Development 133, 3485-3494 (2006) doi:10.1242/dev.02502

IKKβ/2 induces TWEAK and apoptosis in mammary epithelial cells

Fiona O. Baxter[1], Paul J. Came[1], Kathrine Abell[1], Blandine Kedjouar[1], Marion Huth[2], Klaus Rajewsky[3], Manolis Pasparakis[2] and Christine J. Watson[1,*]

The Nuclear Factor-κB (NF-κB) family of transcription factors are ubiquitously expressed and control a wide range of cellular responses, including apoptosis, proliferation, differentiation, inflammation and immunity. Here, we investigated the function of the NF-κB upstream regulator IκB kinase 2/β (IKK2) in apoptosis regulation in the normal physiological setting of regressing mammary gland. Conditional deletion of the gene encoding IKK2 resulted, surprisingly, in delayed apoptosis and remodelling, and abrogation of caspase 3 cleavage. This failure to induce involution was associated with reduced expression, within 24 hours of involution, of the death receptor (DR) ligand TNF and its receptor TNFR1, which are known NF-κB targets. This was associated with elevated levels of active AKT and phosphorylated FOXO3a. Furthermore, we show that expression of TWEAK, another DR ligand, is dramatically downregulated, even in heterozygous IKK2 mammary glands. Unlike other DR ligands, the TWEAK promoter has six consensus FOXO-binding sites, further suggesting that it is differentially regulated. Interestingly, a cleaved form of TWEAK is upregulated during involution. This unexpected function of the IKK2/NF-κB pathway as a regulator of TWEAK expression and inducer of apoptosis has significant consequences for future therapeutic approaches for cancer and inflammatory diseases.

KEY WORDS: Apoptosis, IKK2, Mammary gland, TWEAK, Mouse

INTRODUCTION

Nuclear Factor-κB (NF-κB) is a family of evolutionarily conserved transcription factors that play a vital role in modulating cellular responses to a variety of stimuli (Karin and Ben-Neriah, 2000). In most resting cells, NF-κB is held in an inactive state in the cytoplasm via an interaction with a member of the Inhibitor of κB (IκB) family of proteins. NF-κB is activated in response to a range of factors, and most of these signals are mediated by a multiprotein signalling complex, the IκB kinase (IKK). The IKK complex consists of two catalytic subunits: IKK1/α (CHUK – Mouse Genome Informatics) and IKK2/β (IκBKβ – Mouse Genome Informatics) and a regulatory subunit, NEMO (or IKKγ; IκBKγ – Mouse Genome Informatics). Activation of the IKK complex results in phosphorylation of the IκB proteins; phospho-IκB is rapidly ubiquitinated and degraded via the 26S-proteasome releasing NF-κB and unmasking its nuclear localisation signal, thereby allowing nuclear entry and modulation of target gene expression (Karin and Ben-Neriah, 2000).

In previous studies we showed that NF-κB activity is dramatically regulated throughout mammary gland development (Clarkson et al., 2000). NF-κB DNA-binding activity is high during pregnancy, when there is substantial proliferation of the secretory mammary epithelial cells, is absent during lactation, and is rapidly reactivated during involution, when removal of the pups initiates regression in a highly regulated process in which the epithelial cells are removed by extensive apoptosis (Strange et al., 1992). Apoptosis occurs in two phases: during the first 48 hours cell death is reversible and can be halted by returning suckling pups to the mother. The second, post-48-hour phase, is irreversible and involves breakdown of the extracellular matrix, phagocytosis and tissue remodelling (Furth et al., 1997; Lund et al., 1996).

Using conditional gene targeting, we have shown that apoptosis in the mammary gland is mediated by the transcription factor STAT3 (Chapman et al., 1999) through elevated levels of LIF (Kritikou et al., 2003). Although STAT3 is essential, it is not sufficient for apoptosis, as expression of a constitutively active *Akt* transgene provides an overriding survival signal (Schwertfeger et al., 2001). Thus, while STAT3 provides a powerful apoptotic signal, it is clear that there are additional mechanisms of apoptosis regulation in early involution that synergise to ensure efficient induction of cell death, phagocytosis, suppression of inflammation and remodelling of the architecture of the gland.

In order to identify these regulatory pathways, we carried out a microarray analysis of involution. This analysis identified clusters of genes that are transcriptional targets of either NF-κB or STAT3, or indeed both, during early involution (Clarkson and Watson, 2003). One gene cluster included the NF-κB targets, the TNF superfamily of death receptor (DR) ligands. These ligands can induce apoptosis through binding to their cognate receptor, recruiting caspase 8 (via FADD) and activating the caspase cascade, ultimately resulting in cell death (Ashkenazi and Dixit, 1998).

As NF-κB DNA-binding activity is rapidly induced following the initiation of involution, being detectable within 1 hour after pup removal, this indicates that NF-κB could provide either a pro-apoptotic or anti-apoptotic signal during involution and may have different roles during the different stages of a pregnancy/lactation/involution cycle. Cao et al. demonstrated the dependence on IKK1 kinase activity for maximal NF-κB activation during pregnancy (Cao et al., 2001). These results suggest that IKK2 is not able to compensate completely for the loss of IKK1 kinase activity in the mammary gland during pregnancy. This, in turn, suggests that IKK1 and IKK2 may play different roles in mammary gland development.

[1]Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK. [2]EMBL Mouse Laboratory Programme, Via Ramarini 32, 00016 Monterotondo, Rome, Italy. [3]The CBR Institute for Biomedical Research, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA.

*Author for correspondence (e-mail: cjw53@mole.bio.cam.ac.uk)

Accepted 20 June 2006

The NF-κB family of transcription factors primarily play anti-apoptotic roles. Depending on cell type, extent of NF-κB activation and nature of apoptotic signal, however, NF-κB can also be pro-apoptotic (Fujioka et al., 2004). Furthermore, deletion of IKK2 or IKK1 results in enhanced apoptosis in a number of systems, suggesting that these NF-κB regulators are usually anti-apoptotic. As the NF-κB pathway is likely to be important in apoptosis regulation, and the roles of IKK1 and IKK2 in this process have not been investigated, we generated a mammary epithelial cell-specific knockout of IKK2. This revealed, surprisingly, that IKK2 is a pro-apoptotic factor in mammary epithelium. Furthermore, IKK2 regulates expression of the DR ligand TWEAK (TNFSF12 – Mouse Genome Informatics) through modulating the activity of FOXO3a.

MATERIALS AND METHODS

Generation of conditional IKK2 knockout mice

Mice with mammary gland-specific deletion of IKK2 were generated by crossing floxed IKK2/β mice (Pasparakis et al., 2002) with mice expressing Cre under the control of the mammary-specific β-lactoglobulin milk gene (BLG) promoter. Involution was initiated by removal of pups (normalised to at least six) after 10 days of lactation, and mammary glands were harvested from mice at 0 day lactation and 24, 48, 72 or 144 hours after forced involution. At least three mice of each genotype were analysed for each time point. Glands were either snap frozen for the preparation of RNA, nuclear or protein extracts, or fixed in formalin then embedded in paraffin for sectioning. All animal experiments were performed in compliance with Home Office regulations and approved by the local ethics committee.

Quantification of IKK2 deletion by Southern blotting

Quantification of IKK2 deletion was by Southern blotting as described previously (Pasparakis et al., 2002). DNA was isolated from mammary gland tissue and digested with *Stu*I before electrophoresis and membrane transfer. Hybridisation was with a 700 bp probe that detected a 3.8 kb wild-type allele, a 3.9 kb floxed allele or a 1.8 kb deleted allele. Blots were exposed and bands were quantified using a Bio-Rad Personal Molecular Imager FX System and Quantity One software (Bio-Rad Laboratories Ltd, Hertfordshire, UK).

Immunoblotting

Total protein was extracted from frozen mammary gland tissue as described previously (Kritikou et al., 2003). Samples were resolved in 8-15% SDS-polyacrylamide gels, transferred to PVDF membranes and blocked in PBS with 0.05% TWEEN-20 (PBST) and 5% non-fat powdered milk for 30 minutes before incubation with primary antibody overnight at 4°C. Membranes were quickly washed twice with dH_2O then horseradish peroxidase (HRP)-conjugated secondary antibody was added [goat anti-rabbit HRP, goat anti-rat HRP, or goat anti-mouse HRP (DAKO)] then incubated for 1 hour at room temperature. Following a further two quick H_2O washes membranes were washed twice for 5 minutes in PBST, proteins were detected by enhanced chemiluminescence (ECL).

Primary antibodies were from the following suppliers: AKT, pAKT (ser473), cleaved caspase 3, FOXO3a, pFOXO3a (Ser-253), IKK2, STAT3 and pSTAT3 (Ser-727) from Cell Signalling Technology, Hertfordshire, UK; p50 (SC-7178), p52 (SC-298) and TWEAK (SC-5558) from Santa Cruz Biotechnology Inc, Heidelberg, Germany; α-tubulin and p65 from AbCam, Cambridge, UK; Rb from Neomarkers, Lab Vision Corporation, Suffolk, UK; FOXO3a (used for IHC) was a kind gift from Dr E. Kondo, Department of Pathology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from mammary gland tissue and subjected to EMSA with an NF-κB-specific oligonucleotide probe as described previously (Clarkson et al., 2000).

DAB immunohistochemistry

Mammary gland sections were de-waxed in xylene then rehydrated in ethanol. Antigen retrieval was by pressure cooker for 5 minutes at high pressure in 10 mmol/l citric acid buffer (pH 6.0). Endogenous peroxidase activity was quenched with H_2O_2 before sections were blocked in 10% normal goat serum (Dako, Cambridgeshire, UK) for 2 hours at room temperature. Incubation with diluted primary antibody was overnight at 4°C. Following washes, biotinylated secondary antibody (biotinylated anti-rabbit IgG, Abcam, Cambridge, UK) was incubated on sections for 1.5 hours at room temperature before washing and addition of ABC complex (Vector Laboratories, Peterborough, UK). Following washes sections were developed with DAB chromogen (Vector laboratories) and nuclei counterstained with Carazzi's haematoxylin. Sections were dehydrated in methanol and cleared in xylene before mounting in DPX and viewing.

Fluorescent immunohistochemistry

Sections of paraffin-embedded mammary gland tissue were de-waxed in xylene and rehydrated in alcohol. Antigen retrieval was by pressure cooker for 8 minutes at high pressure in 10 mmol/l citric acid buffer (pH 6.0). KIM-2 cells were grown on plastic chamber slides (Nunc, Fisher Scientific, Leicestershire, UK) and fixed with methanol:acetone (50:50) for 10 minutes at RT. Sections were blocked in 10% goat serum for 1.5 hours at room temperature then primary antibody [cleaved caspase 3 antibody (Cell Signalling Technology) or TWEAK (Santa Cruz Biotech)], diluted in 5% goat serum, was added to the sections and incubated overnight at 4°C. Following PBS washes, secondary AlexaFluor goat anti-rabbit 488 antibody or AlexaFluor donkey anti-rabbit 555 (2 mg/ml in 5% goat serum) (Molecular Probes Europe BV, Leiden, The Netherlands) was incubated on the sections for 1.5 hours. Sections were washed in PBS and mounted in Vectashield mounting medium with DAPI (Vector Laboratories) then imaged. Cleaved caspase 3 staining was visualised with an AxioVertS100TV inverted microscope. Caspase-3-positive cells were counted in five randomly selected fields and percentages were scored relative to nuclear (DAPI) staining. TWEAK staining was visualised with a Leica TCS-NT-UV confocal laser scanning microscope.

TUNEL staining

TUNEL staining was performed using the ApopTag plus peroxidase in situ apoptosis detection kit (Intergen Company, Oxford UK), according to the manufacturer's protocol.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from ground mammary gland tissue using TRI-reagent (Sigma-Aldrich Company Ltd, Dorset, UK) and purified using Absolutely RNA RT-PCR Miniprep Kit (Stratagene, California, USA). cDNA was synthesised using Transcriptor (Roche Diagnostics Ltd, East Sussex, UK) and quantitative PCR performed using Bio-Rad i-cycler platform with i-cycle supermix (Bio-Rad), SYBR green (Sigma-Aldrich), Fluorescein (Bio-Rad) and specific sense and antisense primers (Sigma-Genosys Ltd, Haverhill, UK). Primer sequences were as follows: Cyclophilin A: 5′-CCTTGGGCCGCGTCTCCTT-3′ and 5′-CACCCT-GGCACATGAATCCTG-3′; Fas ligand: 5′-TTCATGGTTCTGGTGG-CTCTGGT-3′ and 5′-CTGGGGTTGGCTATTTGCTTTTCA-3′; *TNF*: 5′-AGGCACTCCCCCAAAAGATG-3′ and 5′-TCACCCCGAAGTTC-AGTAGACAGA-3′; *TRAIL*: 5′-TTAAGAGGCAACTGTATCA-3′ and 5′-GGGCAAGGGAGGAG-3′; *TWEAK*: 5′-TTGGCCTCCTGCTGGT-CGTGGTCA-3′ and 5′-CTCCCGGCGGTCCTCTGCTGTCA-3′; *TNFR1*: 5′-CACGCCGCGCCACGAGGACA-3′ and 5′-CGTGGACGAGGGGGC-GGGATTTCT-3′.

Pharmacological inhibitors

IKK2 inhibitor (15-dPGJ$_2$) was purchased from Cayman Chemicals (IDS Ltd), Tyne and Wear, UK.

RT-PCR

RNA was extracted and cDNA synthesis was as described for quantitative PCR. PCR reactions were performed using Qiagen Taq DNA Polymerase (Qiagen, West Sussex, UK) as outlined by the manufacturer. Specific Fn14

(TNFRSF12a – Mouse Genome Informatics) primers were as follows; Fn14: 5′-GGCCGGGGAGCAAGCACCAG-3′ and 5′-ACCAGCGCCAAAACC-AGGACCAGA-3′.

Bioinformatics

In-silico analysis of the potential *Tweak*, *Tnf*, *Tnfr1*, *FasL* and *Trail* promoter regions was performed using the Eldorado and Matinspector software in the Genomatix suite (www.Genomatix.de), FirstEF (Davuluri et al., 2001) and Genepalette. The sequences used for *Tweak* were NT_096135 and NM-011614, for *Tnf* NT_039649 and NM_013693, for *Tnfr1* AC140324, NM_011609 and AK181531, for *FasL* NT_039185 and NM_010177, and for *Trail* NT_078380 and NM_009425. The transcription start site for *Tweak* was identified using Eponine transcription start site finder (http://servlet.sanger.ac.uk:8080/eponine/), as there is no information on the 5′UTR of this gene. The transcription start site in *Tnfr1* was identified using 5′cap trapped mRNA sequence (AK181531).

RESULTS

Deletion of the Ikk2 gene reduces NF-κB activity

Deletion of the *Ikk2* gene was restricted to mammary epithelial cells by crossing floxed *Ikk2* mice to a transgenic line expressing Cre recombinase under the control of the BLG milk protein gene promoter (BLG-Cre). Crosses of BLG-Cre mice with mice harbouring either one floxed *Ikk2* and one wild-type *Ikk2* allele ($Cre^+/Ikk2^{fl/wt}$), or two floxed *Ikk2* alleles ($Cre^+/Ikk2^{fl/fl}$), reached adulthood with no apparent abnormalities. Fig. 1A shows a representative Southern blot used to characterise the extent of BLG-Cre-mediated recombination of the floxed allele at 24 hours following forced weaning. Varying levels of recombination occurred, with full recombination only obtained with one floxed allele and one null allele. We have previously demonstrated a reproducibly high level of recombination with the BLG-Cre strain and that the expression of Cre, at the levels found with this transgenic line, does not affect mammary gland development and has no influence on the rate of involution (Chapman et al., 1999). This suggests a biological selection against complete recombination of the *Ikk2* allele (Schmidt-Supprian et al., 2003).

We examined the level of IKK2 protein expression in Cre^-, $Cre^+/Ikk2^{fl/wt}$ and $Cre^+/Ikk2^{fl/fl}$ mammary glands at 24 hours' involution by immunoblot analysis. IKK2 protein levels are reduced in mice containing floxed alleles of *Ikk2* and expressing Cre recombinase (Fig. 1B). Analysis of components of the NF-κB pathway by western blotting showed that protein levels of p50 and p65 did not change, and the p52 subunit, which is processed by IKK1 from its p100 precursor, was not detectable in involution (data not shown). This suggests that during involution the non-classical p100-p52 branch of the NF-κB pathway is not active.

As NF-κB subunits translocate to the nucleus only when the IκB inhibitor is degraded (by IKK1/2 phosphorylation) we wished to show the nuclear localisation of these subunits by immunohistochemistry and EMSA of nuclear extracts. In the absence

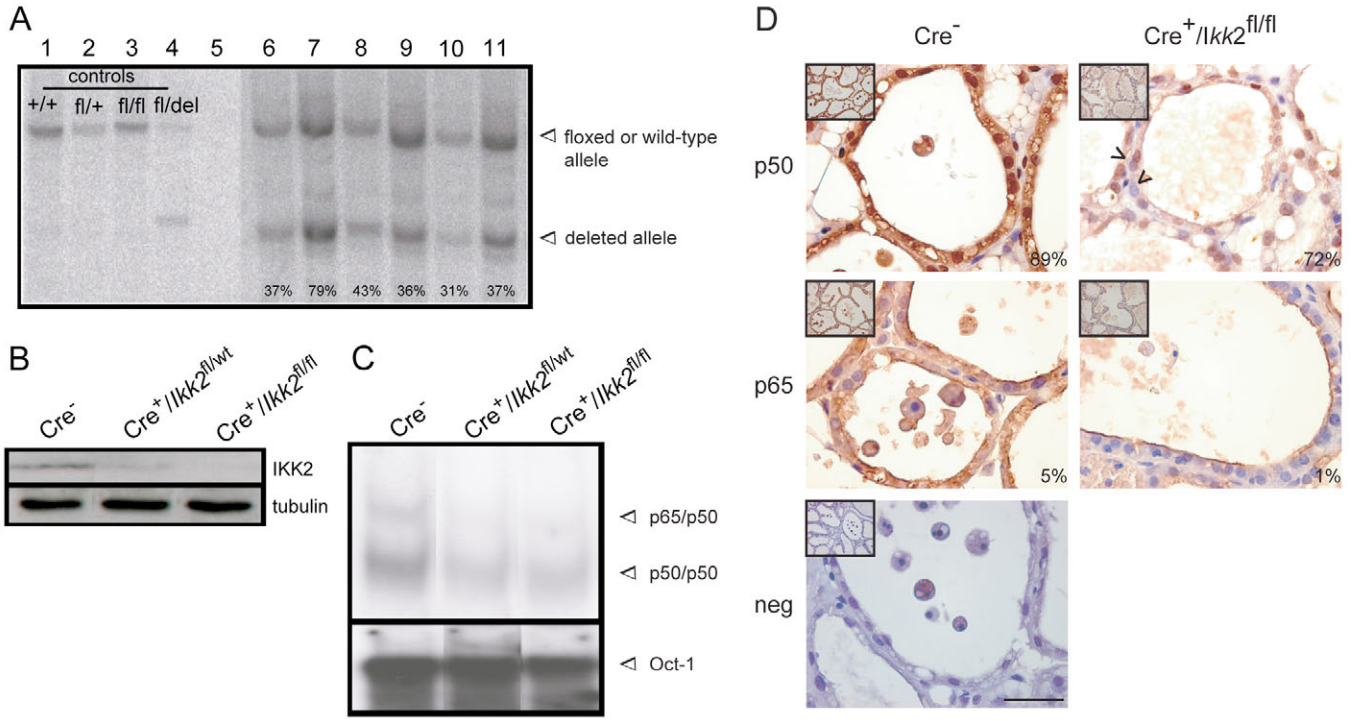

Fig. 1. Analysis of IKK2 expression in mammary glands of floxed mice. (**A**) Southern blot analysis of *Ikk2* locus. Lanes 1-4 controls: lane 1 wild-type DNA; lane 2 DNA from floxed/wild-type tissue (not recombined); lane 3 DNA from floxed/floxed tissue (not recombined); lane 4 DNA from floxed/deleted tissue. Lane 5 empty. Lanes 6-11: mammary gland DNA samples from $Cre^+/Ikk2^{fl/fl}$ mice at 24 hours' involution, showing that varying levels of recombination have occurred (lanes 6-11, respectively: 37, 79, 43, 36, 31 and 37% recombination). (**B**) Protein levels of IKK2 were determined in mammary glands from Cre^-, $Cre^+/Ikk2^{fl/wt}$ or $Cre^+/Ikk2^{fl/fl}$ mice by immunoblotting. At 24 hours' involution, reduced IKK2 levels were seen in $Cre^+/Ikk2^{fl/fl}$ and $Cre^+/Ikk2^{fl/wt}$ mice (loading control was α-tubulin). (**C**) EMSA of mammary gland tissue nuclear extracts for NF-κB and OCT1. NF-κB DNA-binding activity was determined by densitometry and normalised to OCT1. In the absence of IKK2 ($Cre^+/Ikk2^{fl/fl}$), NF-κB DNA-binding activity is reduced by approximately 50%. (**D**) DAB immunohistochemistry for NF-κB p50 and p65 subunits in mammary gland sections from Cre^- or $Cre^+/Ikk2^{fl/fl}$ mice at 24 hours' involution. Compared to Cre^- glands, nuclear p50 staining was reduced in $Cre^+/Ikk2^{fl/fl}$ glands (<). Nuclear p65 staining was weak in Cre^- glands, and undetectable in glands from $Cre^+/Ikk2^{fl/fl}$ mice. Percentage of positively staining nuclei is indicated in the lower right corner of each image. Scale bar: 100 μm.

of IKK2 (i.e. in Cre^{+}/*Ikk2*$^{fl/fl}$ glands), NF-κB DNA-binding activity is diminished by approximately 50%, as determined by EMSA, and is reduced to a lesser extent in Cre^{+}/*Ikk2*$^{fl/wt}$ glands (Fig. 1C). Previous studies using antibody supershifts identified the NF-κB-binding activity as consisting of p65/p50 hetero- and p50 homodimer complexes (Clarkson et al., 2000). This diminished activity was confirmed by immunohistochemistry (Fig. 1D) showing reduced nuclear and cytoplasmic staining for the p50 subunit of NF-κB in Cre^{+}/*Ikk2*$^{fl/fl}$ glands, while p65 nuclear staining was weak in Cre^{-} glands and absent in the Cre^{+}/*Ikk2*$^{fl/fl}$ glands at 24 hours' involution.

Deletion of the Ikk2 gene reduces apoptosis and delays involution

To determine the consequences of reduced IKK2 expression, we examined sections of mammary tissue from Cre^{-} controls, Cre^{+}/*Ikk2*$^{fl/wt}$ and Cre^{+}/*Ikk2*$^{fl/fl}$ mice at day of birth (0dLac), 24, 48, 72 and 144 hours (6 days) of involution (Fig. 2). The alveoli in the Cre^{+}/*Ikk2*$^{fl/fl}$ glands were slightly larger, suggesting that IKK2 may have a minor role in alveolar morphogenesis. Despite these larger alveoli, IKK2-deficient glands produced milk normally.

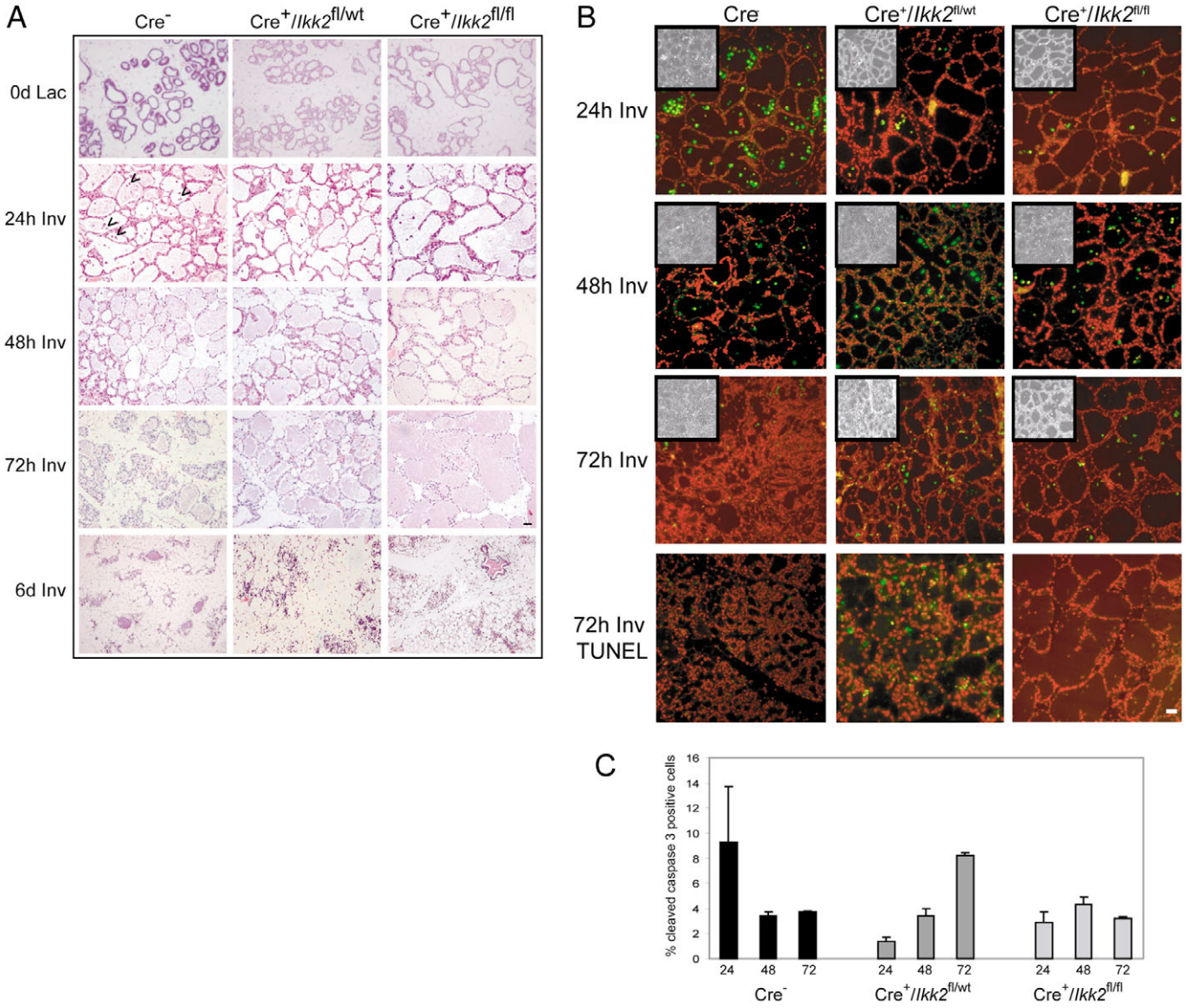

Fig. 2. Deletion of IKK2 in mammary gland results in reduced apoptosis and delayed involution. (**A**) Haematoxylin and eosin staining of mouse mammary gland sections from Cre^{-}, *Cre*$^{+}$/*Ikk2*$^{fl/wt}$ or *Cre*$^{+}$/*Ikk2*$^{fl/fl}$ mice at 0dL, 24, 48, 72 or 144 hours' involution. Apoptosis is apparent in glands from Cre^{-} control mice that subsequently underwent remodelling normally. Apoptotic cells are seen to accumulate in the open lumen of the lobuloalveolar structures (**<**). Involution and remodelling was delayed in the glands from *Cre*$^{+}$/*Ikk2*$^{fl/wt}$ mice. This delay was more pronounced in *Cre*$^{+}$/*Ikk2*$^{fl/fl}$ glands. By 6 days' involution very little difference is seen between mice of the different genotypes. (**B**) Cleavage of caspase 3 was determined by immunofluorescence in mammary gland sections from Cre^{-}, *Cre*$^{+}$/*Ikk2*$^{fl/wt}$ or *Cre*$^{+}$/*Ikk2*$^{fl/fl}$ mice at 24, 48 or 72 hours' involution. (green: cleaved caspase-3-positive cells; red: DAPI-stained nuclei). TUNEL staining at 72 hours' involution showed a higher proportion of positive (green) cells in Cre^{-} and heterozygous glands. (**C**) Cleaved caspase-3-positive cells were expressed as a percentage of total cell number and data presented in graphical format. Approximately 10,000 cells were counted. Cleaved caspase 3 levels peaked at 24 hours' involution in Cre^{-} glands, was delayed by 48 hours in Cre^{+}/*Ikk2*$^{fl/wt}$ mice and remained constant at all involution timepoints in *Cre*$^{+}$/*Ikk2*$^{fl/fl}$ mice. Scale bars: 100 μm.

In Cre$^-$ control mammary glands at 24 hours' involution, apoptotic cells were seen to accumulate in the open lumen of the lobuloalveolar structures (Fig. 2A). Fewer apoptotic cells were observed in Cre$^+$/*Ikk2*$^{fl/wt}$ and Cre$^+$/*Ikk2*$^{fl/fl}$ glands at this time. By 72 hours' involution, the Cre$^-$ glands had undergone extensive tissue remodelling characterised by the collapse of the secretory lobuloalveolar structures and the reappearance of adipocytes (which constitute the majority of the tissue in a resting gland). Fewer collapsed lobuloalveolar structures were observed at 72 hours' involution in the Cre$^+$/*Ikk2*$^{fl/wt}$ glands, and this correlates with a decreased adipocyte population in these glands. The mammary gland therefore displays haploinsufficiency for *Ikk2*. At the same time point in Cre$^+$/*Ikk2*$^{fl/fl}$ glands, almost all lobuloalveolar structures remained open and distended with little evidence of collapse. The gland is primarily composed of secretory epithelial cells (very few adipocytes) and resembles a gland that has not initiated the process of involution. By day 6 of involution, few changes were visible between the mammary glands of the different genotypes, suggesting that in the absence of IKK2 mammary involution is delayed but not completely abrogated.

The delay in involution observed in the absence of IKK2 correlates with a decrease in the cleavage (and thereby activation) of the executioner caspase, caspase 3 (Fig. 2B). In Cre$^-$ glands at 24 hours of involution, there are high numbers of cells positive for cleaved caspase 3 and much lower levels of caspase 3-positive cells at 48 and 72 hours of involution. In the Cre$^+$/*Ikk2*$^{fl/wt}$ and Cre$^+$/*Ikk2*$^{fl/fl}$ glands, this early appearance of cleaved caspase 3 did not occur, suggesting that the initiation of apoptosis requires wild-type levels of IKK2. In the Cre$^+$/*Ikk2*$^{fl/wt}$ glands, cleaved caspase 3 levels increased at 72 hours' involution. However, we did not observe significant cleavage of caspase 3 in any of the time points examined for Cre$^+$/*Ikk2*$^{fl/fl}$ glands (Fig. 2C).

Apoptosis levels were still high at 72 hours in the Cre$^-$ glands, as determined by the TUNEL assay (Fig. 2B, bottom panels). The presence of some TUNEL-positive cells in the Cre$^+$/*Ikk2*$^{fl/wt}$ glands correlated with enhanced caspase 3 cleavage at this time point, and suggests that apoptosis had been initiated. However, in the Cre$^+$/*Ikk2*$^{fl/fl}$ glands, few TUNEL-positive cells were detected, confirming that apoptosis is further delayed.

IKK2 regulates levels of DR ligands, pAkt and pFOXO3a

To characterise this phenotype at the molecular level we examined a number of cellular targets that could be affected by decreased IKK2 levels. Cyclin-D1 is the principle target of NF-κB signalling (via IKK1) during gestation (Cao et al., 2001). Cyclin-D1 protein levels remain unchanged in the glands with decreased NF-κB DNA-binding activity (data not shown). A panel of apoptotic regulators were also examined, including BCL-x_L and BAX (data not shown) and no significant differences were seen in protein levels between mice of the three genotypes. This implies that the perturbation in apoptotic signalling in the mammary gland in the absence of IKK2 is not mediated through these members of the BCL-2 family and that the failed involution phenotype is due to a different mechanism.

This prompted us to examine levels of pAKT, a potent survival signal for involuting mammary gland (Schwertfeger et al., 2001) and its downstream target Forkhead transcription factor (FOXO)3a. In vivo analysis of pAKT and FOXO3a by immunoblotting and immunohistochemistry (Fig. 3A,B) showed that deletion of IKK2 results in a substantial increase in pAKT and a corresponding elevation of serine phosphorylated FOXO3a. Reduced nuclear FOXO3a staining was seen at 24 hours' involution and is less apparent in the Cre$^+$/*Ikk2*$^{fl/wt}$ glands. Furthermore, the normal downregulation of pAKT levels by STAT3 at the onset of involution (Abell et al., 2005) is abrogated in the absence of IKK2 and is diminished by deletion of a single allele. This is independent of pSTAT3 (Fig. 3A), indicating that apoptosis induced by the STAT3/phosphoinositide-3-kinase (PI3K) pathway can be blocked by elevated levels of pAKT induced by loss of a single IKK2 allele. These results show that IKK2 is necessary for the induction of apoptosis in involuting mammary gland by downregulating levels of pAKT, probably via PI3K, and thereby maintaining transcriptional activity of FOXO3a.

Our previous microarray analysis showed a specific upregulation, within 12 hours' involution, of a number of DR ligands belonging to the TNF superfamily (Clarkson et al., 2004). These include FASL (*Tnfsf6*), TNF, TRAIL (*Tnfsf10*) and TWEAK (*Tnfsf12*) (Fig. 3C) and the first three of these genes have previously been reported to be transcriptional targets of NF-κB (Bateu et al., 2001; Kasibhatla et al., 1998; Trede et al., 1995). TWEAK has been shown to be a weak inducer of apoptosis (Chicheportiche et al., 1997), although currently there is little data supporting NF-κB regulation of TWEAK expression.

We therefore examined the levels of these four DR ligand mRNAs in the presence and absence of IKK2 at 24 hours' involution using qRT-PCR (Fig. 3D); levels were compared to an internal control cyclophilin A, a housekeeping gene that is constantly expressed throughout a mammary developmental cycle (K.A., unpublished). Both TNF and TWEAK mRNA levels were significantly decreased (as determined by the paired *t*-test) in Cre$^+$/*Ikk2*$^{fl/fl}$ glands compared with Cre$^-$ control glands, while FASL was reduced (although not significantly) and TRAIL mRNA levels were not altered. As seen from our microarray data, there was a weaker induction of FASL and TRAIL at 12 hours' involution compared with TNF and TWEAK, and this, coupled with the lack of significant change in their expression in the Cre$^+$/*Ikk2*$^{fl/fl}$ glands, suggests that these DR ligands do not play a major role in involution. Furthermore, TRAIL predominantly induces apoptosis in tumour cells (Almasan and Ashkenazi, 2003). It is interesting that levels of the TNF receptor 1 (TNFR1) were also significantly decreased in both Cre$^+$/*Ikk2*$^{fl/fl}$ and Cre$^+$/*Ikk2*$^{fl/wt}$ glands, as the switch between TNF-induced survival and death has been shown to be dependent on the relative levels of TNFR1 and TNFR2 (Maeda et al., 2003). Furthermore, the TWEAK receptor Fn14 (Wiley and Winkles, 2003) lacks a death domain and has been shown to induce apoptosis through interaction with TNF and TNFR1 (Schneider et al., 1999). Thus, TNF and TWEAK may synergise during involution to initiate apoptosis.

To confirm that IKK2 regulates the expression of TWEAK, KIM2 cells (Gordon et al., 2000) were treated with the IKK2 inhibitor 15dPGJ$_2$ and levels of TWEAK mRNA determined by qRT-PCR (Fig. 3E). TWEAK expression was significantly repressed by 4 hours of treatment. This rapid repression of TWEAK mRNA levels suggests that expression may be directly regulated by IKK2, although, unlike IKK1, IKK2 has not been shown to be localised to the nucleus and these data do not exclude the possibility that NF-κB is also a transcriptional regulator of TWEAK.

Full-length TWEAK has a nuclear localisation throughout mammary gland development while the soluble form is upregulated in involution

To further investigate the role of TWEAK, we analysed expression of the TWEAK receptor Fn14 in KIM2 mammary epithelial cells (Fig. 4A). We used this cell line for two reasons: firstly, it is a good model of mammary gland development; secondly, and more

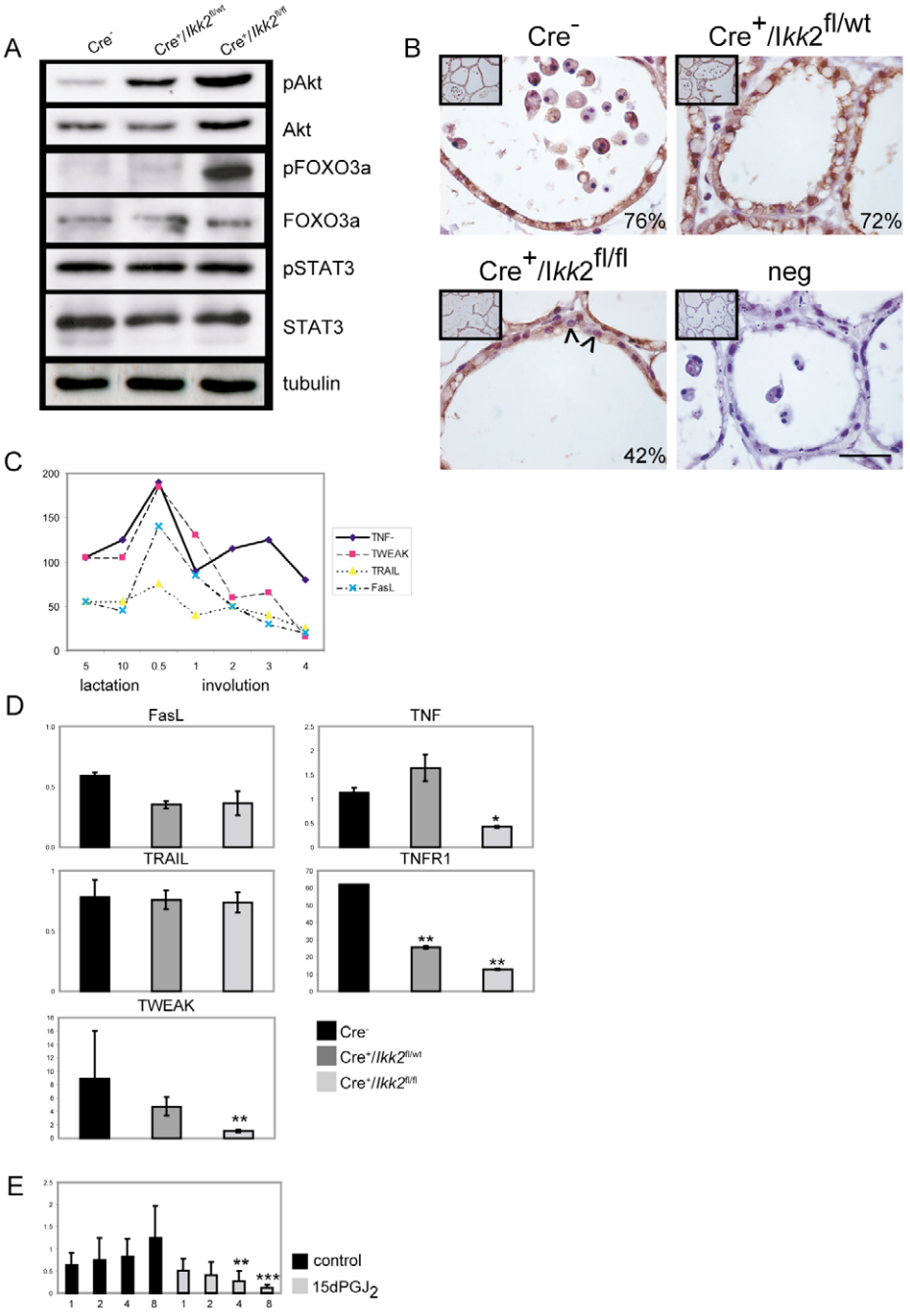

Fig. 3. Changes in gene and protein expression following loss of IKK2. (**A**) Western blot analysis for total and serine phosphorylated levels of AKT, FOXO3a and of STAT3 at 24 hours' involution in mammary glands from Cre^{-}, *Cre*$^{+}$*/Ikk2*$^{fl/wt}$ or *Cre*$^{+}$*/Ikk2*$^{fl/fl}$ mice. Significantly increased levels of pAKT and pFOXO3a were seen in the absence of IKK2. Loading control was α-tubulin. (**B**) DAB immunohistochemistry for FOXO3a in mammary gland sections from Cre^{-}, *Cre*$^{+}$*/Ikk2*$^{fl/wt}$ or *Cre*$^{+}$*/Ikk2*$^{fl/fl}$ mice at 24 hours' involution. Reduced nuclear FOXO3a staining was seen in *Cre*$^{+}$*/Ikk2*$^{fl/fl}$ glands (**<**). Percentage of positively staining nuclei is indicated in the lower right corner of each image. (**C**) A representation of previously obtained microarray data showing expression of DR ligands throughout lactation and early involution (Clarkson et al., 2004). (**D**) qRT-PCR for the DR ligands: FASL, TNF, TRAIL and TWEAK and DR TNFR1. Significant reductions in TWEAK (** t<0.01), TNF (* t<0.05) and TNFR1 as determined by the paired t-test, were seen in glands from *Cre*$^{+}$*/Ikk2*$^{fl/fl}$ mice compared with Cre^{-} control mice. Levels are arbitrary units and have been normalised to cyclophilin A. (**E**) qRT-PCR for TWEAK of control and 15dPGJ$_2$ treated undifferentiated KIM2 cells. Statistically significant decreases in TWEAK mRNA as determined by the paired t-test, were seen following 4 hours (**t<0.01) and 8 hours (***t<0.005) of 15dPGJ$_2$ treatment. Levels are arbitrary units and have been normalised to cyclophilin A. Scale bar: 100 μm.

importantly, using an epithelial cell line allows us to conclude that TWEAK and its receptor are both expressed by luminal mammary epithelium. Tissue from the gland would be contaminated by stromal, endothelial and myoepithelial cells and would complicate the analysis. This shows that Fn14 is expressed in both undifferentiated and differentiated cells. TWEAK exists in two forms, a long form of 35 kDa containing a transmembrane domain and a processed soluble form of 18 kDa (Chicheportiche et al., 1997). Interestingly, full-length TWEAK was found in the nuclear and cytoplasmic compartments of KIM2 cells while the cleaved form of TWEAK was exclusively cytoplasmic (Fig. 4B). This unexpected nuclear localisation was confirmed by immunohistochemistry on a series on mammary gland time points (Fig. 4C) that also reveal the presence of TWEAK on the luminal membrane and in the nuclei of the adipocyte stroma. Confocal analysis in KIM2 cells confirmed cytoplasmic and nuclear localisation of TWEAK, which formed puncta that were distributed throughout the nucleus (Fig. 4D). Furthermore, using PSORTII, we found three nuclear localisation sequences (NLS) at amino acids 90 (RPRR), 96 (PKGRKAR) and 102 (RPRR), while the NLS prediction program CUBIC (http://cubic.bioc.columbia.edu), identified an NLS (QRRRGRR) at the amino terminus of TWEAK. As nuclear staining is evident throughout lactation and pregnancy, we conclude that full-length TWEAK can translocate to the nucleus throughout mammary gland development. In order to bind to Fn14 in involuting mammary gland, TWEAK would need to be processed into its soluble form for both autocrine and paracrine signalling (Winkles et al., 2006). In this context, it is interesting that the 18 kDa soluble form was upregulated from 24-72 hours' involution (Fig. 4E), although low levels of the cleaved form were detectable during lactation.

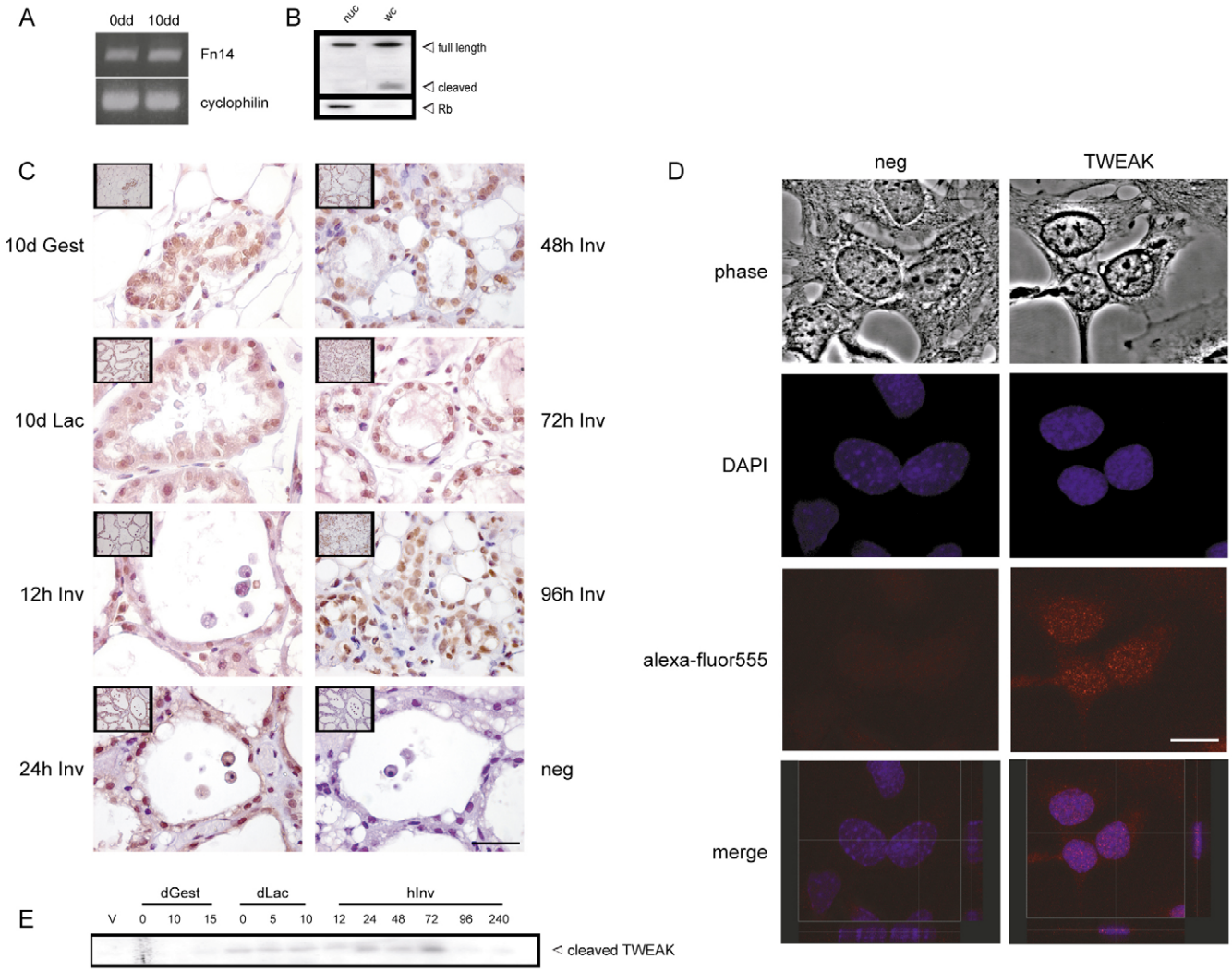

Fig. 4. Expression profile of TWEAK in the mammary gland. (**A**) Expression of Fn14 in mammary epithelial cells. Fn14 was expressed in undifferentiated and 10-day differentiated KIM2 mammary epithelial cells, as determined by qRT-PCR analysis. (**B**) Immunoblotting for TWEAK demonstrated expression of full length (35 kDa) TWEAK in nuclear and cytoplasmic compartments in KIM2 cells, while soluble (18 kDa) TWEAK was exclusively cytoplasmic. Nuclear control was retinoblastoma protein (Rb). (**C**) DAB immunohistochemistry for TWEAK demonstrated nuclear staining at all mammary gland developmental time points examined. Cytoplasmic TWEAK staining was seen late in lactation (10 dl) and early in involution (12 and 24 hours' involution). (**D**) Confocal analysis of TWEAK localisation in KIM2 cells. Undifferentiated KIM2 cells were fixed and stained for TWEAK, using AlexaFlour 555. A single slice of the confocal analysis is shown, with nuclei visualised by DAPI staining. The bottom panel shows a merged image, with sectioning through a nuclear puncta. (**E**) Immunoblotting for TWEAK during mammary gland development showed that the soluble form of TWEAK was detected predominantly at 24-72 hours' involution. Scale bars: 100 μm in C; 20 μm in D.

The TWEAK promoter has multiple FOXO3a-binding motifs

To assess the relative importance of NF-κB and IKK2 in DR ligand expression, we analysed the mouse promoter regions of these death receptors/ligands. Fig. 5A shows a cluster of consensus-binding sites for FOXO in the proximal region of the *Tweak* promoter, while the *Tnf*, *FasL*, *Trail* and *Tnfr1* promoters have NF-κB sites but no FOXO sites. The *Tweak* promoter also has a single NF-κB consensus site. Interestingly, FOXO3a has been shown to regulate the transcription of FasL in human cells (Kauvrma and Khachigian, 2003), although this motif is not present in the mouse promoter.

We suggest that IKK2 has a dual function as a regulator of cell death by regulating both NF-κB and FOXO3a-mediated transcriptional regulation of DR ligands in early involution (Fig. 5B) and that soluble TWEAK plays a major role in the induction of apoptosis.

DISCUSSION

We investigated the function of IKK2 and NF-κB in apoptosis and mammary gland involution by generating a conditional deletion of *Ikk2* in mouse mammary gland. Analysis of the phenotype showed surprisingly that IKK2 either alone, or through NF-κB, is a pro-apoptotic factor for differentiated mammary epithelium, and that in the absence of IKK2, caspase 3 cleavage is abolished and involution delayed.

Although NF-κB factors primarily play anti-apoptotic roles through transcriptional regulation of proteins including the IAPs and BCL-2 homologues (Karin and Ben-Neriah, 2000), it is possible that NF-κB is a pro-apoptotic factor in involution. Indeed, it has previously been shown that NF-κB can also be pro-apoptotic (Fujioka et al., 2004). Furthermore, upstream regulators of this pathway, IKK1 and IKK2 have been shown to

have both NF-κB-dependent and NF-κB-independent roles. Thus, IKK2 and NF-κB could have different roles in apoptosis regulation.

The striking upregulation of DR ligands at 12 hours' involution could be the initiating signal for apoptosis. Indeed, in $Cre^{+}/Ikk2^{fl/fl}$ mice, levels of TWEAK, TNF and TNFR1 transcripts were significantly reduced, and this correlated with a dramatic abrogation of apoptosis. These data implicate the extrinsic (DR) pathway of apoptosis in the initial induction of apoptosis following forced weaning, unlike late involution where the intrinsic pathway is primarily involved.

Indeed, in FASL-deficient mice, involution is slightly delayed (Song et al., 2000). However, this effect is minor compared with deletion of IKK2. Thus, it is likely that FASL does not have a major role in involution, although it may be a contributing factor. Involution in TNF- and TNFR1-deficient mice has not been investigated, although we have previously shown that TNF protects against apoptosis induced by hormone withdrawal in differentiated mammary epithelial cells (Clarkson et al., 2000). This observation, coupled with the strong heterozygous phenotype in the absence of a significant reduction in TNF, suggests that TNF is not the major factor driving apoptosis. We suggest that TWEAK, in association with Fn14 and TNFR1, is an important mediator of IKK2 signalling in involution. Using inhibitor studies, we confirmed that TWEAK is transcriptionally regulated, probably indirectly, by IKK2 in KIM2 mammary epithelial cells.

We have shown that Fn14, the TWEAK receptor, is expressed in mammary epithelial cells in culture and that TWEAK is found in both its full-length and cleaved forms. Interestingly, in vivo, soluble TWEAK is upregulated in involution. Secreted TWEAK could therefore function in a paracrine manner to induce apoptosis in neighbouring luminal cells, thereby amplifying the apoptotic cascade. Such a mechanism could explain the reversibility of first phase involution, when apoptosis can be halted by re-initiation of suckling, which would remove secreted TWEAK. TNF is known to be cleaved by the ADAM family member ADAM17/ TACE (Black et al., 1997); the mechanism by which TWEAK is cleaved has not been established, although it is likely that a membrane-bound protease is also involved. Two possibilities are metalloproteases and calpains, which have recently been suggested to be important for a TNF autocrine cascade (Janes et al., 2006). Alternatively, TWEAK may be cleaved by the lysosomal cysteine protease, cathepsin L, which is dramatically upregulated within 24 hours' involution (Burke et al., 2003). Thus, TWEAK may have a proliferative role during pregnancy but an apoptotic function in involution, resulting from its elevated expression and shedding of its secreted form into the alveolar lumen.

Previous studies by De Ketelaere et al. (De Ketelaere et al., 2004), using overexpression of TWEAK showed that a short form of TWEAK, distinct from the soluble form, localises to the nucleus. This is in contrast to our studies, which show that full-length TWEAK has a nuclear and cytoplasmic localisation. The additional amino-terminal NLS identified in our study further suggests that full-length TWEAK can translocate to the nucleus. The function of nuclear TWEAK is currently unclear but the confocal analysis does suggest that TWEAK is found in specific nuclear domains.

We have recently shown that STAT3 regulates apoptosis by downregulating PI3K activity through modulation of the expression of its regulatory subunits at 48 hours' involution (Abell et al., 2005). We show here that pAKT is regulated also by IKK2, independently of pSTAT3, earlier in involution at 24 hours. This places pAKT as a master sentinel for death in early involution, where it is downstream of both STAT3 and IKK2. We suggest that apoptosis is regulated by STAT3 and NF-κB transcriptional targets and that the balance between cell survival and death, so essential for the reversibility and halting of the apoptotic programme, is controlled

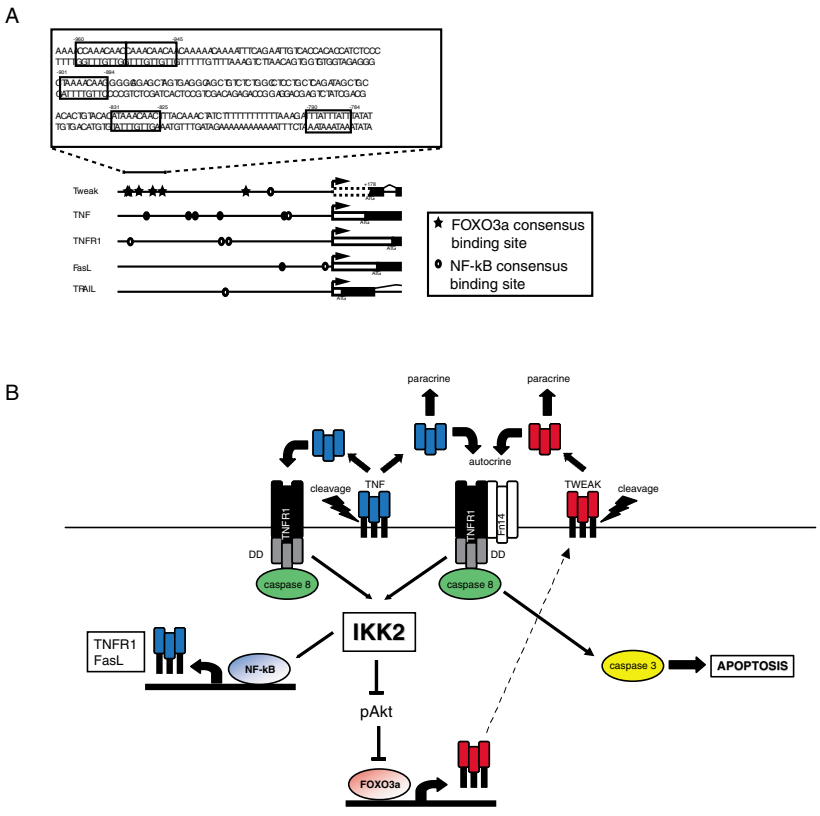

Fig. 5. DR ligands: promoter analysis and putative roles in apoptosis and involution. (**A**) Comparative promoter analysis for members of the TNF superfamily of DR ligands. Promoter analysis was performed as described in Materials and methods. The open boxes are 5′UTR regions of exon1 of the respective genes; the filled boxes are translated parts of the first exons. The dotted box in the *Tweak* promoter represents the putative 5′UTR from the potential transcription start site identified using the Eponine program. This figure shows promoter regions spanning 1 kb upstream of the predicted transcription start sites. Previously published NF-κB consensus-binding sequences are shown as filled circles: TNF (Drouet et al., 1991) and FASL (Matsui et al., 1998). The additional NF-κB-binding sites shown in white circles were identified using the Matinspector program. The FOXO3a consensus sequences used were RTAAAYA (Brunet et al., 1999) or CCAAACAA and TAAAACAA (Tran et al., 2002). (**B**) Proposed model of IKK2 induced apoptosis during early mammary gland involution.

by pAKT. Thus, subtle changes in pAKT levels can determine the fate of individual cells even in the presence of activated STAT3 and NF-κB signalling.

There are a number of possible mechanisms by which AKT can regulate the balance between death and survival signals. AKT is known to phosphorylate and inactivate apoptosis regulators, including BAD, apoptosis signal-regulating kinase 1 (ASK1), caspase 9 and members of the FOXO family of transcription factors (Datta et al., 1999). Phosphorylation of FOXO factors on serine and threonine residues by pAKT sequesters them in the cytoplasm, rendering them transcriptionally inactive (Datta et al., 1999). FOXO3a has been shown to regulate expression of the pro-apoptotic BH3-only protein, BIM. The BIM promoter has a single FOXO consensus-binding site that is conserved between mouse and human (Essafi et al., 2005). However, although we have shown that expression of BIM is regulated by IKK2 in undifferentiated KIM2 cells (data not shown) BIM levels are not reduced in involution, despite elevated levels of pFOXO3a. This suggests that BIM is not important in early involution or that there are sufficient levels of unphosphorylated FOXO3a to regulate its expression.

From promoter analyses, we found a cluster of six FOXO-binding motifs and one NF-κB site in the putative *Tweak* promoter. As expected, the *Tnf* promoter contained multiple NF-κB-binding motifs; however, no consensus FOXO sites were found. The *Tnfr1* promoter proximal region contained three NF-κB sites and the *Trail* promoter a single NF-κB site. Neither of these promoters contained FOXO motifs. We showed by immunoblotting that FOXO3a is highly phosphorylated in the IKK2 null glands. Therefore, we suggest that in IKK2-deficient glands, apoptosis is inhibited by the elevated levels of phosphorylated AKT, inactivation of FOXO3a and reduced expression of TWEAK. This reduction in pro-apoptotic signals results in a failure of caspase 3 cleavage and apoptosis.

The mechanism by which IKK2 reduces phosphorylation of AKT, and in turn FOXO3a, is not clear. It has been shown that IKK2 and AKT transiently associate upon PDGF stimulation and this interaction is dependent on PI3K activity (Romashkova and Makarov, 1999). Phosphorylation of AKT on Thr-308 and Ser-473 preceded IKK activation, suggesting a consecutive activation of these kinases that culminates in the induction of NF-κB-binding activity. We suggest that in the absence of IKK2, this association is abolished and this maintains AKT phosphorylation. Alternatively, IKK2 may regulate the transcription of an unidentified serine/threonine phosphatase at the onset of involution.

Thus, IKK2 may have NF-κB-dependent and independent roles in mammary epithelial cell apoptosis. The diminished NF-κB activity resulting from loss of IKK2 kinase function probably contributes to reduced expression of TNF, TWEAK, FASL and TNFR1 through consensus NF-κB-binding sites leading to a failure to activate caspase 8 and subsequently caspase 3. We present evidence that IKK2 may also regulate TWEAK expression through phosphorylation of FOXO3a to inactivate its transcriptional activity. The elevated expression of TWEAK mRNA at the onset of involution correlates with increased levels of soluble TWEAK and the induction of apoptosis. Thus, IKK2 has a specific function in inducing apoptosis in differentiated mammary epithelial cells through mediating the induction of components of the extrinsic death pathway, particularly TWEAK.

This work was supported by The Wellcome Trust and by a University of Cambridge studentship to PJC. We wish to thank Dr E Kondo, Department of Pathology, Okayama University Graduate School of Medicine and Dentistry, Okayama 700-8558, Japan for kindly providing the FOXO3a antibody used for immunohistochemistry. We also thank Drs Tina Rich, Brian Ferguson and Clare Alexander, Department of Pathology, University of Cambridge for help with the NLS and confocal analysis.

References

Abell, K., Bilancio, A., Clarkson, R. W., Tiffen, P. G., Altaparmakov, A. I., Burdon, T. G., Asano, T., Vanhaesebroeck, B. and Watson, C. J. (2005). Stat3-induced apoptosis requires a molecular switch in PI(3)K subunit composition *Nat. Cell Biol.* **7**, 392-398.

Almasan, A. and Ashkenazi, A. (2003). Apo2L/TRAIL: apoptosis signalling, biology, and potential for cancer therapy. *Cytokine Growth Factor Rev.* **14**, 337-348.

Ashkenazi, A. and Dixit, V. M. (1998). Death receptors: signalling and modulation. *Science* **281**, 1305-1308.

Bateu, T. M., Kwon, H., Sharma, S., Grandvaux, N. and Hiscott, J. (2001). Disruption of NF-kappaB signalling reveals a novel role for NF-kappaB in the regulation of TNF-related apoptosis-inducing ligand expression. *J. Immunol.* **167**, 3164-3173.

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S. et al. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* **385**, 729-733.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J. and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell* **96**, 857-868.

Burke, M. A., Hutter, D., Reshamwala, R. P. and Knepper, J. E. (2003). Cathepsin L plays an active role in involution of the mouse mammary gland. *Dev. Dyn.* **227**, 315-322.

Cao, Y., Bonizzi, G., Seagroves, T. N., Greten, F. R., Johnson, R., Schmidt, E. V. and Karin, M. (2001). IKKa provides an essential link between RANK signalling and cyclin D1 expression during mammary gland development. *Cell* **107**, 763-775.

Chapman, R. S., Lourenco, P. C., Tonner, E., Flint, D. J., Selbert, S., Takeda, K., Akira, S., Clarke, A. R. and Watson, C. J. (1999). Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev.* **13**, 2604-2616.

Chicheportiche, Y., Bourdon, P. R., Xu, H., Hsu, Y. M., Scott, H., Hession, C., Garcia, I. and Browning, J. L. (1997). TWEAK, a new secreted ligand in the tumour necrosis factor family that weakly induces apoptosis. *J. Biol. Chem.* **51**, 32401-32410.

Clarkson, R. W. and Watson, C. J. (2003). Microarray analysis of the involution switch. *J. Mammary Gland Biol. Neoplasia* **8**, 309-319.

Clarkson, R. W., Heeley, J. L., Chapman, R., Aillet, F., Hay, R. T., Wyllie, A. H. and Watson, C. J. (2000). NF-kB inhibits apoptosis in murine mammary epithelia. *J. Biol. Chem.* **275**, 12737-12742.

Clarkson, R. W., Wayland, M. T., Lee, J., Freeman, T. and Watson, C. J. (2004). Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res.* **6**, 92-109.

Datta, S. R., Brunet, A. and Greenberg, M. E. (1999). Cellular survival: a play in three Akts. *Genes Dev.* **13**, 2905-2927.

Davuluri, R. V., Grosse, I. and Zhang, M. Q. (2001). Computational identification of promoters and first exons in the human genome. *Nat. Genet.* **29**, 412-417. Erratum in: *Nat. Genet.* (2002), **32**, 459.

De Ketelaere, A., Vermeulen, L., Vialard, J., Van De Weyer, I., Van Wauwe, J., Haegeman, G. and Moelans, I. (2004). Involvement of GSK-3β in TWEAK-mediated NF-κB activation. *FEBS Lett.* **566**, 60-64.

Drouet, C., Shakhov, A. N. and Jongeneel, C. V. (1991). Enhancers and transcription factors controlling the inducibility of the tumour necrosis factor-alpha promoter in primary macrophages. *J. Immunol.* **147**, 1694-1700.

Essafi, A., de Mattos, S. F., Hassen, Y. A., Soeiro, I., Mufti, G. J., Thomas, N. S., Medema, R. H. and Lam, E. W. (2005). Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. *Oncogene* **24**, 2317-2329.

Fujioka, S., Schmidt, C., Sclabas, G. M., Li, Z., Pelicano, H., Peng, B., Yao, A., Niu, J., Zhang, W., Evans, D. B. et al. (2004). Stabilization of p53: a novel mechanism for proapoptotic function of NF-kappa B. *J. Biol. Chem.* **279**, 27549-27559.

Furth, P. A., Bar-Peled, U. and Li, M. (1997). Apoptosis and mammary gland involution: reviewing the process. *Apoptosis* **2**, 19-24.

Gordon, K. E., Binas, B., Chapman, R. S., Kurian, K. M., Clarkson, R. W. E., Clark, A. J., Lane, E. B. and Watson, C. J. (2000). A novel cell culture model for studying differentiation and apoptosis in the mouse mammary gland. *Breast Cancer Res.* **2**, 222-235.

Janes, K. A., Gaudet, S., Albeck, J. G., Nielsen, U. B., Laufenburger, D. A. and Sorger, P. K. (2006). The response of human epithelial cells to TNF involves an inducible autocrine cascade. *Cell* **124**, 1225-1239.

Karin, M. and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: the control of NF-kB activity. *Annu. Rev. Immunol.* **18**, 621-663.

Kasibhatla, S., Brunner, T., Genestier, L., Echeverri, F., Mahboubi, A. and Green, D. R. (1998). DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. *Mol. Cell* **1**, 543-551.
Kavurma, M. M. and Khachigian, L. M. (2003). Signaling and transcriptional control of Fas ligand gene expression. *Cell Death Differ.* **10**, 36-44.
Kritikou, E. A., Sharkey, A., Abell, K., Came, P. J., Anderson, E., Clarkson, R. W. and Watson, C. J. (2003). A dual, non-redundant, role for LIF as a regulator of development and STAT3-mediated cell death in mammary gland. *Development* **130**, 3459-3468.
Lund, L. R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Dano, K. and Werb, Z. (1996). Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* **122**, 181-193.
Maeda, S., Chang, L., Li, Z. W., Luo, J. L., Leffert, H. and Karin, M. (2003). IKKbeta is required for prevention of apoptosis mediated by cell-bound but not by circulating TNFalpha. *Immunity* **19**, 725-737.
Matsui, K., Fine, A., Zhu, B., Marshak-Rothstein, A. and Ju, S. T. (1998). Identification of two NF-kappa B sites in mouse CD95 ligand (Fas ligand) promoter: functional analysis in T cell hybridoma. *J. Immunol.* **161**, 3469-3473.
Pasparakis, M., Courtois, G., Hafner, M., Schmidt-Supprian, M., Nenci, A., Toksoy, A., Krampert, M., Goebeler, M., Gillitzer, R., Israel, A. et al. (2002). TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* **417**, 861-866.
Romashkova, J. A. and Makarov, S. S. (1999). NF-kappaB is a target of Akt in anti-apoptotic PDGF signalling. *Nature* **401**, 86-90.
Schmidt-Supprian, M., Courtois, G., Tian, J., Coyle, A. J., Israel, A., Rajewsky, K. and Pasparakis, M. (2003). Mature T cells depend on signalling through the IKK complex. *Immunity* **19**, 377-389.
Schneider, P., Schwenzer, R., Haas, E., Mühlenbeck, F., Schubert, G., Scheurich, P., Tschopp, J. and Wajant, H. (1999). TWEAK can induce cell death via endogenous TNF and TNF receptor 1. *Eur. J. Immunol.* **29**, 1789-1792.
Schwertfeger, K. L., Richert, M. M. and Anderson, S. M. (2001). Mammary gland involution is delayed by activated Akt in transgenic mice. *Mol. Endocrinol.* **15**, 867-881.
Song, J., Sapi, E., Brown, W., Nilsen, J., Tartaro, K., Kacinski, B. M., Craft, J., Naftolin, F. and Mor, G. (2000). Roles of Fas and Fas ligand during mammary gland remodelling. *J. Clin. Invest.* **106**, 1209-1220.
Strange, R., Li, F., Saurer, S., Burkhardt, A. and Friis, R. R. (1992). Apoptotic cell death and tissue remodelling during mouse mammary gland involution. *Development* **115**, 49-58.
Tran, H., Brunet, A., Grenier, J. M., Datta, S. R., Fornace, A. J., Jr, DiStefano, P. S., Chiang, L. W. and Greenberg, M. E. (2002). DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science* **296**, 530-534.
Trede, N. S., Tsytsykova, A. V., Chatila, T., Goldfeld, A. E. and Geha, R. S. (1995). Transcriptional activation of the human TNF-alpha promoter by superantigen in human monocytic cells: role of NF-kappa B. *J. Immunol.* **155**, 902-908.
Wiley, S. R. and Winkles, J. A. (2003). TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TweakR/Fn14 receptor. *Cytokine Growth Factor Rev.* **14**, 241-249.
Winkles, J. A., Tran, N. L. and Berens, M. E. (2006). TWEAK and Fn14: new molecular targets for cancer therapy? *Cancer Lett.* **235**, 11-17.