Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1783 (2008) 383–393
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamcr

KIBRA interacts with discoidin domain receptor 1 to modulate collagen-induced signalling

Heidi N. Hilton [a], Prudence M. Stanford [a], Jessica Harris [a,1], Samantha R. Oakes [a], Warren Kaplan [b], Roger J. Daly [c], Christopher J. Ormandy [a,*]

[a] Development Group, Cancer Research Program, Garvan Institute of Medical Research, Sydney, NSW, 2010, Australia
[b] Peter Wills Bioinformatics Centre, Garvan Institute of Medical Research, Sydney, NSW, 2010, Australia
[c] Signal Transduction Group, Cancer Research Program, Garvan Institute of Medical Research, Sydney, NSW, 2010, Australia

Received 5 July 2007; received in revised form 30 November 2007; accepted 3 December 2007
Available online 9 January 2008

Abstract

Mammary gland development is coupled to reproductive events by hormonal cues of ovarian and pituitary origin, which activate a genomic regulatory network. Identification of the components and regulatory links that comprise this network will provide the basis for defining the network's dynamic response during normal development and its perturbation during breast carcinogenesis. In this study KIBRA was identified as a transcript showing decreased expression associated with failed mammary gland development in Prlr knockout mammary epithelium. It is strongly up-regulated during pregnancy, falls during lactation and is again up-regulated during involution of the gland at weaning. A bioinformatic approach was undertaken to identify potential binding partners which interact with the WW domains of KIBRA. We show that KIBRA binds to a WW domain binding motif, PPxY, in the tyrosine kinase receptor DDR1, and dissociates upon treatment with the DDR1 ligands collagen type I or IV. In addition we show that KIBRA and DDR1 also interact with PKCz to form a trimeric complex. Finally, overexpression and knockdown studies demonstrate that KIBRA promotes the collagen-stimulated activation of the MAPK cascade. Thus KIBRA may play a role in how the reproductive state influences the mammary epithelial cell to respond to changing cell-context information, such as experienced during the tissue remodeling events of mammary gland development.

Keywords: Mammary; Progesterone; Cell matrix; PKCζ, ERK MAPK

1. Introduction

The mammary gland reaches its full state of development as a result of hormonal cues that coordinate puberty, pregnancy and parturition with the developmental events that build and activate the mammary secretory epithelium [1–3]. Estrogen and growth hormone cause the formation of the mammary ductal network during pubertal development, and progesterone causes side branches to form with each estrous or menstrual cycle [4–6]. Prolactin and progesterone stimulate the development of the lobuloalveoli during pregnancy [4–7]. Lactation is initiated following parturition by the combined decrease in progesterone levels and increase in prolactin levels. Lactation is maintained by prolactin until weaning causes a combination of reduced nervous stimulation of prolactin release and engorgement of the ductal network, triggering the apoptotic involution of the lobuloalveoli [3]. Developmental events elicited by these hormones initiate transcriptional changes that are programmed by an inheritable and robust genomic regulatory network [8].

During carcinogenesis the genomic regulatory network is perturbed and tumour growth occurs. Defining these regulatory networks and the signalling systems that control them offers an insight into the biology of organ development, provides the foundation to understand how the process is dysregulated during carcinogenesis, and may identify new therapeutic targets. To explore the genomic regulatory network operating during pregnancy in the mammary gland, we transcript profiled prolactin receptor knockout ($Prlr^{-/-}$) and wild type ($Prlr^{+/+}$) mammary

* Corresponding author. Cancer Research Program, Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst, NSW, 2010, Australia.
E-mail address: c.ormandy@garvan.unsw.edu.au (C.J. Ormandy).
[1] Current address: Department of Surgery and Molecular Oncology, University of Dundee, Ninewells Hospital and Medical School, Dundee, UK.

0167-4889/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2007.12.007

epithelium in wild type hosts [9]. We identified KIBRA as a transcript with altered expression in $Prlr^{+/+}$ mammary epithelial transplants compared to $Prlr^{-/-}$ transplants during early pregnancy, with expression confined to the epithelial cells.

KIBRA was originally identified from a yeast two-hybrid screen of a human brain cDNA library as a protein which interacts with the postsynaptic protein Dendrin, and contains two N-terminal WW domains, and a C2 domain [10]. The WW domain is a protein interaction module that recognises specific proline-containing peptide sequences [11]. They are found in a variety of proteins involved in diverse cellular processes including protein degradation, transcription regulation and differentiation, and are also associated with a number of human diseases including muscular dystrophy, Alzheimer's disease and cancer [12]. The C2 domain is a conserved membrane-targeting motif that mediates an array of intracellular processes, the majority of which involve signal transduction and membrane trafficking [13]. The presence of these domains suggested a scaffold protein role for KIBRA in the formation of a signalling complex. To date, the functional role of KIBRA has been implicated in synaptic signalling and memory formation[14,15], and as a transactivator of the estrogen receptor in human breast cancer cells with a potential role in transport along the microtubules, via its interaction with dynein light chain 1 (DLC1) [16].

To further understand how hormonal regulation of KIBRA could influence mammary gland development, we sought to examine the regulation of KIBRA, identify its binding partners and define a signalling pathway endpoint that responds to manipulation of KIBRA expression. Following this approach we have identified Discoidin Domain Receptor 1 (DDR1) as a novel binding partner of KIBRA and show that this occurs in a collagen-regulated manner. Furthermore, we demonstrate that KIBRA is involved in the collagen-stimulated activation of the MAPK cascade.

2. Methods

2.1. Mammary epithelial transplantation and transcript profiling

Mammary epithelial transplants of Prlr wildtype ($Prlr^{+/+}$) and knockout ($Prlr^{-/-}$) epithelium and transcript profiling was performed as previously described by Harris *et al.* [9].

2.2. Quantitative PCR

1 μg RNA was reverse transcribed using AMV reverse transcriptase in a 20 μL reaction (Promega, Madison, WI). PCR reactions were performed in a LightCycler (Roche Diagnostics, Mannheim, Germany) using 1 μL of the cDNA (diluted 1:2), 5 pmol of primers and the FastStart DNA master SYBR Green I enzyme mix (Roche) in a 10 μL reaction volume. Relative quantification was performed by RelQuant software (Roche). Absolute quantification was performed by comparison to a standard curve constructed from serial dilutions of a PCR product purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and analysed using the Second Derivative Maximum method (Roche). Data was normalised to expression of the housekeeping genes β-actin or aminolevulinic acid synthase 1 (ALAS1) [17,18].

2.3. Bioinformatics to identify proteins

Build 33 of the human proteome, containing 37,490 sequences, was downloaded from the NCBI (http://www.ncbi.nlm.nih.gov) and the protein regular expression program "Preg" [19] was used to identify all sequences in the proteome with the PPxY and RxPPxY motifs (where P is proline, R is arginine, Y is tyrosine and x is any amino acid). The GI accession numbers from the matched sequences were used to extract annotations from the 15 June 2003 build of the Affymetrix HGU-133A annotation file. The annotations were then extended by searching batch SOURCE [20] with the LocusLink identity numbers derived from the matched Affymetrix HGU-133A annotations. Protein expression in the mammary gland was determined from the NCBI Unigene database (http://www.ncbi.nlm.nih.gov/unigene).

2.4. Antibody preparation

Anti-KIBRA antiserum was prepared in rabbit (Invitrogen Life Technologies, Carlsbad, CA) using the synthetic peptide sequence, SAQERYRLEEPGTEGKQ, derived from the human KIBRA central portion, conjugated to keyhole limpet haemocyanin (KLH). The KIBRA IgG polyclonal antibody was purified on a peptide affinity column.

2.5. Immunohistochemistry

4 μm tissue sections were mounted on Superfrost Plus adhesion slides (Lomb Scientific, Sydney, Australia) and heated in a convection oven at 75 °C for 2 h to promote adherence. Sections were de-waxed and re-hydrated according to standard protocols. Antigen retrieval was performed using citrate EDTA buffer boiled under pressure (human cells) or Proteinase K enzymatic digestion (human and mouse tissue). Endogenous peroxidase activity was inhibited with 3% H_2O_2. Sections were incubated with the anti-KIBRA primary antibody for 60 min (1:200, human tissue and cells) or 30 min (1:150, mouse tissue) and bound antibody was detected using LINK/LABEL and 3, 3′-diaminobenzidine Plus as substrate. With each run, diluent and rabbit IgG isotype control were the negative technical controls. Counterstaining was performed with haematoxylin and 1% acid alcohol. Cell lines were processed at the same time to enable comparison of signal strength. All immunohistochemistry reagents were obtained from DAKO Corporation (Carpinteria, CA) unless otherwise specified.

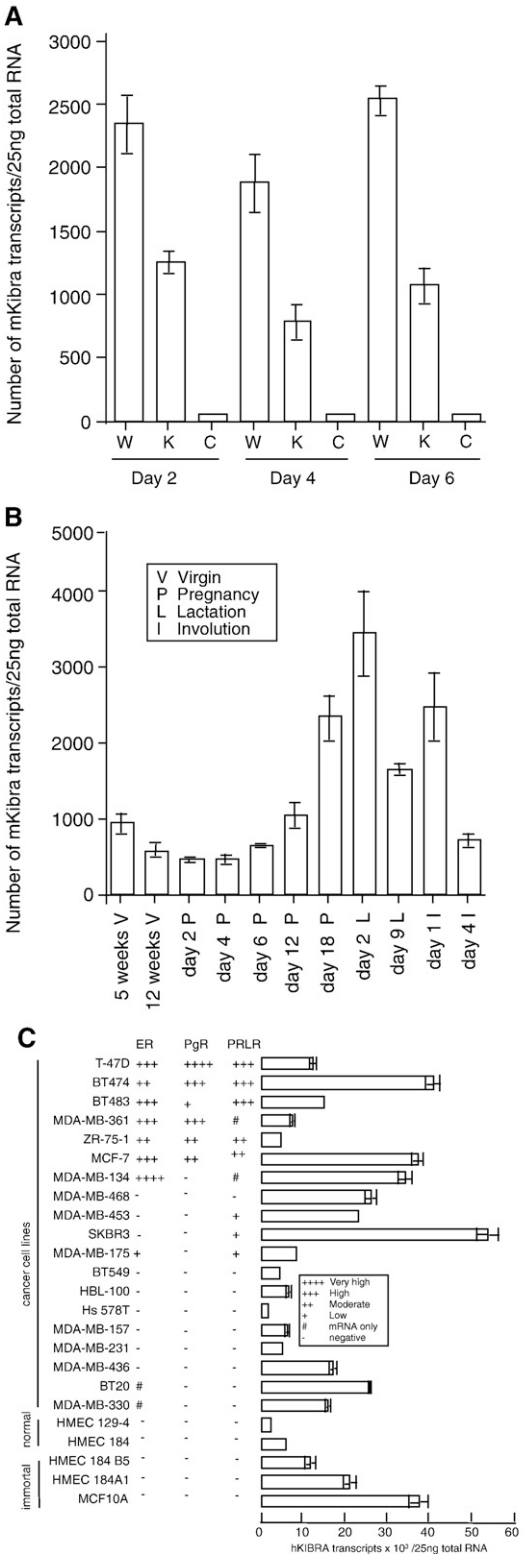
2.6. Plasmids

The human KIBRA cDNA was cloned from HEK-293 cells by PCR into the Gateway entry vector pDONR221 (Invitrogen) using Forward Primer 5′-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TGG AAG ATG CCC CGG CCG GAG C-3′ and Reverse Primer 5′-GGGG AC CAC TTT GTA CAA GAA AGC TGG GTA GAC GTC ATC TGC AGA GAG AGC TGG-3′. Similarly, two overlapping fragments (WW and C2) of the KIBRA cDNA were cloned into pDONR221 by PCR. Fragment 1, containing the two WW domains, was amplified using the Forward Primer described above, and 5′-GGGG AC CAC TTT GTA CAA GAA AGC TGG GTA TTA CGA TAC GGC GGC TGA GAC ACA GG-3′. Fragment 2, containing the C2 domain, was amplified using 5′-GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTC ACC ATG GAG AGG GAC CGG CTG ATC CTT ATC-3′ and the Reverse Primer described above. The KIBRA cDNA and WW and C2 fragments were then cloned into the mammalian Gateway expression vector pcDNA-DEST40 (CMV/C-terminal V5-6xHis) by recombination (Invitrogen). The pRK5-DDR1b expression plasmid was kindly provided by Dr A. Ullrich, Max Planck Institute, Germany. The pRcCMV-PKCζ expression plasmid was kindly provided by Dr T. Biden, Garvan Institute, Australia. Human KIBRA cDNA was also subcloned into the *NotI* site of the untagged expression plasmid pcDNA3.1(+) via the pGEM-T Easy Vector System 1 (Promega) using primers KIBRA5′Forward (GG AAG ATG CCC CGG CCG GAG C) and KIBRA3′Reverse (C TTT TCT GGC GAT TAG ACG TCA TCT GC). For knockdown of mKibra in HC11 cells an siRNA duplex was designed using the algorithm as described in Reynolds et al, 2004 [21]. Double-stranded RNA oligonucleotides against the target sequence GCUUCACUGACCUCUAUUA were synthesised by Dharmacon Research Inc. (Layfayette, CO). For knockdown of hKibra in MCF-10A cells siRNA duplexes were purchased from Dharmacon.

2.7. Site-directed Mutagenesis

The DDR1 mutants were constructed by site-directed mutagenesis with the pRK5-DDR1b plasmid as a template using the QuikChange® II Site-Directed

Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. For each mutant, two complementary synthetic oligonucleotides were designed of which one is indicated: AAPY mutant, 5′-GGT CCT AGA GAG GCA GCC CCG TAC CAG GAG-3′, AAAA mutant, 5′-GA GAG GCA GCC GCG GCC CAG GAG CCC CGG-3′ and PPPF mutant, 5′-GGT CCT AGA GAG CCA CCC CCG TTC CAG GAG-3′. The mutated plasmids were transformed into *Escherichia coli* XL1 Blue Supercompetent cells (Stratagene), and the mutants were verified by sequencing of the plasmid DNA.



2.8. Cell culture and transient transfections

HEK-293 cells were maintained in Minimum Essential Media (MEM) supplemented with 10% foetal bovine serum (FBS) and 0.5 g of sodium bicarbonate per litre. Semiconfluent HEK-293 cells were transiently transfected using Fugene reagent (Roche Applied Science) according to the manufacturer's instructions. After 48 h cells were harvested for immunoprecipitation experiments. T47D human breast cancer cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 10 μg/mL insulin. HC11 murine mammary cells were maintained in RPMI 1640 medium supplemented with 10% heat inactivated FBS, 5 μg/mL insulin and 10 ng/mL epidermal growth factor. Progesterone stimulation experiments involved treating T47D cells with 10 nM ORG2058 (16a-ethoxy-21-hydroxy-19-norpregn-4-en-3,20-dione), which was obtained from Amersham Biosciences, Australia, or 100 nM RU486 [17 beta-hydroxy-11- beta-(4-methylaminophenyl)-17-alpha-(1-propynyl)-etra-4,9-diene-3-one], which was generously provided by Dr J-P Raynaud of Roussel-Uclaf, Romainville, France. MCF-10A cells were maintained as previously described [22]. For collagen treatment, HEK-293 and MCF-10A cells were serum starved overnight, then treated with 10 μg/mL of collagen type IV (human placenta; Sigma, Castle Hill, NSW, Australia) or collagen type I (rat tail; Sigma) for 90 min, unless otherwise specified.

2.9. Immunofluorescence microscopy

Subcellular localisation of KIBRA was assessed using T47D breast cancer cells, and HeLa cells transfected with KIBRApcDNA3.1. Cells were plated onto chamber slides, fixed in 4% paraformaldehyde/PBS, permeablized in 0.2% Triton X-100, and blocked with 1% BSA in PBS. Following incubation with the primary KIBRA antibody (1:100), the cells were stained using rabbit Cy2- or Cy3-labelled secondary antibodies (Jackson ImmunoResearch Laboratories, Pennsylvania, USA), FITC-phalloidin (Sigma) as an actin counterstain, and TOPRO-3 (Molecular Probes OR, USA) as a DNA counterstain. Controls included KIBRA peptide block and secondary antibody only. Cells were visualised using confocal microscopy (Leica Microsystems, Sydney, Australia).

2.10. Immunoprecipitation and Western Blot Analysis

Protein samples were prepared from cell lines lysed in normal lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM pyrophosphate, 100 mM NaF) containing protease and phosphatase inhibitor cocktail tablets (Roche). For analysis of KIBRA phosphorylation T47D lysates were incubated with lambda protein phosphatase (New England Biolabs) according to the manufacturer's instructions. For immunoprecipitation experiments, 1 mg of protein from cell lysates was incubated with antibodies overnight at 4 °C. Protein-G sepharose beads were added to the lysates and incubated for 1 h at 4 °C. The resulting immunoprecipitates were washed four times with lysis buffer and 1 M NaCl, and resuspended in NuPAGE sample buffer and reducing agent (Invitrogen). Immunoprecipitates were separated on NuPAGE 4–12% acrylamide gels (Invitrogen) and transferred to a PVDF membrane. Blots were incubated with the following primary antibodies overnight at 4 °C: α-KIBRA, α-V5 (Invitrogen), α-phosphotyrosine (4G10) (Upstate Biotechnology, Lake Placid, NY), α-DDR1 (C-20) and α-nPKCζ (C-20) (Santa Cruz Biotech), α-phosphorylated and α-total p44/42 MAP kinase (Cell Signaling Technology), and α-βactin (Sigma). Horseradish peroxidase-linked secondary antibodies were used to detect a signal by enhanced chemiluminescent detection.

2.11. Densitometry

This was performed using IP Lab Gel software (Signal Analytics Corp) and statistical analyses were performed using StatView 4.5.

Fig. 1. KIBRA transcript levels in mouse tissue and human breast cancer cell lines. A. Number of mKibra transcripts in each of the $Prlr^{+/+}$ (W) and $Prlr^{-/-}$ (K) mammary epithelial transplants and $Prlr^{+/+}$ fat pads cleared of epithelium (C) during days 2, 4 and 6 of pregnancy. B. Transcript levels of mKibra in wild-type mammary glands at different stages of development. C. hKIBRA transcript levels in human mammary epithelial cells and breast cancer cell lines. Gene expression levels of the prolactin receptor (PRLR), estrogen receptor (ER) and the progesterone receptor (PgR) in each cell line is indicated. Transcript levels were determined by absolute quantitative PCR. Error bars represent the range from duplicate measurements.

3. Results

3.1. Identification of decreased expression of KIBRA in a mouse model of failed mammary gland proliferation and lobuloalveolar development

Prlr$^{+/+}$ and Prlr$^{-/-}$ mammary epithelia were transplanted into the cleared mammary fat pads of 4 week old Rag1$^{-/-}$ animals. We transcript profiled the Prlr$^{+/+}$ and Prlr$^{-/-}$ mammary epithelial transplants during early pregnancy to identify gene expression changes that occur in mammary glands with failed alveolar morphogenesis as a result of the loss of Prlr. We chose early time points of pregnancy to overcome the reduced epithelial cell numbers observed in Prlr$^{-/-}$ mammary glands [9]. To exclude gene expression contributed by the fat pad we also transcript profiled Rag1$^{-/-}$ mammary fat pads cleared of endogenous epithelium. This combined analysis identified a number of genes known to be important for mammary development as decreasing in the Prlr$^{-/-}$ epithelium during pregnancy [9]. Here we report the identification of the murine EST, AI850846, as a mammary epithelium specific transcript that was decreased in Prlr$^{-/-}$ mammary epithelium compared to Prlr$^{+/+}$ mammary epithelium. Sequence database searches identified that AI850846 corresponded to a protein which has since been named KIBRA/WWC1 (GenBank accession no. NM_170779 [mouse]; NM_015238 [human]), and the mouse and human orthologues share 91% amino acid identity. We therefore refer to the mouse and human gene products identified in this study as mKibra and hKIBRA, respectively.

Changes in the MAS 4.0 Average Difference for mKibra from the MGU74A Affymetrix GeneChips were confirmed by absolute quantitative PCR. Each sample for the transcript profiling and the quantitative PCR consisted of RNA pooled from 4 to 6 animals. mKibra mRNA levels were lower in the Prlr$^{-/-}$ mammary epithelial transplants than the Prlr$^{+/+}$ mammary epithelial transplants at days two, four and six of pregnancy (Fig. 1A). mKibra mRNA levels were undetectable above background in the Prlr$^{+/+}$ fat pads cleared of epithelium, confirming KIBRA expression is specific to the mammary epithelium (Fig. 1A).

3.2. KIBRA expression in mammary gland and breast

mKibra mRNA levels were examined in mammary glands from wild-type animals at different stages of development by quantitative RT-PCR. mKibra mRNA was detected at all stages of development in the mammary gland (Fig. 1B). mKibra mRNA in the mammary gland increased during mid pregnancy (day 12) to a

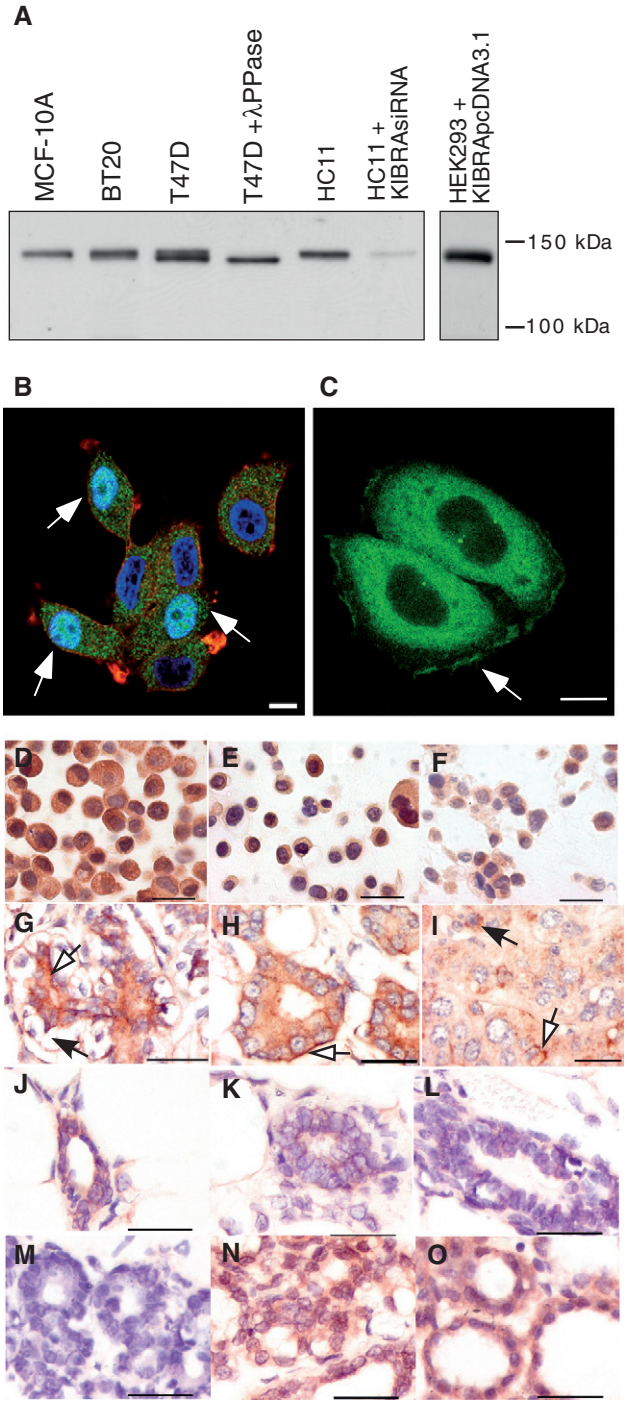


Fig. 2. KIBRA protein expression in mouse and human cells. A. Validation of anti-KIBRA antibody as shown by Western blot. Lysates (20 μg) were loaded, as labelled, of MCF-10A (normal human mammary epithelial cell line), BT20 and T47D +/− lambda protein phosphatase (human breast cancer cell lines), HC11 +/− KIBRA siRNA (normal murine mammary epithelial cell line). Lysate of HEK-293 transfected with KIBRApcDNA3.1 is shown at a shorter exposure due to over-expression. Lysates were blotted with the anti-KIBRA antibody, which detects a band under the 150 kDa marker for both human and mouse samples. B. Localisation of KIBRA as examined by immunofluorescence. T47D breast cancer cells were stained with anti-KIBRA antibody, shown in *green*. Actin is shown in *red*, and the nucleus is shown in *blue.* White arrows indicate where KIBRA co-stains with the nucleus. C. Immunofluorescence of HeLa cells transfected with KIBRApcDNA3.1. Cells were fixed, permeabilised and stained with anti-KIBRA antibody, shown in *green*. The white arrow indicates staining of membrane ruffles. D. KIBRA protein expression in BT474 cells as examined by IHC. E. BT20 cells F. MDA-MB-157 cells G. Normal human breast tissue. Open arrow indicates positive KIBRA staining of luminal epithelial cells. Filled arrow shows negative KIBRA staining of surrounding myoepithelial cells H. and I. High grade IDC. Open arrows indicate KIBRA staining at membranous regions. Filled arrow indicates cytoplasmic staining of KIBRA. J–O. IHC analysis of KIBRA protein expression in mouse mammary tissue throughout development. (J. 12 week virgin K. 2.5 days post coitus L. 6.5 days post coitus M. 12.5 days post coitus N. 18.5 days post coitus O. 1 day post partum.) Scale bars on all images represent 10 μM.

peak at late pregnancy (day 18) and early lactation (day 2) followed by a decrease at later time points (day 9). At involution of the mammary gland (day 1) mKibra mRNA levels increased again to levels observed at day 18 of pregnancy. hKIBRA transcript levels were examined in a panel of primary breast cancer cell lines, as well as normal and immortal basal epithelial cell lines (HMEC) by quantitative RT-PCR. KIBRA expression was low in the normal HMEC cell lines which have a finite lifespan, higher in the immortal mammary cell lines, and showed variable expression in the breast cancer cell lines (Fig. 1C). The expression did not appear to correlate with the expression of the prolactin receptor or steroid hormone receptors (Fig. 1C) [23,24].

In order to analyse mKibra and hKIBRA at the protein level we generated an affinity purified anti-KIBRA antibody. Fig. 2A shows a Western blot of KIBRA expression in various mammary cell lines, both mouse and human, using our KIBRA antibody. We observed specific bands of the KIBRA protein running below the 150 kDa size marker and often as a doublet. When T47D breast cancer cell lysates were treated with lambda protein phosphatase only the lower band of the doublet remained indicating that the upper band is due to post-translational phosphorylation (Fig. 2A). Whether KIBRA is present predominantly as the phosphorylated form or non-phosphorylated form depends on the cell line, for example in normal murine mammary epithelial HC11 cells, mKibra is observed as the phosphorylated form only. KIBRA shows a predominantly cytoplasmic localisation [10], however a significant amount can also be seen in the nucleus of a subset of cells, as shown by immunofluorescence microscopy of endogenous levels of KIBRA in T47D cells (Fig. 2B). KIBRA has previously been reported to have a nuclear localisation signal in the protein sequence [16], and this interesting expression pattern suggests a potential nuclear function of KIBRA. The cellular distribution of KIBRA in transfected HeLa cells is cytoplasmic, as well as showing staining at membranous regions, particularly in membrane ruffle structures (Fig. 2C). The KIBRA transcript levels measured in a panel of breast cancer cell lines by quantitative RT-PCR (Fig. 1C) were reproduced by immuohistochemical signal strength as shown by BT474 cells (Fig. 2D), BT20 cells (Fig. 2E) and MDA-MB-157 cells (Fig. 2F). In human breast tissue, KIBRA protein is present in the luminal epithelium surrounding the ducts in the normal breast, but is not expressed in the myoepithelium, stromal adipocytes or fibroblasts (Fig. 2G). KIBRA protein was also detected in cancerous tissue and showed heterogeneous expression in both the preinvasive (data not shown) and invasive ductal carcinoma (IDC; Fig. 2H and I). Shown also is the expression of KIBRA protein in mouse mammary tissue throughout development (Fig. 2J–O). It is observed as surrounding the alveoli structures and is predominantly cytoplasmic, as seen at time points of 18. 5 days post coitus (Fig. 2N) and 1 day post partum (Fig. 2O), which corresponds to mKIBRA mRNA levels shown in Fig. 1B.

3.3. Effect of hormonal stimulation on KIBRA gene expression

We identified KIBRA as being a gene showing decreased expression in Prlr$^{-/-}$ mammary epithelium and increased expression by MAS analysis in two SCp2 cell experiments following treatment with prolactin, as outlined in Harris *et al.* (2006) (data not shown). We also investigated the effect of other hormones that are required for alveolar morphogenesis. When we treated T47D cells, a progesterone-responsive human breast cancer cell line, with the synthetic progestin ORG2058, we observed a strong up-regulation of KIBRA expression. Fig. 3A shows levels of KIBRA protein expression following ORG2058 treatment over a five day time course. Up-regulation of KIBRA expression is observed from 8 h of treatment and was maintained to at least 5 days. This progestin-induced up-regulation of KIBRA was also observed in BT474 cells, which is another PgR-positive human breast cancer cell line (data not shown). Fig. 3B depicts the relative increase in KIBRA expression from densitometry analysis of 5 experiments. We also co-treated the cells with ORG2058 and the progestin antagonist RU486 for 24 h, and this inhibited the increase in KIBRA expression, indicating this up-regulation occurs in a PgR-specific manner (Fig. 3C). Together these experiments demonstrate that KIBRA

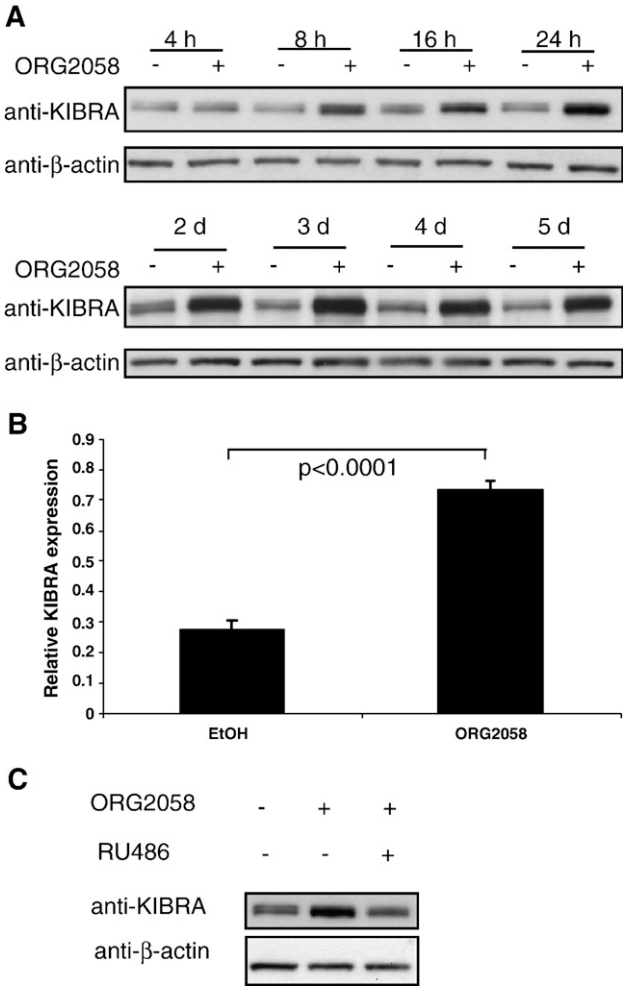


Fig. 3. KIBRA expression is strongly up-regulated by progestin treatment. A. Western blot of time course showing KIBRA protein expression in T47Ds following treatment with 10 nM ORG2058 for between 4 h and 5 days. B. Data generated from densitometry of 5 separate Western blots (each bar is mean ± SE). C. KIBRA protein levels following 24 h 10 nM ORG2058 treatment alone, and 24 h co-treatment with 10 nM ORG2058 and 100 nM RU486.

expression is regulated by two hormones that drive alveolar morphogenesis.

3.4. Identification of KIBRA interacting partners

To gain an understanding of the function of KIBRA in the mammary gland we undertook a bioinformatic approach to identify potential KIBRA interacting proteins. WW domains recognise specific proline-containing peptides, in particular the sequence motif, PPxY (where P is proline, Y is tyrosine, and *x* is any amino acid) [25]. We mined the human proteome for proteins that contained the PPxY motif using the protein regular expression program "Preg" [19]. This list was further refined by selecting proteins that contained the motif RxPPxY (where R is arginine), the preferred putative consensus recognition motif for KIBRA [10], and were expressed in the mammary gland (Table 1). From these candidates we selected DDR1, a collagen activated tyrosine kinase receptor, and ErbB3, a heregulin co-receptor due to their involvement in signalling pathways, as KIBRA was proposed to act as a potential signalling scaffold due to the presence of the WW and C2 domains.

3.5. KIBRA interacts with DDR1 in a collagen-regulated manner

We co-expressed full-length cDNAs of KIBRA and either ErbB3 or DDR1 in HEK-293 cells, and attempted co-immunopreciptation with KIBRA. No signal was observed when we reblotted with antibodies against ErbB3 (data not shown), however we did observe co-immunoprecipitation of KIBRA and DDR1. When we co-expressed KIBRA and DDR1 and treated the cells with collagen type IV, a ligand of DDR1, we observed that more DDR1 co-immunoprecipated with KIBRA in the absence of collagen than in the presence of this ligand (Fig. 4A). Using a second DDR1 ligand, collagen type I, we also observed that less DDR1 co-immunoprecipitated in the presence of this ligand (Fig. 4B). We confirmed that this interaction was collagen-regulated by performing the reciprocal immunoprecipitation, where we observed KIBRA immunoprecipitation with DDR1 antibodies, but not in the presence of collagen IV (Fig. 4C). Furthermore, we performed a kinetic study of DDR1 activation over a 4 h time course and observed a gradual dissociation of KIBRA from DDR1 over time (Fig. 4D). Exposure to collagen causes tyrosine phosphorylation of DDR1 [26], and particularly interesting is the observation that the time point at which DDR1 is first shown to be tyrosine phosphorylated upon collagen treatment ($t=1$ h), is also the first time point at which less DDR1 is observed to co-immunoprecipitate with KIBRA (Fig. 4D). This displacement also occurred in a dose-responsive manner upon collagen treatment (Fig. 4E). Although the sensitivity of the KIBRA antibody precluded detection of an association between endogenous KIBRA and endogenous DDR1, we could detect recruitment of exogenous KIBRA to endogenous DDR1 (Fig. 4G). Together, these data show that KIBRA interacts with DDR1 and that this interaction is regulated by collagens that bind and activate DDR1.

Table 1
List of candidate KIBRA interacting proteins containing RxPPxY motif and are expressed in mammary gland

Sequence derived from	Gene title	Gene symbol	Function	RxPPxY sequence
NM_003035	TAL1 (SCL) interrupting locus	SIL	Cell proliferation	RQPPAY
NM_001447	FAT tumor suppressor homolog 2 (*Drosophila*)	FAT2	Cell proliferation and adhesion	RVPPNY
NM_023038	A disintegrin and metalloproteinase domain 19	ADAM19	Cell–cell and cell–matrix interactions	RPPPDY
NM_005401	Protein tyrosine phosphatase, non-receptor type 14	PTPN14	Signal transduction	RPPPPY
NM_005529	Heparan sulfate proteoglycan 2	HSPG2	Basement membrane molecule	RCPPGY
NM_007039	Protein tyrosine phosphatase, non-receptor type 21	PTPN21	Signal transduction	RPPPPY
NM_007286	Synaptopodin	SYNPO	Cytoskeletal molecule	RSPPSY
NM_014945	Actin binding LIM protein family member 3	ABLIM3	Cytoskeletal molecule	RKPPIY
NM_006720	Actin binding LIM protein 1	ABLIM1	Cytoskeletal molecule	RKPPIY
NM_004393	Dystroglycan 1	DAG1	Muscle contraction	RSPPPY
NM_003494	Dysferlin, limb girdle muscular dystrophy 2B	DYSF	Muscle contraction	RPPPHY
NM_001954	Discoidin domain receptor 1	DDR1	Protein tyrosine kinase receptor	REPPPY
NM_001982	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3	ERBB3	Protein tyrosine kinase receptor	RDPPRY
NM_005157	v-abl Abelson murine leukemia viral oncogene homolog 1	ABL1	Protein tyrosine kinase activity	REPPFY
NM_005642	TAF7 RNA polymerase II	TAF7	RNA polymerase II transcription factor	RLPPEY
NM_005933	Myeloid/lymphoid or mixed-lineage leukemia	MLL	RNA polymerase II transcription factor	RQPPEY
NM_014757	Mastermind-like 1 (*Drosophila*)	MAML1	Transcriptional co-activator	RPPPQY
NM_012245	SKI-interacting protein	SKIIP	Transcription co-activator	REPPPY
NM_012406	PR domain containing 4	PRDM4	Transcription factor	RPPPQY
NM_014345	Zinc finger protein 318	ZNF318	RNA binding	RIPPNY
NM_012091	Adenosine deaminase, tRNA-specific 1	ADAT1	RNA binding and processing	RNPPDY
NM_133330	Wolf–Hirschhorn syndrome candidate 1	WHSC1	Embryogenesis and morphogenesis	RKPPPY
NM_022902	Solute carrier family 30 (zinc transporter)	SLC30A5	Zinc transporter	RLPPEY
NM_003730	Ribonuclease 6 precursor	RNASE6PL	Ribonuclease activity	RDPPDY
NM_018641	Chondroitin 4-*O*-sulfotransferase 2	C4S-2	Transferase activity	RFPPSY
NM_014262	Leprecan-like 2 protein	LEPREL2	Protein metabolism	REPPAY
NM_014427	Copine VII	CPNE7	Lipid metabolism	RIPPKY
NM_001649	Apical protein-like (Xenopus laevis)	APXL	Sodium channel activity	RHPPLY
NM_013423	Rho GTPase activating protein 6	ARHGAP6	Rho GTPase activator activity	RPPPPY

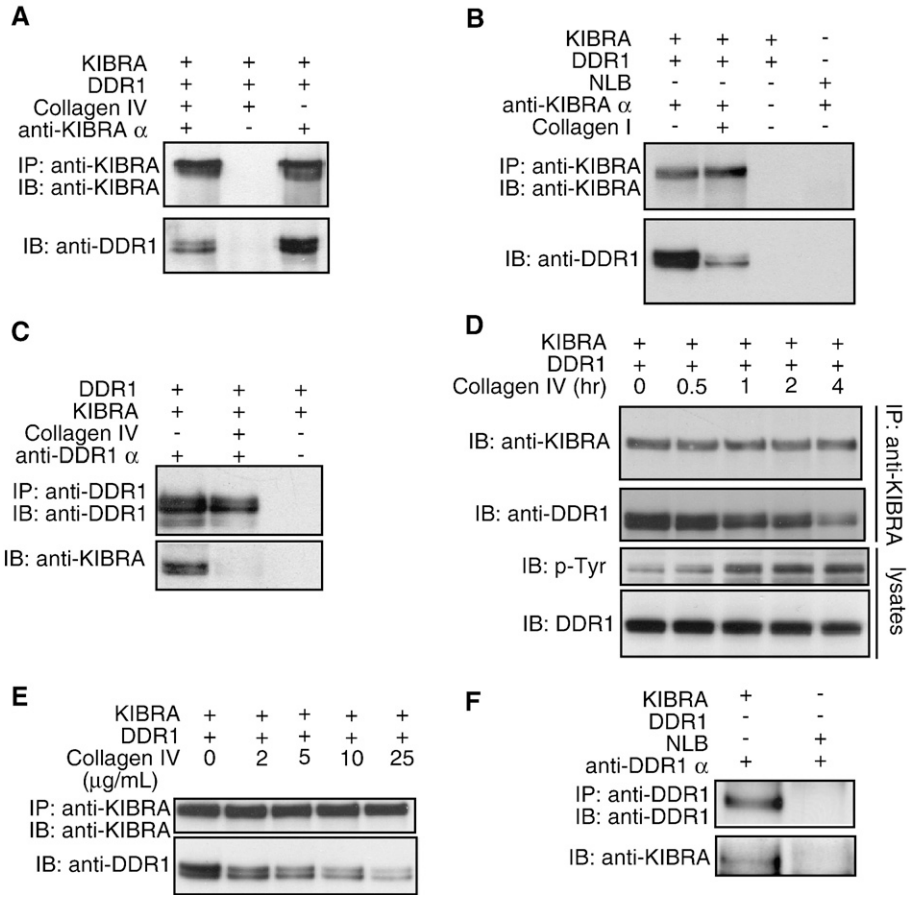


Fig. 4. KIBRA interacts with DDR1 in a collagen-regulated manner. A. KIBRA immunoprecipitates from HEK-293 cells transiently co-transfected with KIBRA and DDR1b cDNAs and blotted with anti-KIBRA and anti-DDR1 showing that DDR1b co-precipitates with KIBRA. B. Co-immunoprecipitation of KIBRA and DDR1 +/− collagen type I. C. DDR1 immunoprecipitates from transfected HEK-293 cells +/− collagen showing that KIBRA co-precipitates with DDR1 in the absence of collagen IV. D. Co-immunoprecipitation of KIBRA and DDR1 over a 4 h time course of collagen treatment. Levels of total and phosphorylated DDR1 in whole cell lysates are shown. E. Co-immunoprecipitation of KIBRA and DDR1 in response to increasing concentrations of collagen IV. F. Endogenous DDR1 immunoprecipitates from T47D cells transiently transfected with KIBRA showing an interaction between KIBRA and endogenous DDR1. NLB = normal lysis buffer control.

3.6. The WW domains of KIBRA bind the PPxY motif in DDR1

To determine whether the KIBRA-DDR1 interaction was occurring via the PPxY motif of DDR1, we performed site-directed mutagenesis on this motif. Three DDR1 mutants were made where the target motif (PPPY) was mutated to AAAA, AAPY and PPPF (where A is alanine, P is proline, Y is tyrosine and F is phenylalanine). Full-length KIBRA was co-expressed with either wild-type DDR1 (DDR1-wt) or the DDR1 mutants. Only DDR1-wt, and not DDR1-AAAA, DDR1-AAPY or DDR1-PPPF, co-immunoprecipitated with KIBRA (Fig. 5A). Similar expression in the protein lysates of DDR1-wt and the mutant constructs is demonstrated in the bottom panel of Fig. 5A. Therefore, the loss of the two proline residues or the tyrosine residues in the PPxY sequence abolishes binding of DDR1 to KIBRA, confirming that integrity of this consensus motif is critical for the interaction to occur.

PPxY motifs bind to WW domains of their target proteins. To determine whether the KIBRA and DDR1 interaction was mediated by the two WW domains of KIBRA, we constructed two overlapping V5-tagged fusion proteins. One included the WW domains of KIBRA, and the other included the C2 domain (Fig. 5B). We co-expressed DDR1 with either WW-V5, C2-V5 or full-length KIBRA-V5. When we immunoprecipitated with the KIBRA antibody, DDR1 was pulled down with full-length KIBRA-V5 and WW-V5, but not with the C2-V5 fusion protein (Fig. 5C). These data indicate that KIBRA and DDR1 interact via a fragment of KIBRA containing the WW domains and the PPPY motif of DDR1.

3.7. KIBRA interacts with DDR1 and PKCζ in a complex

KIBRA has been reported to interact with protein kinase Cζ (PKCζ) [14]. We confirmed this by immunoblotting and showed that endogenous PKCζ co-immunoprecipitates with KIBRA in HEK-293 cells (Fig. 5C). PKCζ is pulled down with C2-V5 KIBRA, which is in agreement with Buther *et al.* (2004) who mapped the KIBRA binding site to a short 44 amino acid fragment downstream of the C2 domain [14]. We observed that both DDR1 and endogenous PKCζ co-precipitate together with full-length KIBRA, but not the C2 domain or WW domain fragments of KIBRA alone, suggesting that KIBRA, DDR1 and PKCζ interact in a complex. When we co-expressed KIBRA, DDR1 and PKCζ in HEK−293 cells we observed co-immunoprecipitation of all three

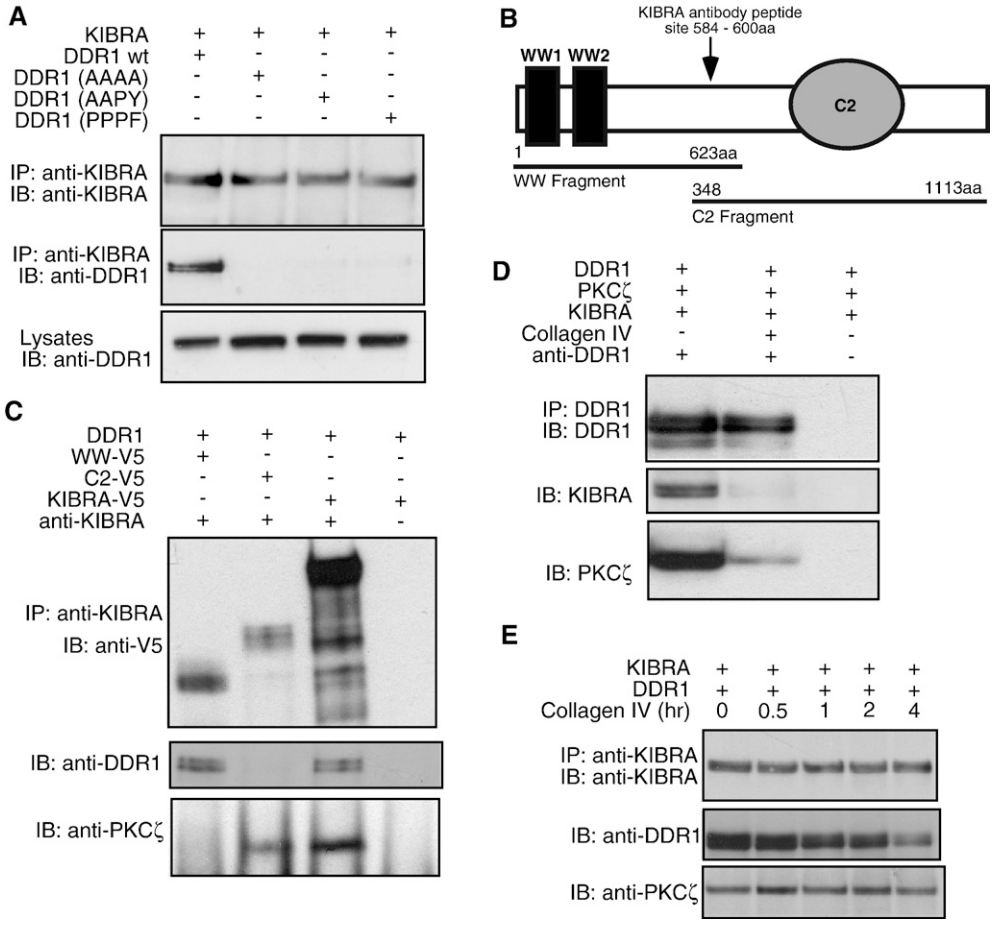


Fig. 5. KIBRA and DDR1 interact via their respective WW domains and PPxY motif. A. KIBRA immunoprecipitates from HEK-293 cells transiently transfected with DDR1b or DDR1 mutants (AAPY, AAAA or PPPF) and blotted with anti-KIBRA and anti-DDR1. Only wild type DDR1b co-precipitates with KIBRA. B. Schematic of KIBRA protein showing the WW and C2 fragments and the KIBRA antibody peptide site. C. KIBRA immunoprecipitates from HEK-293 cells transiently co-transfected with DDR1b and each of WW-V5-6xHIS, C2-V5-6xHIS or KIBRA-V5-6xHIS and blotted with anti-V5 and anti-DDR1. DDR1 co-precipitates with full length KIBRA and the WW fragment but not with the C2 fragment. Endogenous PKCζ also co-immunoprecipitates with full length KIBRA and the C2 fragment. D. DDR1 immunoprecipitates from HEK-293 cells (+/− collagen) transiently co-transfected with DDR1, KIBRA and PKCζ. KIBRA and PKCζ co-precipitate with DDR1 predominantly in the absence of collagen, and only minimal amounts are pulled down following collagen treatment. E. KIBRA immunoprecipitates from HEK-293 cells transiently transfected with DDR1 and KIBRA over a time-course of collagen treatment. The levels of endogenous PKCζ that are pulled down with KIBRA remain constant over the time course.

proteins using DDR1 antibodies (Fig. 5D). The presence of collagen reduced the amount of KIBRA co-precipitating with DDR1, and the amount of associated PKCζ decreased in proportion (Fig. 5D). In addition, collagen treatment has no effect on the KIBRA-PKCζ interaction. Although the levels of DDR1 co-immunoprecipitated with KIBRA decrease over a time course of collagen treatment, the levels of PKCζ remain the same (Fig.5E). These data indicate that KIBRA, DDR1 and PKCζ interact in a tripartite complex, but predominantly in the absence of collagen.

3.8. Modulation of KIBRA expression has downstream effects on the collagen-stimulated activation of ERK MAPK

Having demonstrated that KIBRA interacts in a complex with DDR1 and PKCζ, we searched for common downstream targets of both DDR1 and PKCζ that may provide clues as to the functional role of KIBRA. We identified the ERK MAPK cascade, a ubiquitous cytoplasmic signalling pathway with a critical role in fundamental biological processes such as cell proliferation, differentiation and apoptosis, as being a downstream target of both DDR1 and PKCζ. To investigate whether KIBRA may also be involved in this pathway we transfected HEK-293 cells with KIBRA (KIBRApcDNA3.1) or vector (pcDNA3.1) and stimulated with collagen type IV. As shown in Fig. 6, KIBRA-transfected cells show increased levels of phospho-ERK when exposed to collagen, relative to vector-only cells. No changes were observed in total ERK levels (Fig. 6A). Fig. 6B shows densitometric analysis of a representative Western blot, revealing an approximate 3–4 fold increase in levels of phospho-ERK in cells transfected with KIBRA in the presence of collagen, compared to empty vector controls. To confirm KIBRA is involved in the collagen-regulated stimulation of the MAPK pathway we used the normal human mammary epithelial cell line, MCF-10A, to determine whether there was any effect on the activation of this signal transduction cascade when endogenous KIBRA expression was knocked down by siRNA. Fig. 6C shows that ERK is

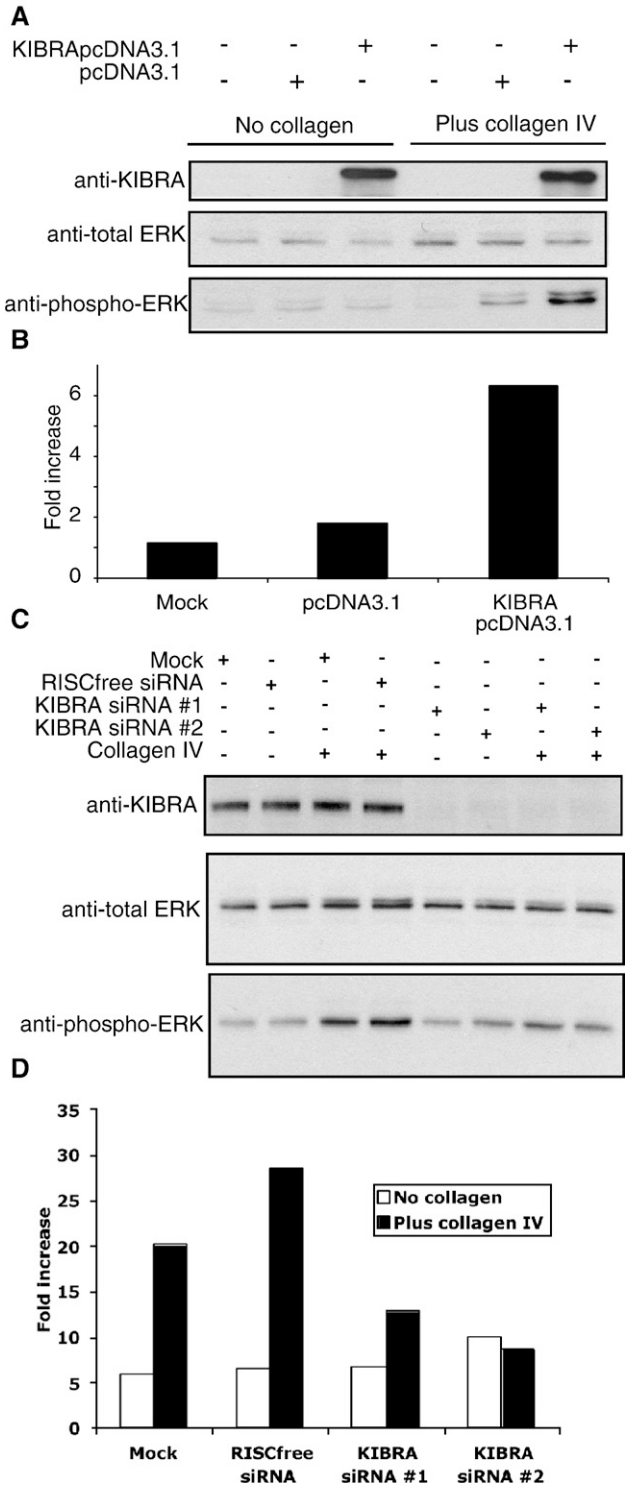


Fig. 6. KIBRA regulates collagen-stimulated ERK MAPK activation. A. Representative immunoblots of lysates from HEK-293 cells transiently transfected with KIBRApcDNA3.1 and pcDNA3.1 and treated +/− collagen for 20 min. B. Corresponding graph depicting fold increase in ERK phosphorylation. C. Western blot of lysates from MCF-10A cells transfected with KIBRA siRNA duplexes and treated +/− collagen for 20 min. D. Corresponding graph depicting fold increase in ERK phosphorylation as determined by densitometric analysis.

activated upon short-term stimulation with collagen in mock and RISCfree siRNA controls, but that this activation is suppressed when KIBRA expression is knocked down using 2 different siRNA duplexes. Fig. 6D shows the corresponding densitometric analysis. These data demonstrate that KIBRA plays a positive role in the collagen-stimulated activation of the ERK MAPK cascade.

4. Discussion

In this study, we have identified KIBRA as a hormonally-regulated epithelial-specific mammary gland transcript that showed decreased expression in the Prlr knockout mammary epithelium and increased expression in response to Pg. We have shown that KIBRA interacts in a collagen-regulated manner with DDR1, a protein known to have an important role in mammary development [27], and that this interaction occurs via the PPxY motif of DDR1. Furthermore, we have demonstrated that KIBRA interacts simultaneously with DDR1 and PKCζ, and that KIBRA is involved in the collagen-regulated stimulation of the MAPK cascade.

KIBRA expression is epithelial-specific with moderate expression levels in the mammary gland and high expression in the kidney and brain. In addition to showing decreased expression in $Prlr^{-/-}$ mice, KIBRA is markedly up-regulated with progesterone treatment. It is interesting to note that a number of other genes were identified in the original study which, similarly to KIBRA, displayed decreased expression levels in $Prlr^{-/-}$ mammary epithelial cells, and are positively regulated by progesterone [9]. In addition to Prlr itself, these genes included amphiregulin, calcitonin and Wnt-4 [28–31]. Both prolactin and progesterone work synergistically in driving alveolar morphogenesis. This has been demonstrated in mammary cells where prolactin causes up-regulation of the progesterone receptor, and conversely progesterone up-regulates the prolactin receptor [28]. This may provide an additional explanation for our detection of KIBRA as differentially regulated in $Prlr^{-/-}$ epithelium compared to wild type.

We have shown here that KIBRA interacts with DDR1, a collagen-activated receptor tyrosine kinase that is essential in mammary gland development [27]. Similarly to KIBRA, DDR1 is epithelial-specific, shows an increase in expression in the mouse mammary gland during pregnancy, and is also over expressed in several primary breast tumours [32]. Female DDR1 knockout mice show defects in blastocyst implantation together with hyperproliferation and abnormal branching of the mammary ducts which result in a lactational defect [27]. DDR1 mutant mice also show an increased amount of collagenous extracellular matrix surrounding the mammary epithelium [27]. This observation suggests a role for DDR1 in mediating extracellular matrix signalling within the mammary gland. The interaction between the mammary epithelium and the extracellular matrix is essential for complete alveolar morphogenesis and in the regulation of processes such as cell motility and adhesion [33], which when dysregulated can drive tumor progression by causing enhanced migration, invasion or metastasis. A number of studies have detected an overexpression of DDR1 in human tumours, particularly in primary breast cancer [32], and pediatric brain cancer [34]. It has also been identified as a dysregulated gene in epithelial ovarian cancer [35], and more recently as a novel marker for differentiation of invasive ductal and lobular breast carcinomas [36]. Since KIBRA binds DDR1 and regulates ERK MAPK

activation in response to the DDR1 ligand collagen, it will be important to determine KIBRA expression in breast and other cancers.

Our data demonstrate that the KIBRA-DDR1 complex dissociates upon activation of DDR1 by collagen, suggesting a role for KIBRA in the downstream signalling pathways induced by the extracellular matrix. It is therefore interesting to note that forced expression and activation of DDR1 with collagen in mouse mammary epithelial HC11 cells resulted in increased activation of Stat5, a downstream target of Prlr, as well as increased β-casein gene expression [37]. This suggests that the collagenous extracellular matrix and DDR1 signalling pathway work in conjunction with the prolactin pathway in order to maintain efficient lactogenesis. As KIBRA expression is regulated by two hormones required for alveolar morphogenesis, we postulate a role for the KIBRA-DDR1 interaction in integrating hormone-stimulated and matrix-derived signals essential in maintaining normal mammary gland function.

Other interacting proteins for both KIBRA and DDR1 have previously been identified. KIBRA has been reported to be a novel substrate for protein kinase Cζ (PKCζ) [14], a member of the family of atypical PKCs, which are characterised by the presence of only one zinc finger module, and do not bind or respond to phorbol esters and diacylglycerol [38]. PKCζ is important in regulating many cell processes, such as proliferation and differentiation, and when stably over expressed in a murine mammary epithelial cell line has been shown to modulate cell proliferation, adhesion and migration, via activation of the ERK MAPK pathway [39]. PKC isozymes have long been implicated in carcinogenesis, and specifically, PKCζ shows increasing expression correlating with increasing tumor grade in urinary bladder cancer [40], is involved in cancer cell motility in pancreatic adenocarcinoma cells [41], and has been shown to be overexpressed in breast carcinomas [42]. With respect to DDR1, other interacting proteins have previously been identified which bind this receptor in a phosphotyrosine-dependent manner. One study reported an interaction between the neuronal phosphoprotein DARPP-32 and DDR1, which similarly to KIBRA was shown to be stronger in the absence of collagen, and their interaction was shown to inhibit the migration of human breast cancer cells [43]. In contrast, previous studies determined that several other cytoplasmic signalling proteins, including Shc, Nck2 and Shp-2, bind DDR1 but only following activation by collagen [26,44].

In addition to the PKCζ pathway, ERK MAPK has also previously been shown to be a downstream effector in the DDR1 pathway [45,46], and so our data demonstrating an interaction between KIBRA, PKCζ and DDR1 in a complex, suggests a potential role for KIBRA in activation of the ERK MAPK cascade. This is further supported by our data showing that overexpression of KIBRA in the presence of collagen causes an increase in ERK phosphorylation, and conversely that knockdown of KIBRA expression inhibits ERK MAPK activation upon collagen treatment. It is interesting to note that while unstimulated DDR1 has been shown to suppress ERK MAPK phosphorylation, collagen-stimulated DDR1 induces ERK MAPK activation [45]. As collagen treatment is also required for ERK phosphorylation when KIBRA is over-expressed, and collagen inhibits the KIBRA-DDR1 interaction, we propose a model whereby these proteins must dissociate in order for activation of the ERK MAPK cascade to occur, and that unstimulated DDR1 potentially interacts with KIBRA to suppress ERK signalling. One possibility of how this activation occurs is either via downstream signalling by the KIBRA–PKCζ complex, or by KIBRA dissociation allowing access to the collagen-activated receptor for molecules that participate in Ras/ERK signalling, such as Shc. These models are not mutually exclusive. It has previously been reported that progestins can stimulate proliferation of mammary epithelial cells derived from adult virgin mice in the presence of collagen type IV [47]. One might speculate that this proliferation is a result of activation of the ERK MAPK cascade due to an up-regulation of KIBRA expression by progestin, in the presence of collagen. And so from the data presented, we hypothesise that in cells which are in contact with the extracellular matrix, modulation of KIBRA expression by progesterone and prolactin is important in activating the ERK MAPK cascade required for tissue remodelling of the mammary gland throughout its cycle of development.

Acknowledgements

We thank Assoc Prof Elizabeth A. Musgrove and Ms C. Elizabeth Caldon for helpful discussion and use of reagents. We also thank Dr Axel Ullrich for supplying the pRK5-DDR1b expression plasmid.

References

[1] L. Hennighausen, G.W. Robinson, Signaling pathways in mammary gland development, Dev. Cell 1 (2001) 467–475.
[2] R.C. Hovey, J.F. Trott, B.K. Vonderhaar, Establishing a framework for the functional mammary gland: from endocrinology to morphology, J. Mammary Gland Biol. Neoplasia 7 (2002) 17–38.
[3] M.C. Neville, T.B. McFadden, I. Forsyth, Hormonal regulation of mammary differentiation and milk secretion, J. Mammary Gland Biol. Neoplasia 7 (2002) 49–66.
[4] C. Brisken, Hormonal control of alveolar development and its implications for breast carcinogenesis, J. Mammary Gland Biol. Neoplasia 7 (2002) 39–48.
[5] W.P. Bocchinfuso, K.S. Korah, Mammary gland development and tumorigenesis in estrogen receptor knockout mice, J. Mammary Gland Biol. Neoplasia 2 (1997) 323–334.
[6] M.I. Gallego, N. Binart, G.W. Robinson, R. Okagaki, K.T. Coschigano, J. Perry, J.J. Kopchick, T. Oka, P.A. Kelly, L. Hennighausen, Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects, Dev. Biol. 229 (2001) 163–175.
[7] R.C. Hovey, J.F. Trott, E. Ginsburg, A. Goldhar, M.M. Sasaki, S.J. Fountain, K. Sundararajan, B.K. Vonderhaar, Transcriptional and spatiotemporal regulation of prolactin receptor mRNA and cooperativity with progesterone receptor function during ductal branch growth in the mammary gland, Dev. Dyn. 222 (2001) 192–205.
[8] T.I. Lee, N.J. Rinaldi, F. Robert, D.T. Odom, Z. Bar-Joseph, G.K. Gerber, N.M. Hannett, C.T. Harbison, C.M. Thompson, I. Simon, J. Zeitlinger, E.G. Jennings, H.L. Murray, D.B. Gordon, B. Ren, J.J. Wyrick, J.B. Tagne, T.L. Volkert, E. Fraenkel, D.K. Gifford, R.A. Young, Transcriptional regulatory networks in *Saccharomyces cerevisiae*, Science 298 (2002) 799–804.
[9] J. Harris, P.M. Stanford, K. Sutherland, S.R. Oakes, M.J. Naylor, F.G. Robertson, K.D. Blazek, M. Kazlauskas, H.N. Hilton, S. Wittlin, W.S. Alexander, G.J. Lindeman, J.E. Visvader, C.J. Ormandy, Socs2 and Elf5

mediate prolactin-induced mammary gland development, Mol. Endocrinol. 20 (2006) 1177–1187.
[10] J. Kremerskothen, C. Plaas, K. Buther, I. Finger, S. Veltel, T. Matanis, T. Liedtke, A. Barnekow, Characterization of KIBRA, a novel WW domain-containing protein, Biochem. Biophys. Res. Commun. 300 (2003) 862–867.
[11] L. Otte, U. Wiedemann, B. Schlegel, J.R. Pires, M. Beyermann, P. Schmieder, G. Krause, R. Volkmer-Engert, J. Schneider-Mergener, H. Oschkinat, WW domain sequence activity relationships identified using ligand recognition propensities of 42 WW domains, Protein Sci. 12 (2003) 491–500.
[12] M. Sudol, K. Sliwa, T. Russo, Functions of WW domains in the nucleus, FEBS Lett. 490 (2001) 190–195.
[13] E.A. Nalefski, J.J. Falke, The C2 domain calcium-binding motif: structural and functional diversity, Protein Sci. 5 (1996) 2375–2390.
[14] K. Buther, C. Plaas, A. Barnekow, J. Kremerskothen, KIBRA is a novel substrate for protein kinase Czeta, Biochem. Biophys. Res. Commun. 317 (2004) 703–707.
[15] A. Papassotiropoulos, D.A. Stephan, M.J. Huentelman, F.J. Hoerndli, D.W. Craig, J.V. Pearson, K. Huynh, F. Brunner, J. Corneveaux, D. Osborne, M.A. Wollmer, A. Aerni, D. Coluccia, J. Hanggi, C.R.A. Mondadori, A. Buchman, E.M. Reiman, R.J. Caselli, K. Henke, D.J.F. dr Quervain, Common Kibra alleles are associated with human memory performance, Science 314 (2006) 475–478.
[16] S.K. Rayala, P. den Hollander, B. Manavathi, A.H. Talukder, C. Song, S. Peng, A. Barnekow, J. Kremerskothen, R. Kumar, Essential role of KIBRA in co-activator function of Dynein Light Chain 1 in mammalian cells, J. Biol. Chem. 281 (2006) 19092–19099.
[17] D.F. Bishop, A.S. Henderson, K.H. Astrin, Human delta-aminolevulinate synthase: assignment of the housekeeping gene to 3p21 and the erythroid-specific gene to the X chromosome, Genomics 7 (1990) 207–214.
[18] A.G. Roberts, G.H. Elder, Alternative splicing and tissue-specific transcription of human and rodent ubiquitous 5-aminolevulinate synthase (ALAS1) genes, Biochim. Biophys. Acta 19 (2001) 95–105.
[19] P. Rice, I. Longden, A. Bleasby, EMBOSS: the European Molecular Biology Open Software Suite, Trends Genet. 16 (2000) 276–277.
[20] M. Diehn, G. Sherlock, G. Binkley, H. Jin, J.C. Matese, T. Hernandez-Boussard, C.A. Rees, J.M. Cherry, D. Botstein, P.O. Brown, A.A. Alizadeh, SOURCE: a unified genomic resource of functional annotations, ontologies, and gene expression data, Nucleic Acids Res. 31 (2003) 219–223.
[21] A. Reynolds, D. leake, Q. Boese, S. Scaringe, W.S. Marshall, A. Khvorova, Rational design for siRNA design for RNA interference, Nat. Biotechnol. 22 (2004) 326–330.
[22] T. Brummer, D. Schramek, V.M. Hayes, H.L. Bennett, C.E. Caldon, E.A. Musgrove, R.J. Daly, Increased proliferation and altered growth factor dependence of human mammary epithelial cells overexpressing the Gab2 docking protein, J. Biol. Chem. 281 (2006) 626–637.
[23] M. Lacroix, G. Leclercq, Relevance of breast cancer cell lines as models for breast tumours: an update, Breast Cancer Res. Treat. 83 (2004) 249–289.
[24] R.L. Sutherland, C.K.W. Watts, C.S.L. Lee, E.A. Musgrove, Breast cancer, in human cell culture, in: J.R.W Masters, B. Palsson (Eds.), Kluwer Academic Publishers, Great Britain, 1999, pp. 79–106.
[25] M. Sudol, T. Hunter, NeW wrinkles for an old domain, Cell 103 (2000) 1001–1004.
[26] W. Vogel, G.D. Gish, F. Alves, T. Pawson, The discoidin domain receptor tyrosine kinases are activated by collagen, Mol. Cell 1 (1997) 13–23.
[27] W.F. Vogel, A. Aszodi, F. Alves, T. Pawson, Discoidin domain receptor 1 tyrosine kinase has an essential role in mammary gland development, Mol. Cell. Biol. 21 (2001) 2906–2917.
[28] C.J. Ormandy, R.E. Hall, D.L. Manning, J.F. Robertson, R.W. Blamey, P.A. Kelly, R.I. Nicholson, R.L. Sutherland, Coexpression and cross-regulation of the prolactin receptor and sex steroid hormone receptors in breast cancer, J. Clin. Endocrinol. Metab. 82 (1997) 3692–3699.
[29] S.K. Das, B.C. Chakraborty, B.C. Paria, X.N. Wang, G. Plowman, S.K. Dey, Amphiregulin is an implantation-specific and progesterone-regulated gene in the mouse uterus, Mol. Endocrinol. 9 (1995) 691–705.
[30] C. Brisken, A. Heineman, T. Chavarria, B. Elenbass, J. Tan, S.K. Dey, J.A. McMahon, A.P. McMahon, R.A. Weinberg, Essential function of Wnt-4 om mammary gland development downstream of progesterone signalling, Genes Dev. 14 (2000) 650–654.
[31] P.M. Ismail, F.J. DeMayo, J.P. Lydon, Progesterone induction of calcitonin expression in the murine mammary gland, J. Endocrinol. 180 (2004) 287–295.
[32] K.T. Barker, J.E. Martindale, P.J. Mitchell, T. Kamalati, M.J. Page, D.J. Phippard, T.C. Dale, B.A. Gusterson, M.R. Crompton, Expression patterns of the novel receptor-like tyrosine kinase, DDR, in human breast tumours, Oncogene 10 (1995) 569–575.
[33] J.E. Fata, Z. Werb, M.J. Bissell, Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes, Breast Cancer Res. 6 (2004) 1–11.
[34] H.L. Weiner, M. Rothman, D.C. Miller, E.B. Ziff, Pediatric brain tumors express multiple receptor tyrosine kinases including novel cell adhesion kinases, Pediatr. Neurosurg. 25 (1996) 64–72.
[35] V.A. Heinzelmann-Schwarz, M. Gardiner-Garden, S.M. Henshall, J. Scurry, R.A. Scolyer, M.J. Davies, M. Heinzelmann, L.H. Kalish, A. Bali, J.G. Kench, L.S. Edwards, P.M. Vanden Bergh, N.F. Hacker, R.L. Sutherland, P.M. O'Brien, Overexpression of the cell adhesion molecules DDR1, Claudin 3, and Ep-CAM in metaplastic ovarian epithelium and ovarian cancer, Clin. Cancer Res. 10 (2004) 4427–4436.
[36] G. Turashvili, J. Bouchal, K. Baumforth, W. Wei, M. Dziechciarkova, J. Ehrmann, J. Klein, E. Fridman, J. Skarda, J. Srovnal, M. Hajduch, P. Murray, Z. Kolar, Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis, BMC Cancer 7 (2007) 55.
[37] E. Faraci-Orf, C. McFadden, W. Vogel, DDR1 signaling is essential to sustain Stat5 function during lactogenesis, J. Cell. Biochem. 97 (2006) 109–121.
[38] J. Moscat, M.T. Diaz-Meco, The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters, EMBO Rep. 1 (2000) 399–403.
[39] A.J. Urtrerger, V.C. Grossini, K.B. Falbo, M.G. Kazanietz, E.D. Bal de Kier Joffe, Atypical protein kinase C-zeta modulates clonogenicity, motility, and secretion of proteolytic enzymes in murine mammary cells, Mol. Carcinog. 42 (2005) 29–39.
[40] A. Varga, G. Czifra, B. Tallai, T. Nemeth, I. Kovacs, L. Kovacs, T. Biro, Tumor grade-dependent alterations in the protein kinase C isoform pattern in urinary bladder carcinomas, Eur. Urol. 46 (2004) 462–465.
[41] C. Laudanna, C. Sorio, C. Tecchio, E.C. Butcher, A. Bonora, C. Bassi, A. Scarpa, Motility analysis of pancreatic adenocarcinoma cells reveals a role for the atypical zeta isoform of protein kinase C in cancer cell movement, Lab. Invest. 83 (2003) 1155–1163.
[42] T. Schondorf, C.M. Kurbacher, M. Becker, M. Warm, H. Kolhagen, U.J. Gohring, Heterogeneity of protein kinase C activity and PKC-zeta expression in clinical breast carcinomas, Clin. Exp. Med. 1 (2001) 1–8.
[43] C. Hansen, P. Greengard, A.C. Nairn, T. Andersson, W.F. Vogel, Phosphorylation of DARPP-32 regulates breast cancer cell migration downstream of the receptor tyrosine kinase DDR1, Exp. Cell Res. (2006) 4011–4018.
[44] D.H. Koo, C. McFadden, Y. Huang, R. Abdulhussein, M. Friese-Hamim, W.F. Vogel, Pinpointing phosphotyrosine-dependent interactions downstream of the collagen receptor DDR1, FEBS Lett. 580 (2006) 15–22.
[45] C.A. Curat, W.F. Vogel, Discoidin domain receptor 1 controls growth and adhesion of mesangial cells, J. Am. Soc. Nephrol. 13 (2002) 2648–2656.
[46] P.P. Ongusaha, J. Kim, L. Fang, T.W. Wong, G.D. Yancopoulos, S.A. Aaronson, S.W. Lee, p53 induction and activation of DDR1 kinase counteract p53-mediated apoptosis and influence p53 regulation through a positive feedback loop, EMBO J. 22 (2003) 1289–1301.
[47] J. Xie, S.Z. Haslam, Extracellular matrix regulates ovarian hormone-dependent proliferation of mouse mammary epithelial cells, Endocrinology 138 (1997) 2466.