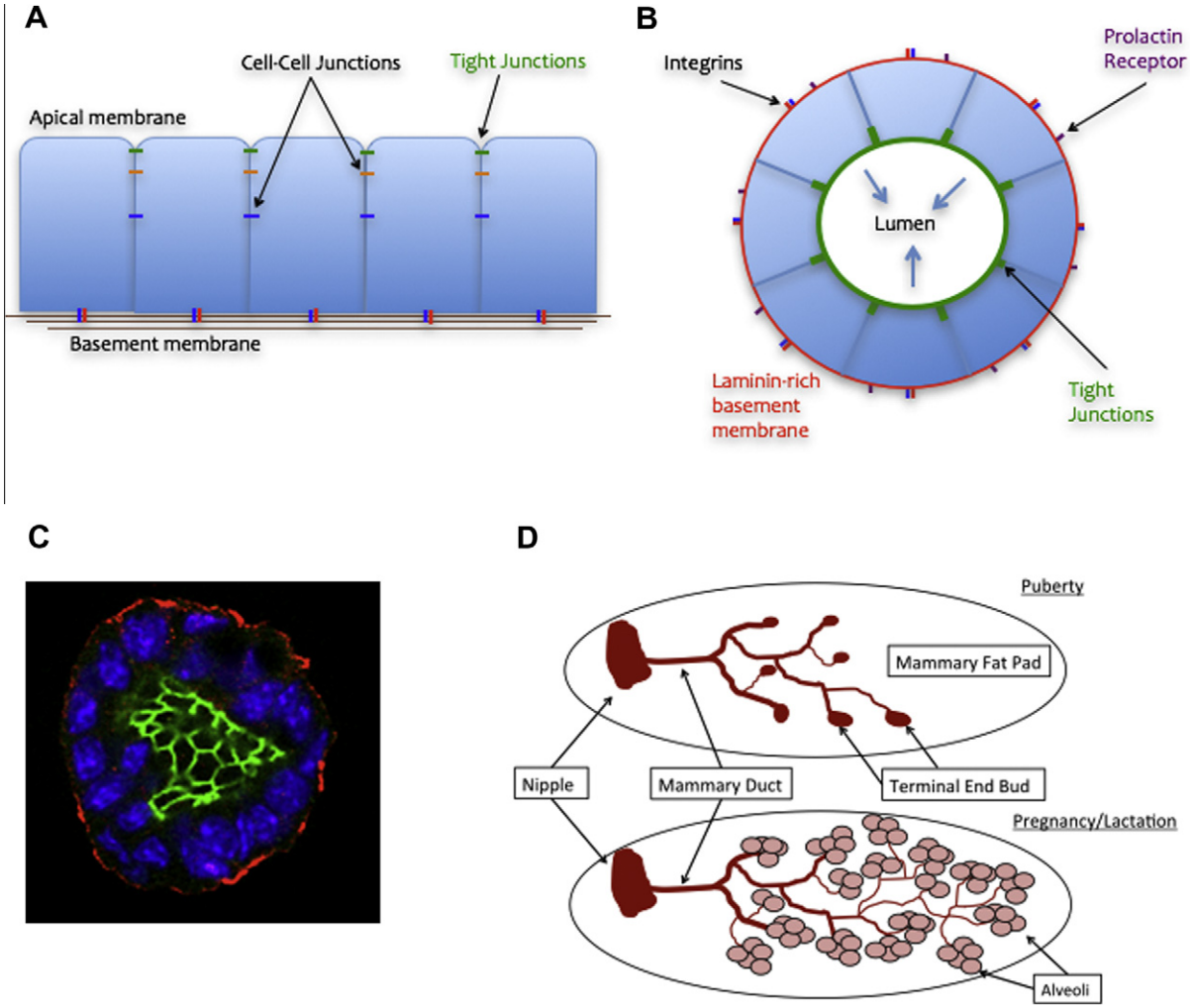

Fig. 1. Mammary gland development. (A) Schematic representation of mammary epithelial cells (MECs) demonstrating distinct apico-basal polarity as a monolayer epithelial sheet grown on collagen. (B) MECs grown in a Laminin rich 3D ECM form hollow acini structures reminiscent of in vivo alveoli with distinct apico-basal polarity. Milk is secreted through the apical membrane (green) into the luminal space. Sparse myoepithelial cells subtend the luminal cells of the acini (not shown). (C) Confocal Z section of a primary mammary acinus grown in culture in Laminin rich ECM, showing the adhesion complex protein ILK at the basal surface (red), the tight junction component ZO-1 at the apical surface (green), and nuclei (blue). (D) Schematic representation of a mammary gland in early puberty (top) and during pregnancy/lactation (bottom).

known about how integrins might control GTPase functions in the context of differentiation and cell polarity. ILK has now been identified as a link between integrins and Rac1 activation in differentiating MECs [8]. The deletion of ILK from pregnant MECs prevents differentiation and production of milk proteins in vitro. $ILK^{-/-}$ MECs have reduced Rac1 activation and are also unable to stimulate Jak2/Stat5 signalling upon treatment with Prl. This ILK-null phenotype is rescued by viral introduction of a constitutively active Rac1, indicating that ILK signalling from integrins is required for downstream Rac1 activation. In vivo analysis of mammary glands from ILK-null mice showed a similar but less severe phenotype than observed in β1-integrin-null mice, where lactation was reduced and pups were undersized and malnourished [8].

In addition to the requirement for ILK in Prl-induced differentiation, $ILK^{-/-}$ MEC's cultured in 3D LnR matrix have a pronounced cell polarity defect similar to β1-integrin-null cells, with a reduced ability to form hollow lumens [8]. Polarity defects were also observed in MECs expressing DNRac [9], which suggests that ILK may be an important transducer of integrin signals to Racin establishing cell polarity. Integrin-mediated adhesion to the ECM influences cell polarity at a cellular and tissue level but little is known about how it does this [30,31]. ILK acting as an integrin effector could play a major role in orientating apico-basal polarity perhaps through activation of GTPases and the actin cytoskeleton. The correct polarisation of MECs into secretory acini is likely to be required for proper PrlR localisation and signalling, and both β1-integrin and ILK are required for this process (Fig. 2B).

Given the requirement for ILK in normal mammary gland function, we argue that this protein provides the critical link between cell adhesion and differentiated function, i.e. polarity and milk protein expression. Moreover since disrupting Rac has similar phenotypes to deleting ILK, an integrin–ILK–GTPase signalling axis may be crucial for the development of differentiated functions in the tissue.

The role of ILK in mammary glands is not well characterised and exactly how ILK modulates small GTPase activation in this context is not known. The availability of $ILK^{fl/fl}$CreER mice now enables inducible ILK-null primary MECs to be isolated and cultured in vitro. Lentiviral delivery of ILK mutants and IPP complex short hairpin ribonucleic acid (shRNA)'s will further enable a molecular dissection of the domains of ILK and specific ILK-interaction partners that are required for Rac activation and/or MEC differentiation. This approach will create a clearer understanding of how ILK and related proteins are required for β1-integrin signalling in the differentiation process.

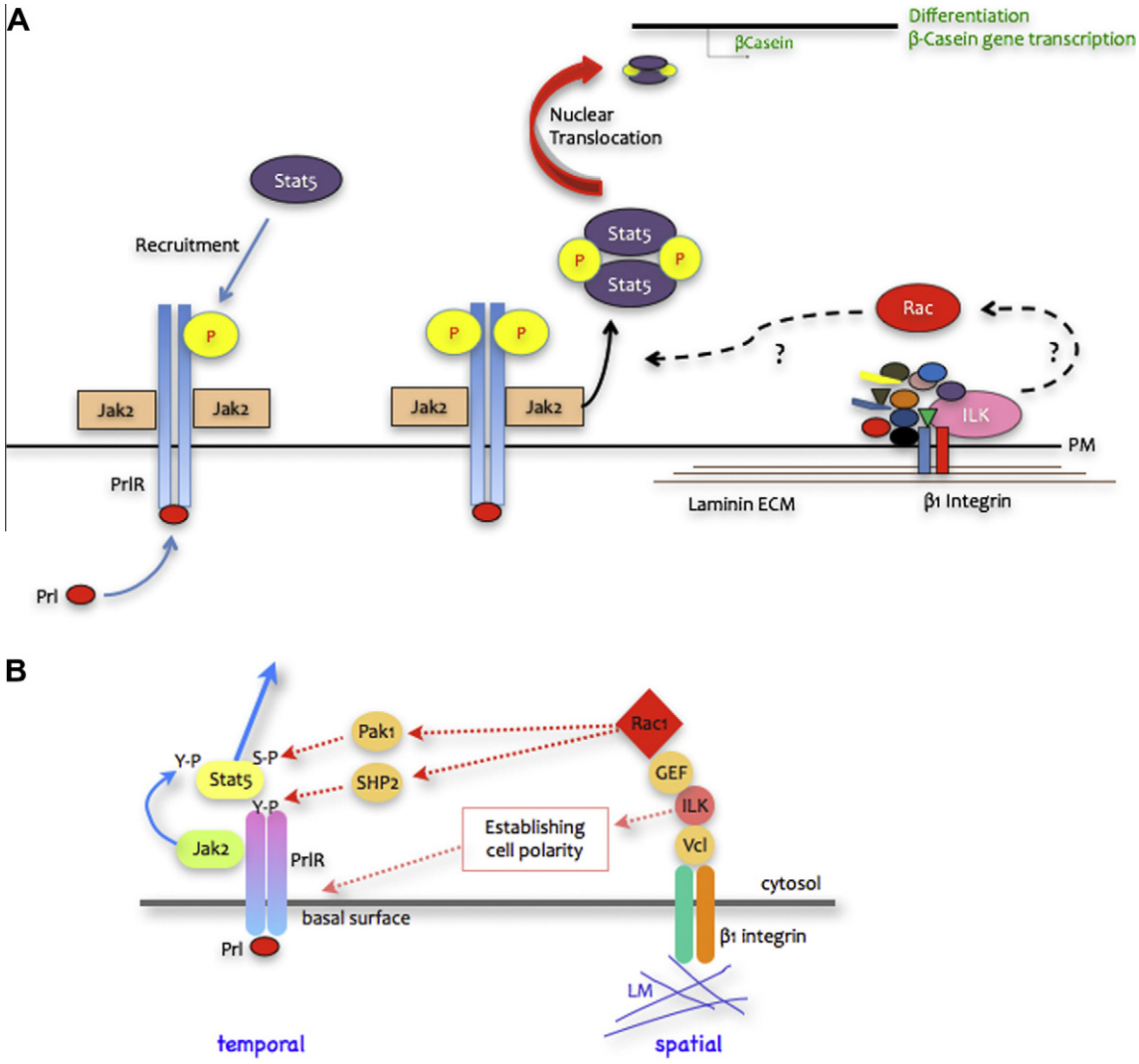

Fig. 2. Prolactin signalling and the requirement for ILK and Rac. (A) Prolactin binding causes PrlR phosphorylation by Jak2, enabling recruitment of Stat5, which becomes phosphorylated and translocates to the nucleus were it initiates the transcription of differentiation genes. The Jak2/Stat5 phosphorylation events at the PrlR are subject to regulation by Rac, which in turn requires ILK for its activity. (B) A schematic representation of the requirement for β1-integrin, ILK and Rac in MEC differentiation and polarisation. Rac functions downstream of ILK and regulates PrlR signalling event, possibly via Pak1 and SHP2. Integrins and ILK are also required for establishing cell polarity, which in turn is needed for the correct basal localization of PrlR and access to ligand.

Here we review the current understanding of the potential mechanisms by which ILK binding partners function downstream of ILK to relay ECM-integrin signals for GTPase activation.

5. Integrin linked kinase

ILK is a 50 kDa, 452 amino acid, multi-domain protein consisting of 5 N-terminal ankyrin repeats, a central Pleckstrin Homology domain (PH) and a C-terminal kinase domain, which may bind directly to β1-integrin tails. The different domains of ILK mediate protein–protein interactions between an increasing number of ILK-interacting partners (Fig. 3). ILK was first identified in 1996 through a yeast 2-hybrid screen for β1-integrin binding partners [32]. ILK's C-terminal region has sequence homology with the catalytic domains of Ser/Thr protein kinases and was characterised as a functional kinase in vitro. However, since the discovery of ILK, there have been consistent findings suggesting that contrary to some evidence, ILK is a pseudo-kinase and functions entirely as an adaptor or scaffolding protein at β1-integrin tails [33–35]. Central to this function is ILK's existence in an IPP complex where it is bound by PINCH1 or 2, and members of the Parvin family (α, β or γ). ILK has been implicated in various different cellular processes the best studied of which is its role in linking integrins to the actin cytoskeleton [36]. The importance of ILK is highlighted *in vivo* by the embryonic lethality of ILK-null mice [37]. In culture, ILK-null cells have marked actin cytoskeletal, cell spreading and motility defects [38].

Exactly how ILK gets to integrin adhesions remains somewhat unclear because prevention of ILK's interactions with numerous binding partners (Parvins, PINCH, Paxillin) ablates FA localisation. One possible mechanism is via interaction with β1 or β3 integrin tails, although very little is known with regard to the detail of this interaction. ILK's interaction with Paxillin is required for ILK localisation to focal adhesions [39]. Paxillin associates with Vinculin, which binds to Talin at integrin tails, and unpublished work from our lab shows that in both Vinculin- and Talin-depleted cells, ILK fails to localise to focal adhesions. A third possible mechanism of localisation concerns ILK's potential interaction with another focal adhesion protein Kindlin-2, which has been suggested because ILK (PAT4) and Kindlin-2 (UNC-112) interact in *Caenorhabditis elegans*

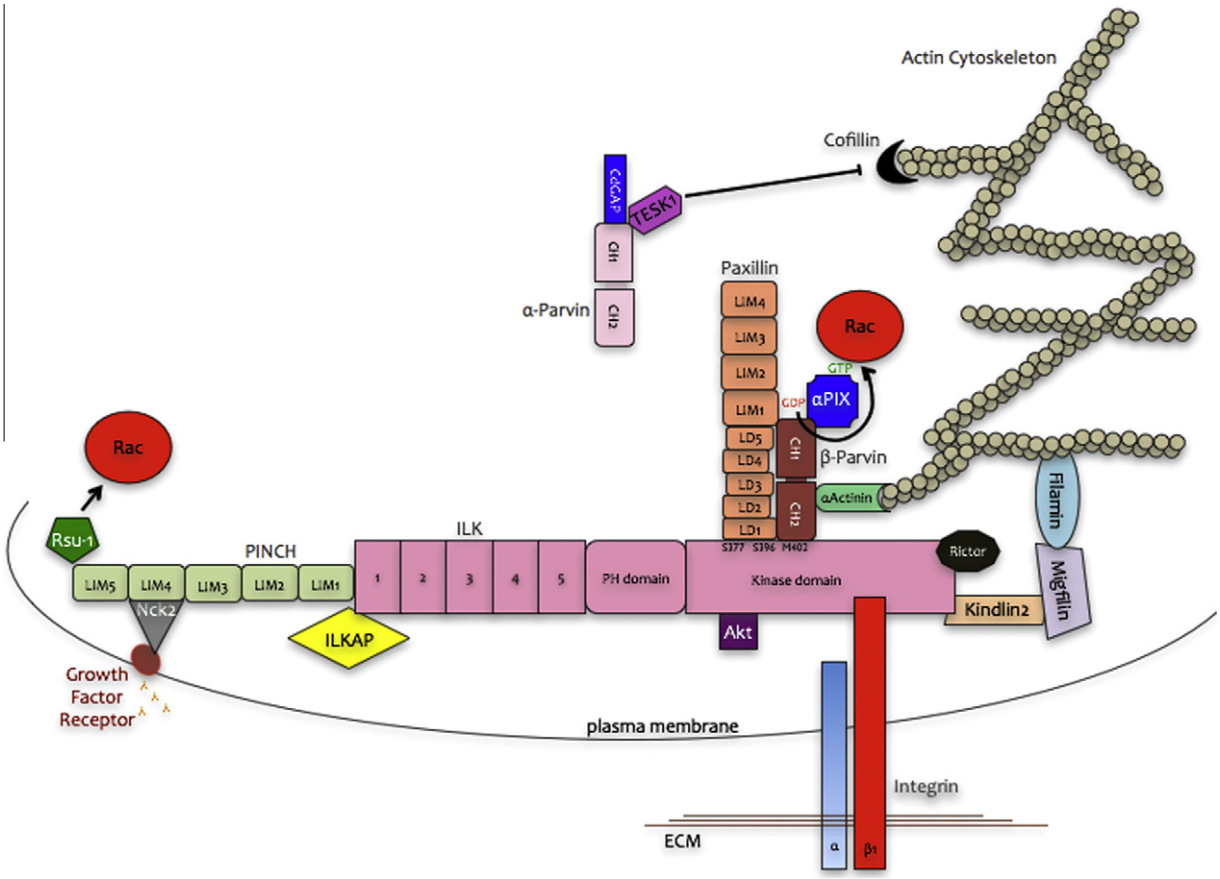

Fig. 3. The IPP complex signalling scaffold. ILK forms an IPP complex with PINCH1/2 and α/β/γ-Parvin. The IPP complex makes extensive interactions with numerous other proteins, some of which are depicted here. Signals from the IPP complex can control Rac activity, via Rsu-1, βParvin-αPix and CdGAP amongst other mechanisms. The IPP complex can also connect to growth factor signalling pathways via Nck2. ILK is also associated with the kinase Akt and the phosphatase ILKAP. Note that the interaction of α and βParvin with ILK is mutually exclusive, and αParvin can associate with ILK if βParvin is not present.

[40]. Kindlin-2 may bind to ILK via an unidentified location in ILKs C-terminus and a point mutation at F438 in the C-terminus ablates ILKs localisation to focal adhesions, perhaps by preventing the Kindlin-2 interaction [41].

A significant proportion of previous research on ILK has focussed on how ILK links integrins to the actin cytoskeleton. ILK cannot bind to actin itself, but provides a structural link between integrins and actin via the IPP complex protein αParvin, which binds to F-actin microfibers. ILK also connects to actin via a PINCH1–Nck2–N-WASP interaction, via Paxillin (which binds to the actin binding protein Vinculin), and via a Kindlin-2–Migfillin–Filamin interaction network [42]. Of most relevance to the current discussion, there have been numerous reports demonstrating that ILK and the IPP complex are able to regulate the activity of Rac in the context of cell spreading/migration. These mechanisms of Rac regulation could be how ILK is responsible for differentiation in the mammary gland.

ILK is required for transducing signals from the ECM that enable cellular differentiation in several tissue types, for example ILK deficient hepatocytes don't differentiate correctly in the presence of ECM [43]. High expression of ILK correlates with increased differentiation in various different normal tissue and tumour types [44]. In the brain, ILK is required for cerebellar development downstream of β1-integrins via a mechanism that involves Cdc42 activation[45]. In the skin dermal myofibroblasts differentiate into fibroblasts in response to injury, which requires transforming growth factor β1 (TGFβ1) signalling and integrin-mediated mechanotransduction. ILK is required for both these events, as ILK$^{-/-}$ myofibroblasts have reduced Smad2/3 activation and abnormal F-actin stress fibre organisation [46].

6. The ILK–PINCH–Parvin complex

The IPP complex is fundamental to ILKs functions. For instance ILK is dependent on PINCH and Parvin for its stability, and knockdown of components of the IPP complex causes reduction in the protein levels of the other components [38]. This means that ILK, PINCH and Parvin probably exert almost all of their functions while associated with each other, thus any modulation of Rho family GTPase activity is likely to occur through interactions mediated by the IPP complex as a whole entity. The IPP complex forms in the cytoplasm prior to ECM engagement by integrins [41].

At integrins, the IPP complex acts as a scaffold to which numerous proteins bind (Fig. 3). The IPP complex is evolutionarily conserved in *Drosophila* and *C. elegans* and indispensible for the proper assembly of integrin–actin adhesion structures [36]. In mammals there is the capacity to form distinct IPP complexes composed of different Parvin and PINCH proteins. Little is known about what dictates the particular PINCH or Parvin that is incorporated into the complex, and the functional significance of these different complexes is unknown. Potentially, ILK could respond to different cues and form different IPP complexes for different functions. Research into this aspect of IPP complex formation and function is urgently required and will yield interesting insights into the

structural/functional heterogeneity of β1-integrin signalling complexes. The IPP complex recruits proteins that are capable of directly regulating Rac activity, representing various mechanisms by which ILK may be required for MEC differentiation.

6.1. The PINCH proteins

ILK interacts with the PINCH (particularly interesting cysteine–histidine rich) proteins, PINCH1 and PINCH2, which are encoded by different genes. They are Lin11, Isl-1 & Mec-3 (LIM)-only proteins that contain five LIM domains followed by a short C-terminal region [47,48]. The crystal structure of the interaction between ILK and PINCH has been solved and is mediated by the N′-terminal most Ankyrin domain of ILK and the 1st LIM domain of PINCH1 [49]. PINCH1-null cells exhibit slower cell spreading/migration and this defect is similar to that seen when the PINCH–ILK interaction is impaired [50]. Reintroduction of a ΔC′-terminal ILK binding-deficient version of PINCH1 fails to rescue PINCH1-null spreading defects [51]. Depletion of ILK or PINCH1 reduces Rac1 activation and further suggests that the IPP complex mediates regulation of Rac [52]. PINCH's role in regulating actin cytoskeletal behaviour might therefore be due to regulation of Rho GTPases such as Rac (Figs. 3 and 4).

PINCH1 interacts with other proteins via its other LIM domains, for example, the LIM4 domain binds the 3rd SH3 domain of Nck2 (Grb4) [53,54] (Fig. 4). Nck2 is an adaptor protein that is able to interact with key components of growth factor receptor signalling pathways including epidermal growth factor receptor (EGFR) and son of sevenless homologue 1 (SOS) [55,56]. The ILK–PINCH1–Nck2 interaction represents an exciting potential linkage between integrins and both growth factor receptor signalling pathways, and Rac1/Cdc42 regulation. Nck2 can interact with DOCK180, which is a Rac GEF, thus providing a connection from the IPP complex to Rac activity [57].

ELMO proteins are scaffold proteins required for recruiting and enabling DOCK180 Rac GEF activity [58,59]. In polarised cells, ILK binds to ELMO-2, forming a complex involving RhoG [60]; RhoG has previously been shown to activate Rac through an ELMO–DOCK180 mechanism [61]. Together this provides the possibility of an associated network of Rho family GTPase regulating proteins centered on ILK. Thus an ILK–PINCH1–Nck-2–DOCK180–ELMO2–ILK complex could form at β1-integrin tails and modulate Rac activity, required for Prl induced milk production (Fig. 4). However, this 'interconnected network' of interactions has not been functionally observed and is inferred from published interactions in the literature.

PINCH1 may also regulate Rac1 activity via its interaction with Ras suppressor 1 (Rsu-1) (Fig. 4). Rsu-1 is a Leucine Rich Repeat protein that inhibits Ras and anchorage independent growth and localises (with PINCH and ILK) to integrin tails, enhancing cell attachment. Rsu-1 binds to PINCH1 (but not PINCH2) at its C-terminus and 5th LIM domain [62]. The PINCH1–Rsu-1 interaction is specifically required in MCF10A mammary cells for activation of Rac leading to cell spreading [63]. Rsu-1 is dependent on the ILK–PINCH1 interaction for its localisation, and impairment of this interaction by (siRNA or expression of truncated PINCH) prevents Rac activation. This is a clear example of how the formation of the IPP complex at integrin tails can lead to modulation of Rac activity. Interestingly however, Rsu-1 in Ras transformed breast cancer cell lines appears to inhibit Rac activation and cell spreading suggesting that the IPP complex via its Rsu-1–PINCH1–ILK interaction can negatively regulate cell migration and invasiveness in can-

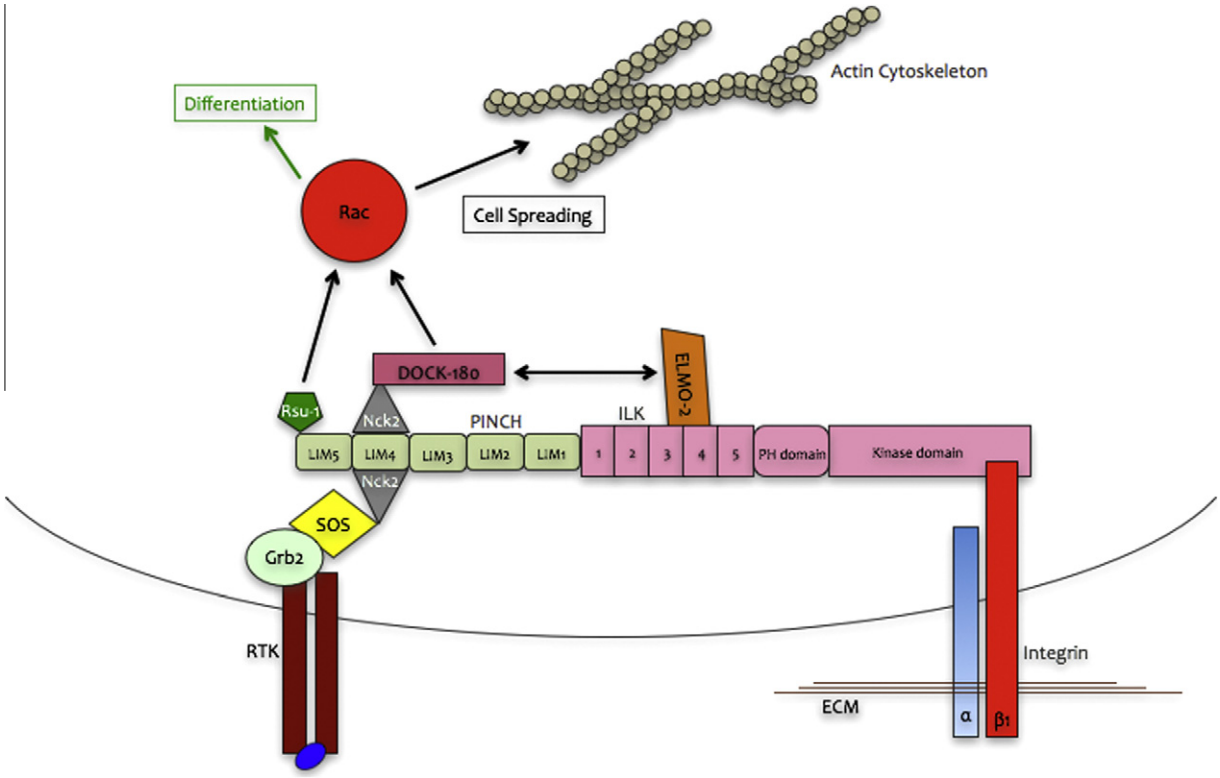

Fig. 4. The ILK–PINCH1 interaction influences Rac1 via DOCK180 or Rsu-1. Nck2 binds to the LIM4 domain of PINCH1 and can also interact with SOS, which provides a functional link between integrins and growth factor receptor signalling pathways. Nck2 binds the Rac GEF DOCK-180, which can activate Rac. ILK also interacts with ELMO-2, which associates with DOCK180 and enables its GEF activity. Note that the ELMO-2 binding site on ILK has not been properly determined. PINCH1 also interacts with Rsu-1 via its short C-terminal region. The Rsu-1–PINCH1 interaction leads to Rac activation and increased cell spreading via an as yet unidentified mechanism.

cer [64]. Taken together these findings show that Rsu-1 via it interaction with PINCH1 can regulate Rac activity levels in normal and transformed cells, but in an as yet undefined manner.

PINCH2's functional role is less well characterised than PINCH1, and PINCH2-null mice are phenotypically normal and develop into fertile adults [65], suggesting some PINCH1 compensation for PINCH2 loss. When PINCH2 is expressed in PINCH1-null cells it binds to ILK and rescues the IPP instability and localisation defects of PINCH1 depletion. However, it cannot functionally compensate for PINCH1 in cell spreading/migration [66]perhaps due to the inability to bind to Rsu-1. This suggests that PINCH2 is not able to modulate Rac/Cdc42 activity. Structural analysis of competition between PINCH1 and 2 binding to ILK showed that they both bind to the same site, with the same affinity, suggesting that expression levels dictate the PINCH composition of the IPP complex [67]. There is no information on the ratio of PINCH1 IPP complexes to PINCH2 IPP complexes and the functional significant of this in cells. Understanding the different PINCH composition of the IPP complex and the effect thereof, represents an interesting area for future research into IPP complex function.

Thus, the obligate interaction between ILK and PINCH1 might provide a mechanistic link through which integrins activate Rac in MECs. Two possibilities appear to exist, either via Nck2 and Dock180, or through Rsu-1 (Fig. 4). Some of these interactions have been shown to occur in breast cancer cell lines, but have not yet been investigated in primary MECs or in mammary glands in vivo.

6.2. The Parvins

The Parvins are a family of ILK binding focal adhesion proteins consisting of α, β and γParvin. They contain a variable N-terminus followed by 2 Calponin Homology (CH) domains, separated by a 60 amino acid linking region. α and βParvin are co-expressed ubiquitously while γParvin is not, consequently much less is known about γParvin (68). The interaction between ILK and α, β or γ Parvin is mutually exclusive [52]. α and β Parvin share 74% sequence identity at the amino acid level, while they have reduced homology with γParvin at 42% and 67%, respectively [68].The Parvin's second CH domain is required and responsible for binding to ILK, (via ILKs C-terminal kinase domain)[69,70] and for localisation to integrin adhesions [71]. This interaction is crucial because either a ILK K220A or M mutation, which prevents Parvin binding, or an αParvin gene knockout, cause kidney agenesis and postnatal lethality [35]. The precise role of this residue in Parvin binding is not clear, but the 3D structure of the ILK-Parvin interaction indicates that another region in the ILK pseudo-kinase domain, centered around M402/K403, provides the direct contact sites for Parvin [33].

Like ILK, αParvin contains a Paxillin binding subdomain (PBS), which is required for its interaction with Paxillin [72]. αParvin associates with Paxillin and ILK via different sites in its CH2 domain and both of these interactions are required but not sufficient for its FA localisation. Interestingly, mutation of the Parvin PBS prevents FA localisation and causes a reduction in cell spreading and migration [73]. βParvin does not differ markedly from αParvin at the amino acid and structural level, however, the functional differences are quite pronounced. While βParvin is a positive regulator of Rac/Cdc42 induced cell spreading/migration, αParvin appears to be a negative regulator [52,74].

6.2.1. βParvin

βParvin can regulate the activation state of Rac1 via an interaction between its CH1 domain and the guanine nucleotide exchange factor (GEF) αPix (also known as ARHGEF-6 or Cool-2) [75] (Fig. 5A). The Pix family of GEFs consists of αPix and numerous splice variants of βPix, which promote Rac and Cdc42 activation [76]. αPix binds to βParvin and co-localises with both βParvin and ILK at membrane ruffles and lamellipodia. Interestingly, two pathologically relevant mutations in αPix that cause 'non-specific mental retardation' prevent the interaction with βParvin [76].Exogenous expression of the αPix binding CH1 domain of βParvin alone, causes increased Rac/Cdc42 activation, which leads to increased actin cytoskeletal reorganisation [77].

Mammary epithelial cells ectopically overexpressing ILK have enhanced cell spreading and cytoskeletal reorganisation, which is Cdc42/Rac dependent [78,79]. Expression of a dominant negative version of αPix prevents any ILK-βParvin mediated increase in Rac activation and cell spreading [74]and overexpression of a ΔCH1 version of βParvin that cannot bind to αPix reduces cell spreading. Interestingly however, overexpression of full-length βParvin reduces its observed interaction with αPix [77]. This suggests that the CH2 domain of βParvin may modulate its interaction with αPix and subsequent ability to activate Rac. Therefore it seems possible that βParvin's ability to activate Rac via αPix requires an interaction with ILK and perhaps some other regulatory events mediated by ILK. Nonetheless, the observed ILK-βParvin-αPix-Rac1 pathway is an important linkage from integrins to Rho family GTPases that affects the actin cytoskeleton in cell migration (Fig. 5A).

In addition,Akt is required for STAT5 activation in response to Prl, and Akt has been shown to associate with ILK and βParvin [80,81]. ILK knockout in primary MECs has no effect on Akt phosphorylation, and the role of ILK and Parvins in association with Akt remains to be established in differentiating MECs [8].

Together these studies have highlighted a further potential mechanism for the IPP complex to influence Rac1 activation. This pathway of ILK–βParvin–αPix interactions could be how ILK regulates Rac1 activity in differentiation of MECs in response to Prl stimulation (Fig. 5A).

6.2.2. αParvin

One of the main functions of Parvin is to regulate actin cytoskeletal dynamics, which in turn affects cell spreading and migration. siRNA mediated knockdown of αParvin causes increased cell spreading and increased Rac activation, while overexpression of the ILK binding CH2 domain of αParvin inhibits cell spreading (perhaps by preventing βParvin from binding ILK) [52].So in contrast to βParvin, αParvin may be a negative regulator of Rac-induced cell spreading. In keeping with this hypothesis αParvin can interact with the Rac/Cdc42 GTPase activating protein CdGAP via its N′-terminus (amino acids 21–25) [82] (Fig. 3). This is functionally important because expression of a αParvin mutant that does not bind to CdGAP significantly increases cell spreading probably via preventing CdGAP inhibition of Rac1. This is consistent with the observed effect of both αParvin and CdGAP siRNA depletion and suggests that the ILK–αParvin–CdGAP interaction provides a negative influence on Rac/Cdc42 and cell spreading.

Together, the above discussion highlights how the Parvins mediate several interactions with proteins related to the actin cytoskeleton and small GTPase regulation. Parvins have two different functions, with βParvin encouraging Rac activation and cell spreading (Fig. 5A), and αParvin inhibiting these processes (Fig. 3). βParvin via its interactions with the Pix family of Rac/Cdc42 GEFs seems to be a likely candidate required for β1-integrin/ILK signalling to Rac in MEC differentiation. It is clear that there are numerous ways in which ILK acting through IPP mediated interactions influences the actin cytoskeleton and Rac activity, which may be why ILK is required for MEC differentiation and polarity.

7. ILK and Paxillin

In addition to ILK's interactions within the IPP complex, ILK also binds to other integrin adhesion proteins. Paxillin is an important

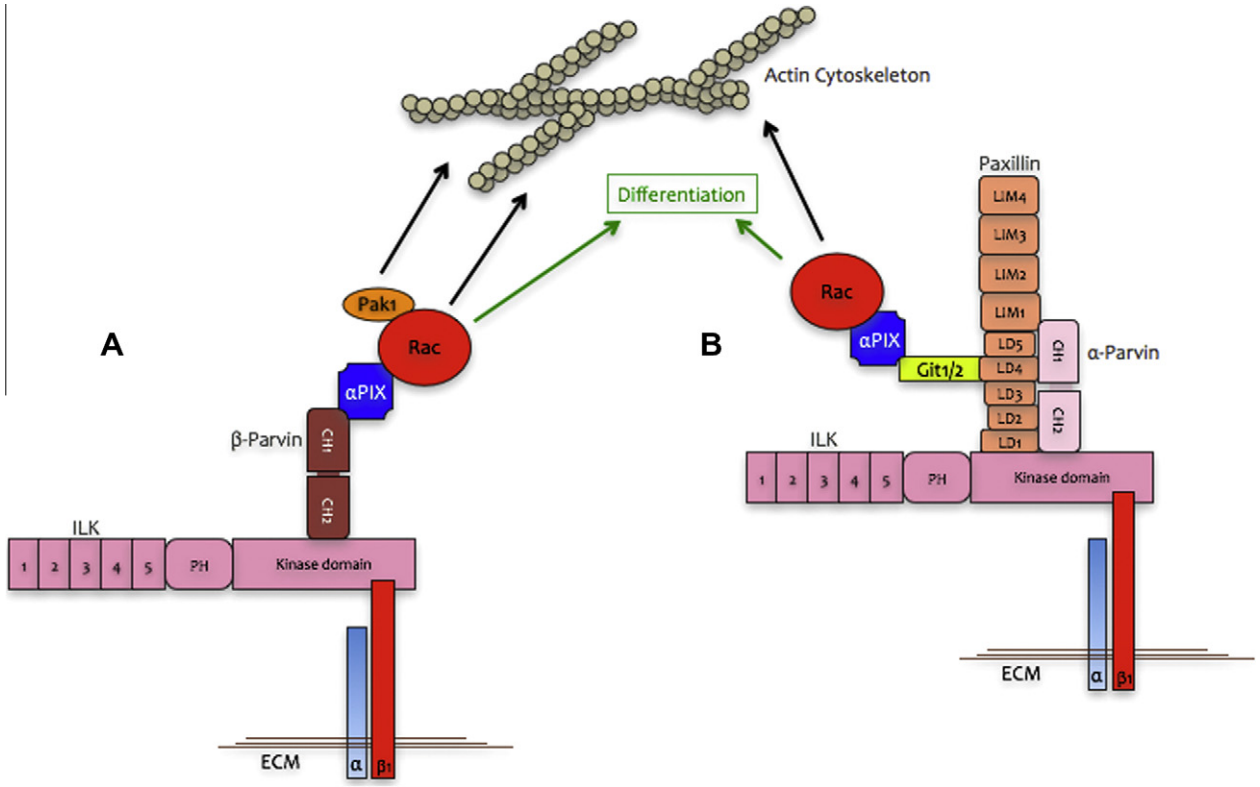

Fig. 5. The ILK–βParvin and ILK–Paxillin interaction regulate Rac GTPase activity via the Pix GTPase activating proteins. (A) βParvin interacts with the Rac/Cdc42 GEF αPix and localises to the membrane in spreading cells. αPix activates Rac and Cdc42, which affect the actin cytoskeleton. Active Rac activates Pak1, which can regulate cell spreading and can modulate the αPix–Rac interaction. (B) Paxillin interacts with the IPP complex via ILK and αParvin. The LD4 motif of Paxillin interacts with Git1 and Git2/Pkl and recruits α or βPix. Thus a Paxillin–Git–Pix complex can be recruited to the IPP complex, which activates Rac.

scaffold proteinat integrin adhesions, which undergoes extensive phosphorylation by numerous tyrosine kinases such as FAK (Focal adhesion kinase) [83]. Paxillin's N-terminus contains 5 leucine and aspartic acid rich motifs (LD motifs) that make extensive interactions with the IPP complex. The LD1 domain interacts with the PBS in ILKs kinase domain (from Ser377–Ser396) [39] (Fig. 3) and the LD1, 2 and 4 motifs bind to αParvin [84] (Fig. 5B). The importance of the ILK–Paxillin interaction is highlighted by the observation that both ILK and αParvin need to interact with Paxillin for their correct FA localisation [73].

Paxillin is a platform for signalling to Rho family GTPases and provides another potential link from ILK, via Paxillin to Rac/Cdc42 activation. Paxillin associates with the Pix family of GEFs via an interaction between its LD4 motif with the GIT family of Arf-GAP proteins (G-protein coupled receptor kinase interacting protein) [85] (Fig. 5B). GIT1 and GIT2 (a.k.a. Paxillin Kinase Linker, PKL) bind strongly to α or βPix and form an oligomeric complex of GIT-Pix proteins [86]. Paxillin interacts with the GIT1/2 proteins to form a Paxillin–GIT1/2–Pix complex that can also associate with Pak and Nck, and which is recruited to FAs [85]. This complex regulates Rac and Cdc42 signalling via the Pix proteins or via PAK, which is a downstream effector of Rac/Cdc42.

The exact nature of the interactions within the Paxillin–GIT1/2-Pix–PAK–Nck complex and the functional significance thereof remains to be properly established. However, it represents another way in which ILK, via its interaction with Paxillin, is potentially associated with signalling complexes that regulate Rac/Cdc42 activity. Deletion of ILK may alter the localisation and ability of this complex to regulate Rac activity. For example, in carotid arteries under vascular oxidative stress, ILK's interaction with Paxillinis required for βPix recruitment to the plasma membrane leading to activation of Rac [87]. The effect of ILK and the IPP complex on the formation and function of the Paxillin based GIT/Pix signalling complex is not known and remains to be investigated. However, because Paxillin interacts with both ILK and αParvin, it is possible that they could crosstalk with and influence Paxillin's recruitment of the GIT1/2-Pix proteins and subsequent Rac activation. Interestingly, a dominant negative form of βPix reverses ILK-induced activation of Rac, which suggests that ILK itself can also signal via GIT2/PKL directly to βPix to modulate Rac activity [88]. This study highlights a direct link between ILK and the Paxillin–Git–Pix complex in Rac activation. It is important to note, however, that Paxillin can also signal to Rho family GTPases via other mechanisms that do not involve the IPP complex.

8. Conclusion

It now emerges that the ILK scaffold has a key role in integrin-mediated Rac activation. We have discussed at least three potential routes for this signalling pathway; via PINCH1, βParvin and Paxillin, and it will now be crucial to determine which of these pathways is functionally relevant in the physiology of the mammary gland. Whilst we suspect that one or more of the pathways likely controls mammary development of lactation, it is increasingly apparent that they are involved in normal homeostasis as well. Several components have been found to be associated with breast cancer, and thus pinpointing their roles in breast biology may help to understand and treat this chronic condition.

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