

Molecular dissection of integrin signalling proteins in the control of mammary epithelial development and differentiation

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Cell-matrix adhesion is essential for the development and tissue-specific functions of epithelia. For example, in the mammary gland, β 1-integrin is necessary for the normal development of alveoli and for the activation of endocrine signalling pathways that determine cellular differentiation. However, the adhesion complex proteins linking integrins with downstream effectors of hormonal signalling pathways are not known. To understand the mechanisms involved in connecting adhesion with this aspect of cell phenotype, we examined the involvement of two proximal β 1-integrin signalling intermediates, integrin-linked kinase (ILK) and focal adhesion kinase (FAK). By employing genetic analysis using the *Cre-LoxP* system, we provide evidence that ILK, but not FAK, has a key role in lactogenesis in vivo and in the differentiation of cultured luminal epithelial cells. Conditional deletion of ILK both in vivo and in primary cell cultures resulted in defective differentiation, by preventing phosphorylation and nuclear translocation of STAT5, a transcription factor required for lactation. Expression of an activated RAC (RAS-related C3 botulinum substrate) in ILK-null acini restored the lactation defect, indicating that RAC1 provides a mechanistic link between the integrin/ILK adhesion complex and the differentiation pathway. Thus, we have determined that ILK is an essential downstream component of integrin signalling involved in differentiation, and have identified a high degree of specificity within the integrin-based adhesome that links cell-matrix interactions with the tissue-specific function of epithelia.

KEY WORDS: Adhesome, Breast, Differentiation, Focal adhesion kinase, Glandular development, Integrin, Integrin-linked kinase, Mammary, Mouse

INTRODUCTION

The development and function of epithelial tissues requires integration of signals from the local cellular micro-environment and from further afield via hormones. Among the different types of local cues for epithelial fate decisions, cell adhesion to the extracellular matrix (ECM) is of central importance. Integrins are a major class of receptors that adhere cells to the ECM and organise cell shape through the cytoskeleton, and they also influence cell fate decisions, including proliferation, survival, migration and differentiation (Streuli, 2009). An emerging concept is that integrins monitor the cellular ECM environment, thereby equipping cells with context-dependent checkpoints for growth factor and hormone signalling (Giancotti and Tarone, 2003; Katz and Streuli, 2007). However, a full understanding of normal developmental mechanisms, and abnormalities in epithelial diseases such as carcinoma, depends upon learning how cell-ECM interactions link with downstream endpoints, such as differentiation, and dissecting the specific intermediates in this process remains an important challenge.

To determine the molecular mechanisms by which integrins control the development and tissue-specific functions of epithelia, our laboratory is using the mammary gland system where the spatial (i.e. adhesive) signals that permit differentiation integrate with temporal (i.e. hormonal) developmental cues (Naylor and Streuli, 2006). We have previously shown that post-pregnancy differentiation of the luminal epithelial cells that synthesise milk proteins is influenced by converging signals derived from the ECM and the endocrine hormone prolactin. Indeed, β 1-integrin is crucial for mammary gland development and epithelial cell function (Faraldo et al., 1998; Klinowska et al., 1999; Li et al., 2005; Streuli et al., 1991). Furthermore, genetic approaches have indicated that β 1-integrin is required for differentiation, and that it cooperates via RAC1 (RAS-related C3 botulinum substrate 1) with prolactin signalling to control STAT5, a major regulator for mammary cell fate (Akhtar and Streuli, 2006; Naylor et al., 2005). The aim of the current work was to extend these previous studies and identify proximal β 1-integrin effectors that control cellular differentiation.

Integrins bind ECM proteins when in an extended active conformation, and simultaneously promote the formation of multiprotein adhesion complexes (adhesomes) at the plasma membrane, thus acting as both focal centres for cytoskeletal assembly and as signalling platforms (Humphries et al., 2003; Zaidel-Bar et al., 2007). Despite the importance of integrins in determining cell fates, it is not well understood which proximal integrin adaptor and signalling proteins are involved in controlling specific phenotypes. Two key mediators of β 1-integrin signal transduction are integrin-linked kinase (ILK) and focal adhesion kinase (FAK), and these proteins have different downstream effectors (Hannigan et al., 2005; Schatzmann et al., 2003). Integrin signalling through focal adhesions has not widely been studied in

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the context of a multicellular 3D environment, and here we have used a genetic analysis to determine whether these proteins are involved in glandular differentiation, both in vivo as well as in a 3D culture model of primary mammary epithelial cells (MECs).

We demonstrate that ILK and FAK are located within the same adhesion complexes; however, only one of these proteins, ILK, but not FAK, is required for the tissue-specific function of MECs. This indicates for the first time that one specific adhesome component mediates this key role for integrin, i.e. the control of cellular differentiation. Moreover, ILK has not previously been studied in any glandular epithelium in vivo, and our study also provides new insights into its involvement in tissue development, as well as pointing to a novel differentiation function for this integrator of adhesion signals.

MATERIALS AND METHODS

Conditional knockout mice

The *Ilk^{flx/flx}*, *FAK^{flx/flx}*, *Blg-Cre^{Tg/+}* and *Wap1Cre^{Tg/+}* mice have been previously described (McLean et al., 2004; Naylor et al., 2005; Selbert et al., 1998; Terpstra et al., 2003; Wintermantel et al., 2002). For the in vivo analysis, Cre-mediated specific *Ilk* or *Fak* gene deletion in luminal mammary epithelial cells was as described for the β 1-integrin gene (Naylor et al., 2005). Cre-mediated specific *Ilk* gene deletion in luminal mammary epithelial cells was achieved by crossing *Ilk^{flx/flx}* mice with *Cre^{Tg/+}* mice to produce *Ilk^{flx/+}; Cre^{Tg/+}* mice. Double heterozygous *Ilk^{flx/+}; Cre^{Tg/+}* mice were then crossed with *Ilk^{flx/flx}* mice to produce offspring that carried *Ilk^{flx/+}*, *Ilk^{flx/+}; Cre^{Tg/+}*, *Ilk^{flx/flx}* or *Ilk^{flx/flx}; Cre^{Tg/+}* alleles. To avoid problems in nursing pups that were caused by mothers with potentially defective mammary glands, only the male mice of the breeding pairs carried the *Cre* transgene. The genotypes of offspring were determined by PCR amplification of ear genome, and *Ilk^{flx/flx}; Cre^{Tg/+}* females were used. Among the controls, *Ilk^{flx/+}*, *Ilk^{flx/+}; Cre^{Tg/+}* and *Ilk^{flx/flx}* were apparently indistinguishable from wild type, so we used *Ilk^{flx/flx}*. An identical strategy was employed to generate FAK-null mammary epithelium. Cre expression is usually highly penetrant, although there is some degree of variability, and in control mice with the *Cre* transgene crossed to *Rosa26* mice, X-gal stained most of the epithelial component of the glands blue. To confirm gene deletion in the *Ilk^{flx/flx}* and *Fak^{flx/flx}* crosses, PCR was carried out on DNA isolated from glands of each mouse used in the in vivo study.

Female mice were mated between 8 and 12 weeks of age. Growth rate analysis of their pups was performed by standardising litter sizes to eight pups/litter, and weighing pups at least every second day through lactation. Mouse lines were maintained by repeated backcrossing with C57Bl/6 mice in a specific pathogen-free environment. Mice were housed and maintained according to the University of Manchester and UK Home Office guidelines for animal research.

Primary cell culture and virus infection

Primary MECs were isolated and cultured from pregnant mice as described (Pullan and Streuli, 1996). Cells were cultured in growth media containing 5 μ g/ml insulin, 1 μ g/ml hydrocortisone (Sigma), 3 ng/ml epidermal growth factor (EGF), 10% foetal calf serum (Biowest), 50 U/ml penicillin/streptomycin, 0.25 μ g/ml fungizone and 50 μ g/ml gentamycin in Ham's F12 medium (Gibco). Differentiation medium, DMEM/F12 (Gibco) containing insulin and hydrocortisone with or without 3 μ g/ml prolactin (Sigma), was added for 24 hours before harvest.

For viral infection in situ, the required amount of virus was diluted into a minimal volume of low- Ca^{2+} Ham's F12 and incubated on cells cultured in monolayer for 2 hours. Low Ca^{2+} disruption of intercellular junctions in confluent cultures aided viral infection by allowing viruses to penetrate all surfaces of the cells. The same volume of growth media containing twice the concentrations of additives and Ca^{2+} as above was then added onto the virus-containing medium and incubated overnight for maximal infection.

For *Ilk* or *Fak* gene deletion, primary MECs from 15.5- to 17.5-day pregnant were infected with adenovirus to enable up to 90% infection rates. This strategy was used because the logistics of obtaining sufficient *Ilk^{flx/flx}; Cre^{Tg/+}* females precluded primary culture experiments from *Ilk^{-/-}*

glands. *Ilk^{flx/flx}* or *FAK^{flx/flx}* cells were infected with either Ad-Cre (Ad5 viruses expressing Cre recombinase under the control of mouse CMV promoter; Ad-CreM1 from Microbix Biosystems) or Ad-*lacZ* (Ad5 viruses expressing β -galactosidase from Dr Hazel Weir and Dr Anne Ridley) and plated onto reconstituted BM-matrix (Matrigel, BD Biosciences) as described previously (Watkin and Streuli, 2002). In control experiments, infection of primary cultures of mammary cells isolated from wild-type mice with Ad-Cre or Ad-*lacZ* had no effect on the levels of ILK or β -casein (see Fig. S2 in the supplementary material).

For rescue experiments, cells were first infected in situ in monolayer with control Ad-*lacZ* or with Ad-Cre in order to allow sufficient time for endogenous ILK protein to be removed. Cells were trypsinised and re-infected in suspension for 1 hour with either (1) Ad-ILK-GFP encoding a wild-type ILK fused to GFP containing a farnesylation sequence (Boulter et al., 2006) and control Ad-GFP (both Ad5 viruses made by Vector Biolabs, see below); or (2) Ad-V12Rac and control Ad-*lacZ* (Ad5 viruses derived from pJM17) (Wojciak-Stothard and Ridley, 2002). It was not possible to use co-infection because the kinetics of de novo protein expression by Ad-virus was not compatible with the time required for ILK protein turnover.

In each case, cells were plated onto BM-matrix (Matrigel) in growth media. After 48 hours, medium was changed to differentiation medium with or without prolactin and incubated for a further 24 hours before harvest for immunoblotting and immunofluorescence.

Generation of ILK-GFP adenovirus

Recombinant adenoviruses (serotype5 de1/E3) expressing ILK-GFP and GFP were constructed using the pDUAL-CCM adenoviral cloning vector (Vector Biolabs, PA). The first-generation viral stock was generated using the Ad-HQ system by Vector Biolabs. Membrane-targeted ILK-GFP was subcloned from pcdna3.1-ILK-GFP-F (Boulter et al., 2006) into pDUAL-CCM using *HindIII* restriction sites to create pDUAL-CCM-ILK-GFP. To generate the GFP vector, GFP was amplified by PCR from pCDNA-3GFP (Akhtar et al., 2000) with a quadropeptide linker using following primers: forward, 5'-CCCAAGCTTCCCACCCATGAGTAAAGGAGAAGAAGACTT-3'; and reverse, 5'-ACCGGTACCCCGGGTTTGTATAGTTCATCCATGCC-3'. The product was cloned into pDUAL-CCM using *HindIII* and *KpnI* restriction sites to create pDUAL-CCM-GFP. In each case, the localisation of the expressed proteins was checked by transfection into 293 cells prior to generating virus. Large-scale adenoviral stocks were amplified in E1-competent 293 human embryonic kidney cells and purified on a caesium chloride gradient as previously described (Watkin and Streuli, 2002).

Morphological and histological analysis

Whole-mount analysis was performed by spreading inguinal mammary glands on polysine slides, fixing in 10% neutral buffered formalin overnight, defatting in acetone before staining with carmine alum (0.2% carmine, 0.5% aluminium sulphate) overnight. The whole mount was dehydrated using a graded ethanol series followed by immersion in Slide Bright (Genta Medical) for 1 hour and stored in methyl salicylate before photography using a Leica dissecting microscope. Whole mounts were subsequently paraffin embedded before sectioning (5 μ m) and subjected to standard Haematoxylin and Eosin staining. Histology was imaged with a Zeiss AxioScop2 microscope using PlanNeoFluar 40 \times lens (numerical aperture 0.75) fitted with a Zeiss AxioCam colour camera, and analysed with Openlab (3.1.7) software (Improvision).

ORO staining was performed by staining 6 μ m mammary cryosections in freshly diluted ORO solution [six parts 0.5% ORO stock solution (Sigma) and four parts water] for 15 minutes. Sections were rinsed twice with 60% isopropanol, once with water and then counterstained with Mayer's Haematoxylin for 1 minute before photography as above.

Protein analysis

Proteins were extracted using 1 \times NP-40 lysis buffer (10% w/v glycerol, 50 mM Tris-HCl, 100 mM NaCl, 1% w/v Nonidet-P40, 2 mM MgCl_2 and fresh protease/phosphatase inhibitors; pH 7.5) for cells in monolayer or 2 \times NP-40 buffer for cells on BM-Matrix. Equal amounts of proteins were used and equivalent loading assessed by referral to controls, such as Calnexin (Bioquote SPA-860). Immunoblotting was as described (Zoubiane et al.,

2004) and specific binding was detected using horseradish peroxidase secondary antibodies (Jackson ImmunoResearch) with SuperSignal West Pico Chemiluminescent substrate (Pierce) and Biomax Light film (Kodak).

Primary antibodies were β -casein (Streuli and Bissell, 1991), β -galactosidase (Promega #z3783), Cre (Chemicon #MAB3120), ILK (BD Transduction #611802), FAK (a gift from A. Ziemiecki, University of Bern, Bern, Switzerland), pT202/Y204-p42/44-MAPKinase (Cell Signaling #9101), ERK2 (Santa Cruz #sc-154), pS473-PKB (Cell Signaling #9271), pT308-PKB (Cell Signaling #9275), PKB (Cell Signaling #9272), pS9-GSK3 β (Cell Signaling #9336), GSK3 β (BD Transduction #610201), RAC1 (23A8; Upstate #05-389), Myc (9E10, Roche #1-667-203), Actin (Sigma #A2066), STAT5A (Santa Cruz #sc-1081), pY694/Y699-Stat5 (Upstate), Adipophilin (Progen Biotechnik #GP40).

RAC activity assay was performed as previously described using Pak1-PBD agarose (Akhtar and Streuli, 2006; Benard and Bokoch, 2002).

Immunostaining

Cells cultured in monolayer or BM-Matrix were fixed with 4% formaldehyde (Polysciences) in PBS, stained as described and imaged after mounting with Prolong Anti-Fade (Molecular Probes) by conventional or confocal microscopy (Akhtar and Streuli, 2006). Conventional microscopy was on a Zeiss Axioplan2 microscope using PlanApochromat 100 \times , 63 \times and 40 \times lenses (numerical aperture 1.40) with Immersol 518F oil. Z sections (0.2 μ m) were captured with a Hannamatsu Orca ER camera and deconvolved with Velocity (3.5.1) software. In some cases, acini were visualised by confocal imaging using Leica SP2 AOBs confocal microscope, with the pinhole set to 1 Airy Unit. Immunofluorescence of mammary tissue was imaged by confocal microscopy and analysed with Velocity. Immunohistochemistry of mammary gland was performed on paraffin-embedded tissue using DakoCytomation EnVision + System-HRP (DAB) kit for immunohistochemistry and protein expression was detected with a Zeiss Axioscop2 microscope.

STAT5 nuclear translocation was performed by infecting primary mammary epithelial cells grown in monolayer with viruses for 24 hours, using the EHS overlay assay (Streuli et al., 1995b). After this time, virus containing media was replaced with differentiation medium containing BM-Matrix (1:50 dilution) without prolactin for a further 24 hours. Fifteen minutes before harvest, cells were stimulated with prolactin, fixed and immunostained with STAT5A, β -galactosidase and Cre (Naylor et al., 2005).

Antibodies different to those used in immunoblotting were: β 1-integrin (Klinowska et al., 1999), mouse-ILK (Upstate Biotechnology #05-575), rabbit-ILK (Cell Signaling Technology), phospho-FAK Y397 (Biosource International #44-624G), rhodamine-RX conjugated phalloidin (Molecular Probes), Alexa Flour 488-conjugated wheat germ agglutinin (Molecular Probes #W11261) and β -catenin (BD Biosciences #C19220). Secondary antibodies were conjugated to Cy2, rhodamine-RX and Cy5 (Jackson ImmunoResearch).

RESULTS

ILK is required for normal post-pregnancy mammary gland development

In primary cultures of MECs, ILK and FAK both localise to the same adhesion complexes (Fig. 1A). Moreover, deleting β 1-integrin from MECs alters the location or activation of these integrin-signalling proteins (Naylor et al., 2005). β 1-Integrin is required for milk protein expression, suggesting that either ILK or FAK might mediate the differentiation signals. We therefore tested this hypothesis using the *Cre-LoxP* system.

Initially, we examined the role of ILK in mammary development and differentiation in vivo by deleting its (*LoxP*-flanked) gene with *Cre* driven by promoters for the milk proteins β -lactoglobulin (BLG) or whey acidic protein (WAP), both of which are specific for luminal MECs. ILK gene deletion was analysed in glands of *Ilk^{fl/fl}; Blg-Cre^{Tg/+}* or *Ilk^{fl/fl}; Wap-Cre^{Tg/+}* (hereafter called *Ilk^{-/-}*) mice, and the corresponding wild-type littermates (*Ilk^{fl/fl}*). PCR analysis identified the recombined 230 bp product in *Ilk^{-/-}* glands and the floxed *Ilk*

allele (fx, 2.1 kb) in both wild-type and *Ilk^{-/-}* tissue (Fig. 1B; the *Ilk* gene is still intact in myoepithelial and stromal cells of *Ilk^{-/-}* glands). Consistent with excision of *Ilk^{fl/fl}* alleles, the ILK protein level was lower in the *Ilk^{-/-}* glands than those of wild-type littermates (Fig. 1C). ILK was not expressed in the luminal epithelial cells within *Ilk^{-/-}* glands (Fig. 1D) but was retained in myoepithelial cells localised at the periphery of alveoli (see Fig. S1 in the supplementary material), demonstrating that its deletion was specific to luminal MECs.

To determine whether loss of ILK affects mammary gland function, we examined whether the dams were able to nurse their pups efficiently. The pups from *Ilk^{-/-}* dams gained weight slower than those suckling on wild-type mothers (Fig. 1E), suggesting that ILK is required for normal post-pregnancy mammary gland development.

To ascertain the mechanism of the nursing deficiency, we performed whole-mount and histological analyses. Normal ductal elongation, side branching and lobuloalveolar development occurred in *Ilk^{-/-}* glands in pregnancy and at lactation (Fig. 1F). Mammary alveoli formed in both *Ilk^{fl/fl}; Blg-Cre^{Tg/+}* or *Ilk^{fl/fl}; Wap-Cre^{Tg/+}* mice, indicating that ILK is not required for the cell fate decision leading to alveolargenesis. However, histological examination revealed two major defects in alveolar development in *Ilk^{-/-}* transgenics (Fig. 1G). First, the alveoli displayed morphological imperfections. In late pregnancy, the lumina were smaller, and by lactation day 2 the epithelial cells protruded into the lumen, with continued abnormalities visible at lactation day 8. This is similar to the phenotype of β 1-integrin-null mammary glands. Second, there was an absence of cytoplasmic lipid droplets during mid/late pregnancy (as in the β 1-integrin-null mammary glands), and the alveolar lumina were smaller throughout lactation, suggesting a lactation defect. The basal distribution of laminin-1, α 6-, β 1- and β 4-integrins was normal (not shown), indicating that the morphological and differentiation deficiency occurred downstream of integrins.

These data show that the phenotype of the *Ilk^{-/-}* and *Itg β 1^{-/-}* mammary glands is similar, although the nursing defect is not as marked as following integrin deletion. In order to identify proximal β 1-integrin effectors for differentiation, we focused on the role of ILK in the functional differentiation of MECs.

ILK is required for the functional differentiation of epithelia

To investigate whether ILK is necessary for MEC differentiation in vivo, we examined the ability of *Ilk^{-/-}* alveoli to make milk products including fat and protein. ILK ablation had a severe effect on synthesis and secretion of milk fat, shown by small quantities of intra-luminal fat droplets (Fig. 1G) and Oil Red O staining (Fig. 2A). The size and numbers of adipophilin-coated cytoplasmic lipid droplets were also reduced in *Ilk^{-/-}* alveoli (Fig. 2B), concomitant with less adipophilin protein (Fig. 2C). The levels of WAP were lower in the absence of ILK, indicating that milk protein synthesis was defective in vivo (Fig. 2D).

To confirm that ILK is required for milk synthesis, we examined 3D cultures of cells isolated from *Ilk^{fl/fl}* mammary glands, where the *Ilk* gene was deleted using *Cre*-expressing adenovirus (Ad-*Cre*). This resulted in loss of ILK, but no changes in the levels of FAK (Fig. 2E) or the basal location of β 1-integrin. MECs resemble in vivo alveoli when plated on basement membrane (BM)-matrix, and differentiate in the presence of prolactin. Neither the infection procedure nor *Cre*-expression in control MECs adversely affected milk protein expression (see Fig. S2 in the supplementary material). Prolactin treatment led to β -casein synthesis in the mock-infected *Ilk^{fl/fl}* cells (Fig. 2F); similarly, control *Ilk^{fl/fl}* acini infected with Ad-

lacZ synthesised milk proteins (Fig. 2G). However, deletion of ILK prevented milk protein synthesis (Fig. 2F,G), indicating that the defect observed *in vivo* is a direct consequence of ILK-loss in the luminal MECs.

We also examined whether expression of a wild-type ILK fused to farnesylated-GFP (hereafter called ILK-GFP, see Materials and methods) could rescue the milk protein defect after ILK deletion (Boulter et al., 2006). ILK-GFP localised to focal adhesions of monolayer-grown wild-type MECs and the basal surface of acini in 3D culture (not shown). ILK was first deleted in *Ilk^{fx/fx}* MECs with Ad-Cre and then those cells were re-infected with either control or ILK-GFP-expressing viruses. β -Casein was synthesised in the controls (Fig. 3F, lane 2) and cultures infected with either Ad-GFP or Ad-ILK-GFP (Fig. 3A,B,F, lanes 3,4). As above, ILK deletion prevented β -casein synthesis, and the expression of GFP was unable to restore the defects (Fig. 3C,D,F, lanes 5,6). However, the

expression of ILK-GFP in the ILK-null cells restored basally located ILK (Fig. 3E) and rescued the ability of cells to synthesise milk proteins (Fig. 3E,F, lane 7).

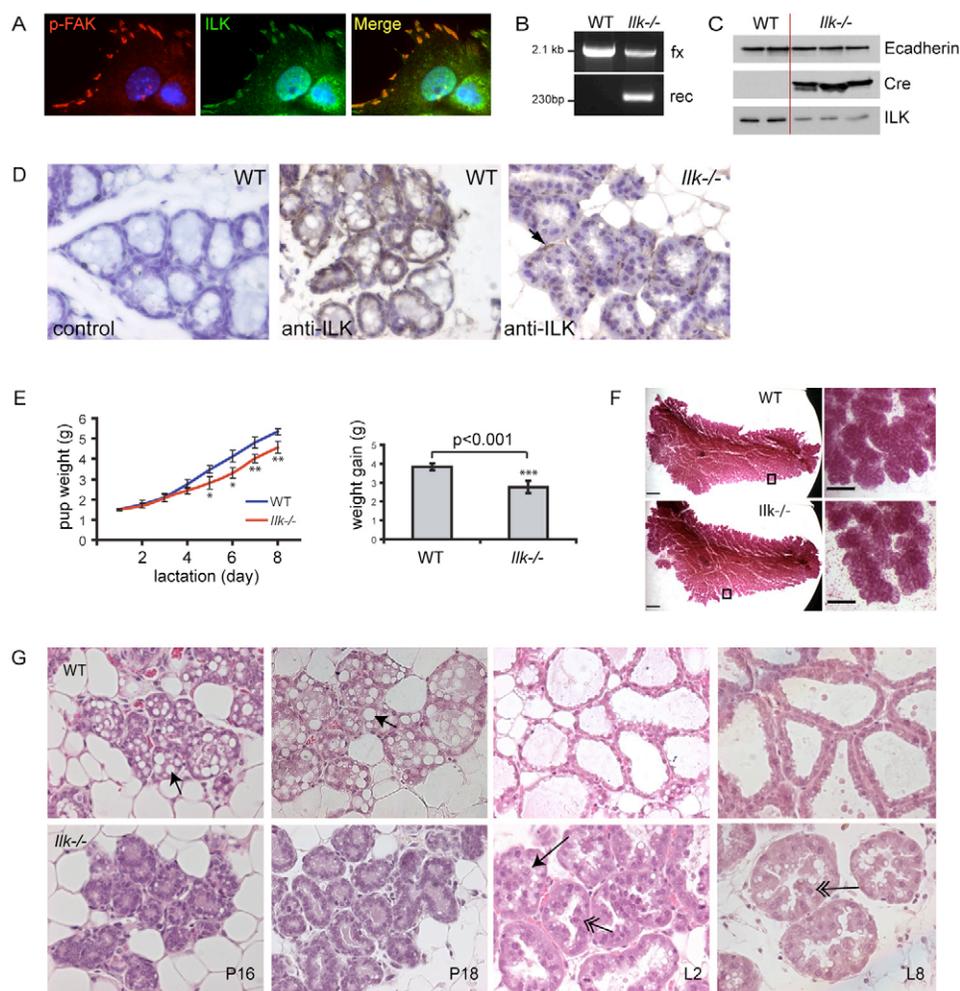
These results demonstrate that ILK is necessary for efficient epithelial differentiation. *In vivo*, ILK deletion leads to the impairment of both milk fat/protein production and a mild reduction in pup growth rates, whereas in primary cell culture, MECs lacking ILK are unable to synthesise milk proteins and this function can be rescued by ILK-GFP.

Specific integrin signalling enzymes are required for the functional differentiation of epithelia

ILK is a key mediator of integrin signalling; thus, its requirement for mammary differentiation might just reflect the essential role of β 1-integrin in this process. To test the specificity for ILK in linking integrins with differentiation, we examined the role of an equally

Fig. 1. Conditional depletion of ILK in mammary epithelia *in vivo*.

(A) Colocalisation of ILK (green) and p-FAK (red) in the same adhesion complexes of primary MECs. (B-G) Mice harbouring conditional *LoxP*-flanked alleles of the *Ilk* gene (*Ilk^{fx/fx}*) were crossed with transgenic lines expressing Cre recombinase under the control of mammary-specific promoters for the milk proteins, β -lactoglobulin (BLG) or whey acidic protein (WAP). This causes *Ilk* gene deletion in nulliparous mice between 8 and 12 weeks of age (BLG-Cre), or following activation of luminal cell differentiation during pregnancy (WAPICre). Data from *Ilk^{fx/fx};Blg-Cre^{Tg}* glands were similar to *Ilk^{fx/fx};WapiCre^{Tg}* glands. The *Ilk* gene is still intact in myoepithelial and stromal cells of *Ilk^{-/-}* mice. Wild type refers to *Ilk^{fx/fx}* control littermates. (B) Genomic PCR of wild-type and *Ilk^{fx/fx};WapiCre^{Tg}* mammary glands. The bands correspond to the floxed allele (fx, 2.1 kb) and the recombined allele (rec, 230 bp). Similar data were obtained from glands of *Ilk^{fx/fx};Blg-Cre^{Tg}* mice. (C) Immunoblot of wild-type and *Ilk^{fx/fx};WapiCre^{Tg}* mammary gland extracts shows reduced levels of ILK protein in *Ilk^{-/-}* compared with wild type. Protein extracts were normalised to luminal epithelial content using E-cadherin as an epithelial marker. (D) Immunohistochemistry shows ILK in luminal MECs of alveoli in wild-type mammary gland but not in sections stained without primary antibodies (control) or in ILK-null MECs (*Ilk^{fx/fx};WapiCre^{Tg}* shown). ILK staining at edges of alveoli (arrow) corresponds to myoepithelial cells where ILK is not deleted (also see Fig. S1 in the supplementary material). (E) (Left panel) Weight analysis shows that pups nursing on *Ilk^{fx/fx};Blg-Cre^{Tg}* mothers (four litters with eight pups in each) gain weight slower than those nursing on wild type ($n=5$); error bars=s.e.m.; * $P<0.05$ (Student's paired *t*-test); ** $P<0.01$. (Right panel) The data reanalysed to show weight gain/pup from day 1 to day 8; error bars=s.e.m.; *** $P<0.001$ ($P=0.0008$). (F) Carmine staining of whole-mounted mammary gland of wild-type and *Ilk^{fx/fx};Blg-Cre^{Tg}* mice at lactation day 2. ILK deletion does not affect the overall morphology of these or *Ilk^{fx/fx};WapiCre^{Tg}* glands (not shown). Scale bars: 2.6 mm (insets, 0.44 mm). (G) Haematoxylin and Eosin staining of mammary gland at days 16 and 18 of pregnancy (P16, P18) and days 2 and 8 of lactation (L2, L8). In pregnancy, alveoli still form in *Ilk^{fx/fx};Blg-Cre^{Tg}* glands, but lipid droplets do not accumulate (wild-type alveoli, arrows). During lactation, some alveoli in *Ilk^{-/-}* glands have cells that fill the lumina (arrow), whereas others show cells protruding into the luminal space (double-headed arrows).



(A) Colocalisation of ILK (green) and p-FAK (red) in the same adhesion complexes of primary MECs. (B-G) Mice harbouring conditional *LoxP*-flanked alleles of the *Ilk* gene (*Ilk^{fx/fx}*) were crossed with transgenic lines expressing Cre recombinase under the control of mammary-specific promoters for the milk proteins, β -lactoglobulin (BLG) or whey acidic protein (WAP). This causes *Ilk* gene deletion in nulliparous mice between 8 and 12 weeks of age (BLG-Cre), or following activation of luminal cell differentiation during pregnancy (WAPICre). Data from *Ilk^{fx/fx};Blg-Cre^{Tg}* glands were similar to *Ilk^{fx/fx};WapiCre^{Tg}* glands. The *Ilk* gene is still intact in myoepithelial and stromal cells of *Ilk^{-/-}* mice. Wild type refers to *Ilk^{fx/fx}* control littermates. (B) Genomic PCR of wild-type and *Ilk^{fx/fx};WapiCre^{Tg}* mammary glands. The bands correspond to the floxed allele (fx, 2.1 kb) and the recombined allele (rec, 230 bp). Similar data were obtained from glands of *Ilk^{fx/fx};Blg-Cre^{Tg}* mice. (C) Immunoblot of wild-type and *Ilk^{fx/fx};WapiCre^{Tg}* mammary gland extracts shows reduced levels of ILK protein in *Ilk^{-/-}* compared with wild type. Protein extracts were normalised to luminal epithelial content using E-cadherin as an epithelial marker. (D) Immunohistochemistry shows ILK in luminal MECs of alveoli in wild-type mammary gland but not in sections stained without primary antibodies (control) or in ILK-null MECs (*Ilk^{fx/fx};WapiCre^{Tg}* shown). ILK staining at edges of alveoli (arrow) corresponds to myoepithelial cells where ILK is not deleted (also see Fig. S1 in the supplementary material). (E) (Left panel) Weight analysis shows that pups nursing on *Ilk^{fx/fx};Blg-Cre^{Tg}* mothers (four litters with eight pups in each) gain weight slower than those nursing on wild type ($n=5$); error bars=s.e.m.; * $P<0.05$ (Student's paired *t*-test); ** $P<0.01$. (Right panel) The data reanalysed to show weight gain/pup from day 1 to day 8; error bars=s.e.m.; *** $P<0.001$ ($P=0.0008$). (F) Carmine staining of whole-mounted mammary gland of wild-type and *Ilk^{fx/fx};Blg-Cre^{Tg}* mice at lactation day 2. ILK deletion does not affect the overall morphology of these or *Ilk^{fx/fx};WapiCre^{Tg}* glands (not shown). Scale bars: 2.6 mm (insets, 0.44 mm). (G) Haematoxylin and Eosin staining of mammary gland at days 16 and 18 of pregnancy (P16, P18) and days 2 and 8 of lactation (L2, L8). In pregnancy, alveoli still form in *Ilk^{fx/fx};Blg-Cre^{Tg}* glands, but lipid droplets do not accumulate (wild-type alveoli, arrows). During lactation, some alveoli in *Ilk^{-/-}* glands have cells that fill the lumina (arrow), whereas others show cells protruding into the luminal space (double-headed arrows).

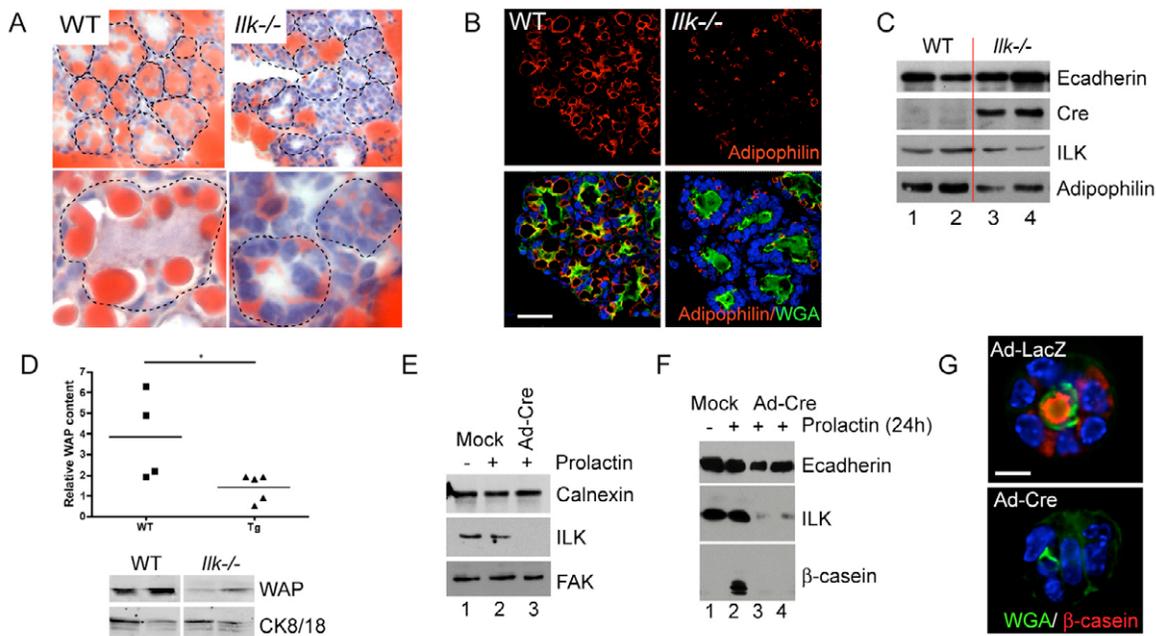


Fig. 2. ILK is required for milk protein synthesis. (A) ORO staining of tissue sections, with broken lines indicating alveolar edges. In comparison with wild type, *Ilk*^{-/-} glands do not contain significant quantities of milk fat in alveolar lumina. (B) Immunofluorescence with anti-adipophilin antibody (red) reveals large CLDs in wild-type glands but these are significantly reduced in *Ilk*^{-/-} glands. Wheat germ agglutinin (WGA-488; green) was used to detect the luminal surface. Scale bar: 40 μ m. (C) *Ilk*^{-/-} glands display lower levels of adipophilin compared with wild-type littermates, shown by immunoblotting. Remaining ILK is in myoepithelial and stromal cells. (D) The amount of WAP in lysates of whole mammary gland (P18) determined by immunoblotting (chemifluorescence), with a representative blot shown underneath. Relative expression calculated using the luminal MEC marker. Each triangle represents the WAP levels in a different mouse. *The median *Wap* content of *Ilk*^{-/-} glands (Tg; triangles) is significantly reduced ($P=0.0238$, Mann Whitney test) compared with that of wild type (squares). (E) Ad-Cre infection of primary *Ilk*^{fl/fl} MECs results in the absence of ILK protein. FAK levels are similar in wild-type and ILK-null cultures. (F) Immunoblotting shows defective β -casein synthesis in primary *Ilk*^{fl/fl} MEC infected with Ad-Cre, in response to 24 hours of prolactin treatment. (G) Immunofluorescence of β -casein (red), WGA-488 (green) and nuclei (DAPI; blue) in Ad-*lacZ*- and Ad-Cre-infected *Ilk*^{fl/fl} primary cultures. These are deconvoluted images through the centre of 3D acini cultured on BM-matrix. Scale bar: 10 μ m.

important integrator of integrin signalling, FAK. We employed a similar strategy to that described above, using Cre recombinase to ablate the *Fak* gene from mammary epithelia both in vivo and in the culture model.

Heterozygous pups suckling from *Fak*^{-/-} mothers gained weight at the same rate as those feeding from wild-type dams, indicating that, in contrast to ILK, FAK is not necessary for lactational differentiation (Fig. 4A). Despite the reduction of FAK protein (Fig. 4B), the *Fak*^{-/-} gland histology was similar to that of wild type (Fig. 4C), and there were no differences in alveolar content, as judged by Haematoxylin and Eosin or ORO staining (Fig. 4D). This suggested that FAK was not involved in milk protein expression. We therefore deleted the *Fak* gene in primary MECs isolated from *FAK*^{fl/fl} mice with Ad-Cre, and examined whether *Fak*^{-/-} cells plated in 3D culture were able to synthesise β -casein. Although the equivalent experiment in cells from *Ilk*^{fl/fl} mice prevented milk protein expression (Fig. 2F), the loss of FAK had no detrimental effect (Fig. 4E).

Thus, two key integrators of integrin signalling that are located within the same supra-molecular adhesion complexes have distinct functions, and only one of them, ILK, is required to deliver signals that lead to MEC differentiation.

Signals downstream of ILK for mammary differentiation

To explore the mechanism underlying the requirement for ILK in mammary differentiation, we examined the potential involvement of signalling pathways downstream of ILK. One pathway activated

downstream of ILK is that of the small GTPase RAC (Boulter et al., 2006). Indeed, ILK-null MECs had reduced levels of active RAC (Fig. 5A). RAC1 integrates integrin-mediated adhesion with lactational differentiation, and we therefore reasoned that it might be an intermediate linking ILK with the differentiation response (Akhtar and Streuli, 2006). To test this hypothesis, we examined whether an activated V12Rac1 could rescue differentiation in *Ilk*-null cells. We first generated *Ilk*^{-/-} cells by infecting primary MECs from *Ilk*^{fl/fl} mice with Ad-Cre virus. When these cells were re-infected with Ad-V12Rac1 or a control Ad-*lacZ* adenovirus and plated in 3D culture to form acini, we found that V12Rac1 was able to restore the lactation defect, leading to β -casein expression (Fig. 5B, compare lanes 5 and 6). These results suggest that a RAC1 pathway transmits the ILK-mediated signals required for milk protein expression.

ILK also has serine/threonine kinase activity in vitro, and can direct the phosphorylation of GSK3 β at S9 and PKB/Akt at S473 in certain cultured cell types (Hannigan et al., 2005). We found that ILK deletion in mammary acini did not alter steady-state phosphorylation of GSK3 β or PKB/Akt, or their transient phosphorylation after insulin stimulation (see Fig. S3 in the supplementary material). The loss of ILK also did not alter ERK phosphorylation in the steady state or after acute stimulation with EGF. These data suggest that, at least in primary MECs, ILK does not influence Akt or Erk signalling.

As β 1-integrin is required for prolactin signalling, we also examined whether ILK is necessary for the phosphorylation and nuclear translocation of STAT5, a key transcription factor for

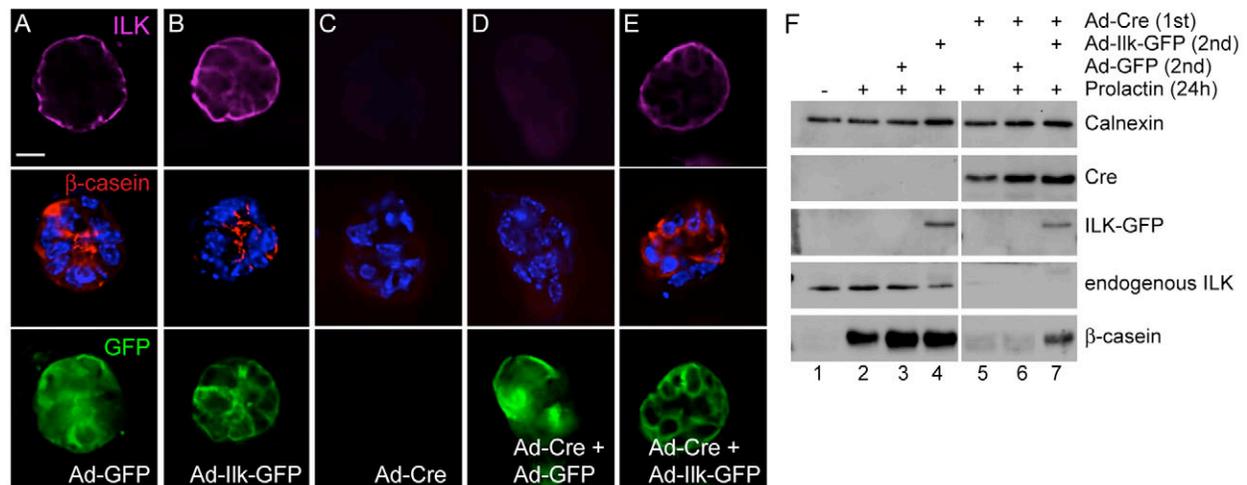


Fig. 3. Rescue of lactational differentiation in ILK-null acini by ILK-GFP. *Ilk^{flx/flx}* MECs were either untreated or infected with Ad-Cre, then rescued with Ad-GFP or Ad-ILK-GFP, and treated with prolactin, then immunostained or immunoblotted. (A,B) Acini expressing GFP or ILK-GFP (green) display ILK (pink) at their basal domains and synthesise β -casein (red). (C) Infection with Ad-Cre results in loss of ILK and prevention of β -casein synthesis. (D) Re-infection with Ad-GFP virus does not rescue lactation defect in ILK-null acini. (E) Expression of ILK-GFP in ILK-null acini results in basal localisation of ILK, and in β -casein synthesis. (F) Prolactin induces β -casein synthesis in wild type (lane 1 versus lane 2) and control cells infected with Ad-GFP or Ad-ILK-GFP (lanes 3,4). In the cells that were initially infected with Ad-Cre (lanes 5-7), ILK deletion results in loss of β -casein synthesis, which is not rescued after a second infection with Ad-GFP (lane 6). However, a second infection with Ad-ILK-GFP does rescue β -casein expression (lane 7). Viral infections are shown with antibodies to GFP (ILK-GFP runs at 78 kDa, compare with endogenous ILK at 50 kDa) and Cre.

mammary differentiation (Oakes et al., 2006). Immunofluorescence analysis revealed nuclear STAT5 in the alveoli of wild-type mice in vivo, but this was diminished significantly within *Ilk^{-/-}* alveoli (Fig. 5C). In primary MECs, prolactin triggered STAT5 nuclear translocation and phosphorylation within 15 minutes in Ad-*lacZ* infected *Ilk^{flx/flx}* cells, but was unable to do so in the majority of Ad-Cre-infected cells (Fig. 5D,E). Moreover, the levels of STAT5-dependent transcripts encoding β -casein and WAP were lower in Ad-*lacZ*-infected *Ilk^{flx/flx}* cells than in wild-type controls (Fig. 5F).

These results show that the mechanistic link between ILK and the differentiation response resides in the ability of ILK ability to activate RAC1 and to licence STAT5 signalling. The data advance previous work on β 1-integrin function by identifying a specific mediator of the adhesion requirement for glandular differentiation, which we show to be ILK.

DISCUSSION

Precisely how integrins control cell behaviour is not well understood. The current model is that they recruit adaptor proteins and thereby transduce signals. This raises two issues: (1) the adhesome is widely regarded as having a large number of distinct components, but which ones are involved with different aspects of integrin control, e.g. cell cycle or differentiation, is not known (Zaidel-Bar et al., 2007); (2) there are numerous studies examining how integrins control for example migration, but virtually nothing is known about how they regulate cellular differentiation (DeMali et al., 2003; Giancotti and Tarone, 2003).

Here, we show that in both in vivo and in 3D culture models of mammary gland behaviour, ILK, but not FAK, acts downstream of integrins to control lactational differentiation. Thus, we have (1) identified a high degree of specificity within the integrin-based adhesome that links cell-matrix interactions with the function of epithelia, and (2) determined a key downstream component of integrin signalling involved in differentiation.

Specific integrin adhesome proteins control lactational capacity and tissue function

β 1-integrins control the terminal differentiation of MECs through a permissive interaction with endocrine signalling (Faraldo et al., 1998; Naylor et al., 2005). This indicates a direct influence of integrins on pathways driving transcriptional endpoints (Streuli et al., 1995a). Here, we extend this model by identifying one proximal integrin-containing adhesion complex protein, ILK, that is required for this differentiation pathway. Our results show that the ability of dams to nurse is diminished in the absence of ILK. This is not due to a delay in mammary development, but rather to the inability to synthesise and secrete adequate quantities of milk proteins and lipids. Interestingly, ILK is not required for specification of alveoli, because lobuloalveolar development occurs even when Cre is expressed prior to the initiation of pregnancy using the β -lactoglobulin promoter.

A novel aspect of the integrin control on differentiation regards the synthesis of milk lipids. We had previously observed that β 1-integrins are required for efficient lipid production in mammary epithelium, as judged by ORO staining, and here we extend those data by showing the involvement of ILK. One possible mechanism may be through transcriptional or post-transcriptional control of adipophilin, which stabilises the triglyceride core in cytoplasmic lipid droplets, and we observed that both were decreased in *Ilk^{-/-}* glands. An alternative might be the defective expression or regulation of key lipid metabolic proteins, e.g. Glut-1 transporter, fatty acid desaturase, fatty acid elongase and sterol regulatory element binding protein (SREBP1), which are normally upregulated during pregnancy and lactation (Russell et al., 2007). We are currently examining both these as targets for integrin/ILK signalling.

Although the loss of ILK and β 1-integrin result in very similar phenotypes in terms of STAT5 activation, milk protein synthesis and altered morphology in 3D cultures of MECs, these phenotypes are not

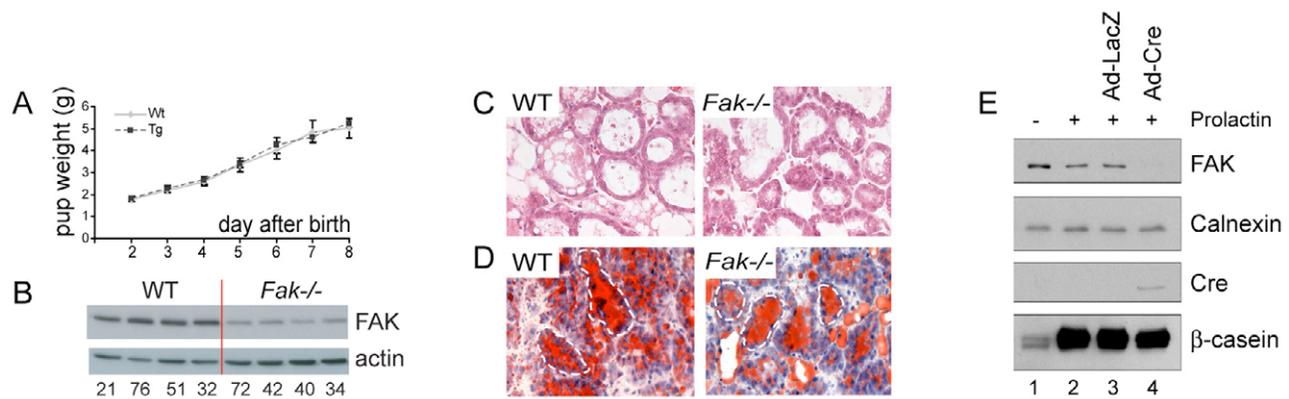


Fig. 4. Specificity of integrin signals for differentiation. (A) Growth of pups nursing on *Fak^{fl/fl};Blg-Cre^{Tg/+}* mothers is not different from pups nursing on wild-type controls. (B) Extracts from wild-type and *Fak^{fl/fl};Blg-Cre^{Tg/+}* mammary glands shows loss of FAK protein in vivo. Numbers refer to different individual mice. Similar to *Ilk*-null glands, there is residual FAK present in other cell types of the tissue. (C,D) Both wild-type and *Fak*-null glands show similar (C) histology of the alveolar structures and (D) ORO staining; broken lines circumscribe individual alveoli. (E) Immunoblot of mock, Ad-*lacZ* or Ad-*Cre*-infected *Fak^{fl/fl}* cells, showing that although FAK protein is removed, β-casein production is not prevented.

as severe in the *Ilk^{-/-}* glands in vivo as the β1-integrin-null glands. In mammary tissue, the morphology of the alveoli is altered similarly in the absence of ILK and β1-integrin, and there is also a similar decrease in milk fat. However, the reduction in STAT5 activation and milk protein synthesis is less marked than in the β1-integrin-null glands, and the pups are underweight, whereas most do not survive when nursing from β1-integrin-null dams. This suggests that factors other than ILK are involved in permitting full lactation to occur in vivo.

In contrast to the need for ILK, FAK is not required for lactational differentiation. Interestingly, our results using the BLG promoter to drive FAK gene deletion differ from those using the MMTV promoter, where a delay of mammary development in early pregnancy was so severe that alveoli did not form properly (Nagy et al., 2007). This phenotype was ascribed to severe hypoplasia, and our in vitro data extend those results by demonstrating that FAK deletion does not compromise the ability of MECs to differentiate.

Importantly, our data provide insights into the way that adhesion complexes work. They show that signalling platforms within the adhesion complex have completely different functions, because FAK, which resides within the same adhesion complexes as ILK as determined by immunostaining, is dispensable for milk protein expression both in vivo and in 3D culture. There are distinct effectors downstream of ILK and FAK, and our data suggest that those specific to ILK control differentiation. For example, The FAK/Src complex is a crucial tyrosine kinase that activates several effectors through phosphorylation, and it is also an adaptor for other adhesion complex proteins such as paxillin and CAS (Schatzmann et al., 2003). By contrast, ILK binds numerous other proteins, including pinch and parvins, and is a serine/threonine kinase in some cell types (Hannigan et al., 2005). We are currently screening for specific effectors using genetic approaches.

Possible mechanisms for ILK to control differentiation

There are several possible mechanisms underlying the requirement for ILK in differentiation. One relates to the altered morphology acquired by the ILK-null glands and 3D cell cultures (Fig. 1G, Fig. 3C). We are currently investigating the mechanism of this disruption, although it is unlikely to be due to disturbed cell-ECM

interactions because laminin 1 in the basement membrane surrounding alveoli and the β1-integrins are both located basally, in vivo and in culture (data not shown). We have previously shown that the morphology of mammary acini is not linked to their ability to express milk proteins, suggesting that altered cellular organisation of ILK-null acini does not directly compromise differentiation but rather that it may be due to modified signalling downstream of ILK (Streuli et al., 1991; Zoubiane et al., 2004).

In the context of ILK-mediated signalling pathways, we found no evidence to associate ILK with PKB signalling in mammary epithelia, indicating that it most probably functions as an adaptor protein to recruit other signalling moieties rather than as a kinase itself.

Instead, our evidence indicates that RAC1 acts downstream of ILK in the differentiation response. In previous studies we have shown that RAC is involved with mammary differentiation by rescuing the lactation defect of integrin-deficient cells with an activated V12Rac1 (Akhtar and Streuli, 2006). A similar strategy employed here also rescued milk protein expression after ILK ablation, thus confirming that the tissue-specific differentiation response is under the control of small GTPases. It seems likely that a function of adhesion is to activate RAC1 in a spatially restricted context, e.g. via an ILK-signalling complex, which is in turn required for prolactin signalling, at least in MECs. In addition, the appropriate RAC effectors might not be recruited in the absence of ILK, compounding the effect on differentiation (Kawashima et al., 2006). Thus, ILK may provide a structural platform for localised activation of GEFs, as well as the recruitment of RAC effectors, which together contribute to downstream signals.

A current focus of our attention is to identify the ILK-binding partners required to activate RAC1, and thereby influence differentiation. A candidate is parvin, which binds the guanine nucleotide exchange factor αPIX in some cell types, and in doing so it regulates the activity of RAC1 (Rosenberger and Kutsche, 2006; Sepulveda and Wu, 2006). Other potential links from ILK to RAC include associations with the Arf GAP, PKL/GIT2 and βPIX, or with pinch, NCK2 and DOCK180 (Boulter and Van Obberghen-Schilling, 2006; Legate et al., 2006). Future studies knocking down each of these components in MECs, in combination with gene transfer and rescue approaches will elucidate the details of how the ILK-scaffold controls epithelial polarity and morphogenesis.

Prolactin is a key player in post-pubertal mammary development, as it is required for the specification of alveoli, their growth and proliferation (Oakes et al., 2006). The JAK-STAT pathway is an effector of prolactin signalling, culminating in the phosphorylation, dimerisation and nuclear translocation of STAT5. In terms of the alveolar differentiation response, it is known that β 1-integrins exert their control through the prolactin-STAT5 pathway. Our analysis

now extends this conclusion by demonstrating that ILK has a novel role in licensing signalling through this cytokine receptor, because STAT5 activation is diminished in both the mammary gland in vivo and the primary culture model in its absence. A possible consequence of reduced lactational differentiation in ILK-null mammary epithelia might be altered acinar morphology, because there are not enough milk products to expand the lumens to their

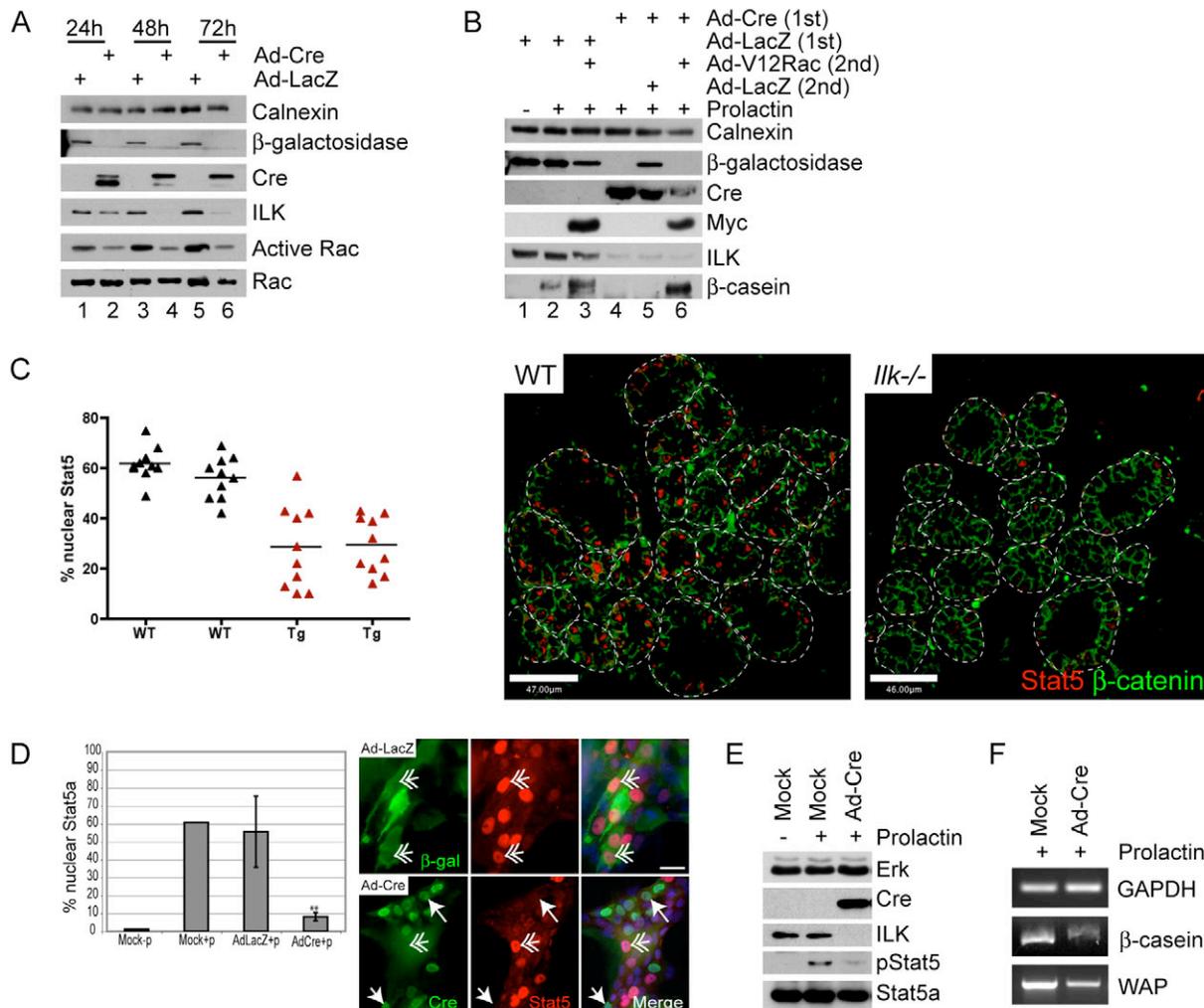


Fig. 5. Signals linking ILK to differentiation. (A) RAC activity was assessed by Pak1-PBD pull-down in primary *Ilk^{fl/fl}* MECs, following infection for the indicated times. (B) *Ilk^{fl/fl}* MECs were infected with Ad-*lacZ* or Ad-Cre then rescued with either Ad-*lacZ* or Ad-V12Rac1 and induced to differentiate with prolactin. Expression of V12Rac1 in ILK-null cells (i.e. Ad-Cre primary infection) rescues β -casein synthesis (compare lanes 4 and 6); expression of *lacZ* does not cause a rescue (lane 5). (C) Wax sections of mammary glands were immunostained for STAT5 (red) and counterstained for β -catenin (green), and the percentage of nuclei with STAT5 was determined from two independent wild-type and *Ilk^{fl/fl}; Blg-Cre^{Tg}* mice. Each triangle represents the % nuclear STAT5 levels in fields of view cumulatively containing >200 luminal epithelial cells. The median % nuclear STAT5 of *Ilk^{-/-}* glands (red triangles) is significantly reduced compared with that of wild type (black triangles); unpaired *t*-test ($P < 0.0001$). The photomicrographs show representative fluorescence images. Broken lines indicate alveolar extremities. Scale bars: 47 μ m in wild type; 46 μ m in (*Ilk^{fl/fl}; Blg-Cre^{Tg}*). (D) Primary *Ilk^{fl/fl}* MECs were infected with Ad-*lacZ* or Ad-Cre, cultured in monolayer and incubated in differentiation medium containing BM-matrix before a 15-minute prolactin stimulation. On the left are shown the % of cells undergoing STAT5 nuclear translocation in mock-infected cells without (Mock-p) or with (Mock+p) prolactin, and in cells infected with Ad-*lacZ* (AdLacZ+p) or Ad-Cre (AdCreM1+p). Approximately 60% of cells responded to prolactin by showing STAT5 translocation, but this was reduced to ~10% in the absence of ILK ($n=4$ independent experiments). Error bars are standard deviation from mean, and a pair of asterisks indicates significance at $P < 0.01$ (Student's paired *t*-test). A representative example of immunofluorescence staining for β -galactosidase or Cre (green), STAT-5 (red) and DAPI (blue) is on the right; double-headed arrows show either Ad-*lacZ*-infected (top panels) or Cre-negative cells (bottom panels), where STAT5 translocates to nuclei; the single arrows show Ad-Cre-infected cells, where there is no STAT5 translocation. Scale bars: 32 μ m. (E) Immunoblots of primary *Ilk^{fl/fl}* MECs show that Ad-Cre infection prevents efficient STAT5 tyrosine phosphorylation (Y694/699) by prolactin. (F) RT-PCR of primary *Ilk^{fl/fl}* MECs shows that Ad-Cre infection results in lower levels of STAT5 transcriptional activity as determined by levels of β -casein and WAP transcripts.

maximum extent. We are currently examining whether there is a direct effect of ILK deletion on ion channels and thereby fluid movement. Interestingly, both prolactin receptor-null and STAT5-null mammary epithelia show a similar phenotype with disorganised alveolar structures and only small lumina (Miyoshi et al., 2001).

In summary, the work presented here shows that the permissive role of $\beta 1$ -integrin for glandular epithelial differentiation resides in the ability of adhesion complexes to recruit specific signalling components, in this case ILK. Not all adhesion complex proteins (i.e. FAK) have equal roles in this aspect of integrin signalling, suggesting that the adhesome circuitry is wired specifically for different cell fate decisions.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/1136/6/1019/DC1>

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