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Genetic inactivation of *Par4* results in hyperactivation of NF-κB and impairment of JNK and p38

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The *Par4* gene was first identified in prostate cells undergoing apoptosis after androgen withdrawal. PAR4 was subsequently shown to interact with, and inhibit, atypical protein kinase C isoforms, functioning as a negative regulator of the NF-κB pathway. This may explain its pro-apoptotic function in overexpression experiments. To determine the physiological role of PAR4, we have derived primary embryonic fibroblasts (EFs) from *Par4*^{−/−} mice. We show here that loss of PAR4 leads to a reduction in the ability of tumour necrosis factor-α (TNF-α) to induce apoptosis by increased activation of NF-κB. Consistent with recent reports demonstrating the antagonistic actions of NF-κB and c-Jun amino-terminal kinase (JNK) signalling, we have found that *Par4*^{−/−} cells show a reduced activation of the sustained phase of JNK and p38 stimulation by TNF-α and interleukin 1. Higher levels of an anti-apoptotic JNK-inhibitor protein, X-chromosome-linked inhibitor of apoptosis, in *Par4*^{−/−} EFs might explain the inhibition of JNK activation in these cells.

INTRODUCTION

Programmed cell death, or apoptosis, is a physiological process that is induced in response to environmental stresses and cellular signals, and is important in development, tissue remodelling and in diseases such as cancer and neurodegeneration. *Par4* was first detected in a differential screen of genes induced in prostate cells undergoing apoptosis (Sells *et al.*, 1994). Subsequent studies showed that ectopic expression of PAR4 triggers apoptosis in NIH-3T3 cells and sensitizes prostate cancer and melanoma cells to apoptotic stimuli (Diaz-Meco *et al.*, 1996; Berra *et al.*, 1997; Sells *et al.*, 1997). There is evidence that induction of PAR4 contributes to the pro-apoptotic effects of non-steroidal

anti-inflammatory drugs in human colon carcinomas (Zhang & DuBois, 2000) and to the induction of neuronal cell death by the amyloid-β protein or by trophic-factor withdrawal (Guo *et al.*, 1998). PAR4 binds the zinc-finger domains of the atypical protein kinase C isoforms (aPKCs) through its coiled-coil carboxy terminus, thus inhibiting aPKC enzymatic activity (Diaz-Meco *et al.*, 1996). This is important because the ability of PAR4 to induce apoptosis is abolished by the overexpression of the aPKCs (Diaz-Meco *et al.*, 1996), which suggests that the inhibition of aPKC activity by PAR4 is a crucial event in apoptotic cell death. In fact, blocking the function of ζPKC (Diaz-Meco *et al.*, 1996) or λ/ιPKC (Diaz-Meco *et al.*, 1996; Murray & Fields, 1997) by using dominant-negative mutants or antisense oligonucleotides is sufficient to trigger apoptosis. In addition, embryonic fibroblasts (EFs) and mouse B cells in which the gene encoding ζPKC has been knocked out are more sensitive to apoptotic stimuli, demonstrating an anti-apoptotic role for this kinase (Leitges *et al.*, 2001; Martin *et al.*, 2002). Together, these results suggest a model in which PAR4 inhibits aPKCs, an event that is required for apoptosis to proceed.

The aPKCs are essential for the activation of the anti-apoptotic transcription factor NF-κB by inflammatory cytokines and the *ras* oncogene (Moscat & Diaz-Meco, 2000). Overexpression of PAR4 inhibits NF-κB and makes cells sensitive to the induction of apoptosis by tumour necrosis factor-α (TNF-α) (Diaz-Meco *et al.*, 1999). These results suggest that at least one of the mechanisms by which PAR4 controls apoptosis is by blocking the aPKC–NF-κB signalling cascade. Other studies have shown that overexpression of PAR4 leads to the activation of stress-activated kinases, such as p38 (Berra *et al.*, 1997), suggesting that inhibition of ζPKC is required for the full activation of the stress-activated MAP (mitogen-activated protein) kinases. Here, we have made *Par4*-deficient mice by homologous recombination and have investigated the effect of an absence of the PAR4 protein in several signalling pathways in EFs.

RESULTS AND DISCUSSION
Generation of *Par4*^{−/−} mice

To analyse the role of PAR4 *in vivo*, we disrupted this gene in mice by homologous recombination (Fig. 1A). Chimaeric germline-transmitting males were crossed to females and produced heterozygous

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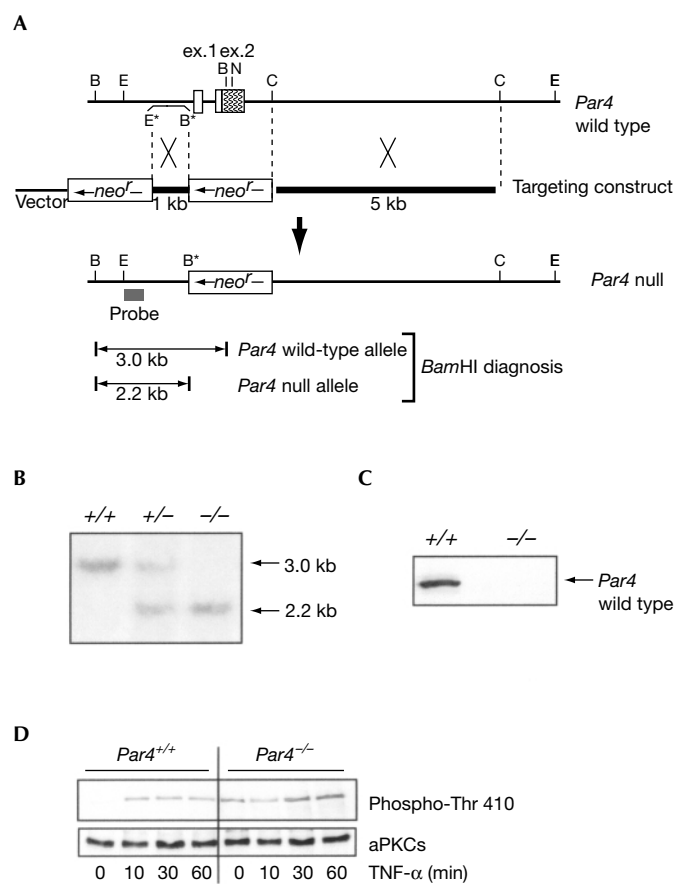*These authors contributed equally to this work
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F_1 progeny. Intercrosses of these F_1 mice were carried out to produce *Par4^{-/-}* mice. Wild-type and mutant alleles were identified by Southern blot analysis, as shown in Fig. 1B. Primary cultures of EFs were obtained from heterozygous intercrosses, and it was shown that *Par4^{-/-}* EFs do not express the PAR4 protein (Fig. 1C). The basal activity of aPKCs in *Par4^{-/-}* EFs is increased as compared with wild-type controls, and is increased further by the addition of TNF-α to the medium (Fig. 1D). These results show that PAR4 is a negative regulator of aPKCs *in vivo*.

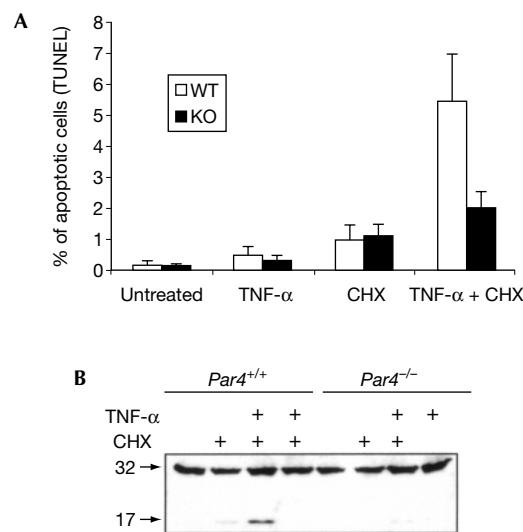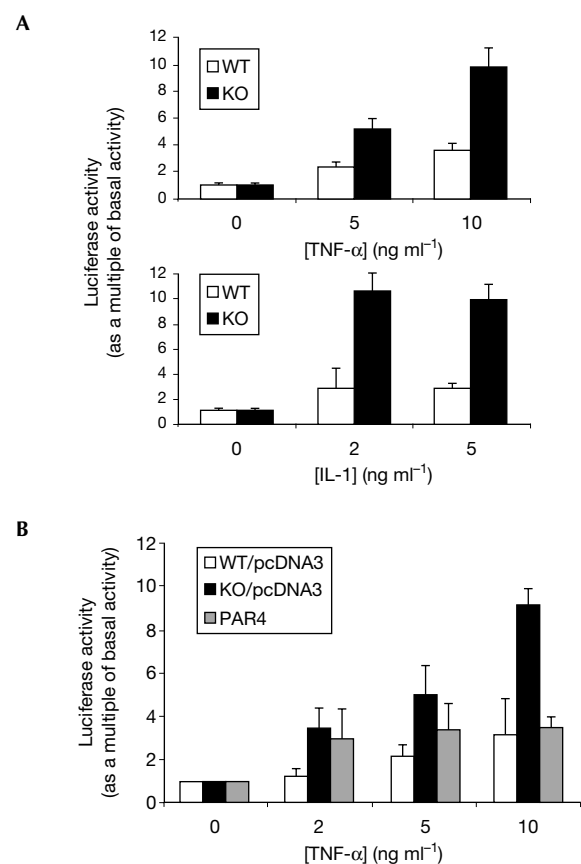## Apoptosis in *Par4^{-/-}* embryonic fibroblasts

Because the overexpression of PAR4 leads to an increase in the sensitivity of EFs to the pro-apoptotic actions of TNF-α (Diaz-Meco *et al.*, 1999), we analysed whether the absence of PAR4 from *Par4^{-/-}* EFs affects the ability of these cells to undergo TNF-α-induced apoptosis. Wild-type and *Par4^{-/-}* EFs were incubated with TNF-α, cycloheximide or a combination of both, and the occurrence of apoptosis was detected by TUNEL (TdT-mediated dUTP nick end-labelling) analysis. Under these conditions, neither TNF-α nor cycloheximide alone was sufficient to trigger apoptosis (Fig. 2A). However, incubation with both together triggered apoptosis in wild-type and *Par4^{-/-}* cells. The extent of the apoptotic response was significantly reduced in *Par4^{-/-}* cells as compared with that in the control cells. This suggests that the presence of PAR4 in wild-type cells contributes to the apoptotic response. To support this finding, extracts were made from cultures of wild-type and *Par4^{-/-}* EFs, and the processing of caspase-3 levels was analysed by immunoblotting. The addition of TNF-α and cycloheximide together induced the processing of caspase 3 at higher levels in wild-type EFs than in *Par4^{-/-}* EFs (Fig. 2B), consistent with the TUNEL analysis.

NF-κB activation in *Par4^{-/-}* embryonic fibroblasts

The aPKCs are important for NF-κB activation (Lallena *et al.*, 1999), and overexpression of PAR4 inhibits this pathway (Diaz-Meco *et al.*, 1999). We therefore analysed whether an absence of PAR4 has any effect on the activation of NF-κB in response to TNF-α. EFs from wild-type and *Par4^{-/-}* mice were transfected with a κB-dependent luciferase reporter and then either left untreated or stimulated with suboptimal concentrations of TNF-α or interleukin-1 (IL-1). The ability of these cytokines to activate κB-dependent transcription was

Fig. 1 | *Par4^{-/-}* genotypic analysis. (**A**) Restriction map of the *Par4* locus and generation of the *Par4^{-}* mutant allele; the targeting vector was integrated into the endogenous locus by homologous recombination to produce the mutant allele (*Par4* null). The two boxes in the upper part indicate exons one (ex. 1) and two (ex. 2) of *Par4*, and the shaded area indicates the coding region. Restriction enzyme abbreviations are as follows: B, *Bam*HI; E, *Eco*RI; N, *Not*I; C, *Cla*I. Restriction sites with an asterisk were introduced to enable cloning and genotyping. (**B**) Southern blot analysis of litters derived from a heterozygote intercross. +/+, wild-type; +/−, heterozygous; −/−, homozygous. (**C**) Immunoblot analysis of extracts from wild-type (^{+/+}) or *Par4* null (^{−/−}) embryonic fibroblasts (EFs). (**D**) Wild-type or *Par4^{-/-}* EFs were stimulated with 10 ng ml^{−1} of tumour necrosis factor-α (TNF-α) for varying times, after which cell extracts were prepared and the activity of the atypical PKCs (aPKCs) was determined by immunoblotting with an anti-phospho-aPKC antibody.
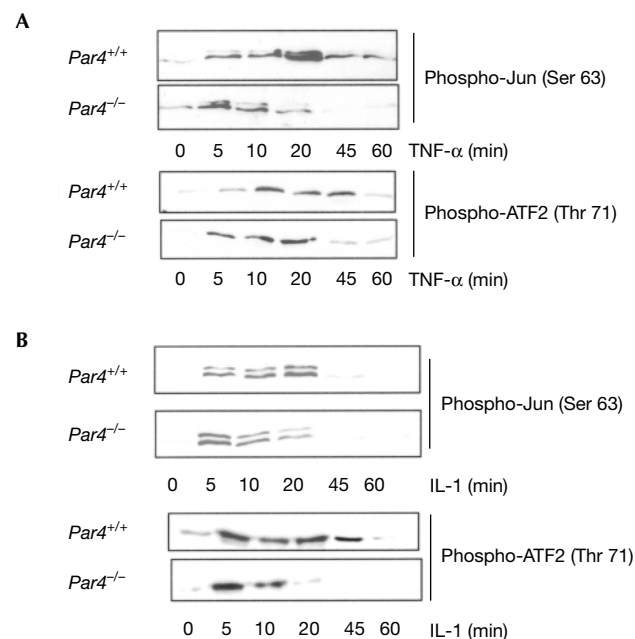

Fig. 2 | Apoptosis in *Par4^{-/-}* embryonic fibroblasts. (**A**) Wild-type (WT) and *Par4* null (KO) embryonic fibroblasts were incubated with 10 ng ml^{−1} of tumour necrosis factor-α (TNF-α), 10 μg ml^{−1} cycloheximide (CHX) or both for 72 h, after which the percentage of apoptotic cells was determined by TUNEL (TdT-mediated dUTP nick end-labelling) analysis. Results are the mean ± s.d. of three independent experiments with incubations in duplicate. (**B**) Cell extractions were carried out from cultures incubated for 12 h as above and the processing of caspase 3 was analysed by immunoblotting. The data shown are representative of three independent experiments.

A

B

Fig. 3 | NF-κB signalling in *Par4*-deficient cells. (**A**) Wild-type and *Par4*⁻/⁻ embryonic fibroblasts (EFs) were transfected with a κB–luciferase reporter plasmid and the control reporter plasmid pRL–CMV. After transfection (24 h) cells were treated with varying concentrations of tumour necrosis factor-α (TNF-α) or interleukin-1 (IL-1) for 6 h; cell extracts were then made and luciferase activity was analysed. (**B**) Wild-type and *Par4*⁻/⁻ EFs were transfected with the same reporter plasmids as above, but *Par4*⁻/⁻ cells were also transfected with 0.5 μg of either a PAR4 expression vector or a control plasmid (pcDNA3). Wild-type EFs were transfected, in parallel, with the control plasmid. Cells were stimulated with TNF-α and luciferase activity was analysed. Results are the mean ± s.d. of two independent experiments with incubations in duplicate.

increased greatly in the *Par4*⁻/⁻ cells as compared with that in the wild-type control cells (Fig. 3A). When cells were transfected with a mutant luciferase reporter lacking the κB-recognition sites, no differences were observed between wild-type and *Par4*⁻/⁻ cells (data not shown). These results show that PAR4 is a negative regulator of NF-κB signalling. When PAR4 was re-introduced into *Par4*⁻/⁻ EFs the enhanced activation of κB-dependent transcription in response to TNF-α (Fig. 3B) or IL-1 (not shown) was abolished. Recent studies have shown that the absence of ζPKC in EFs derived from knockout mice severely impaired κB-dependent transcription, with no effects on IκB kinase (IKK) activation (Leitges *et al.*, 2001). As PAR4 binds and inhibits ζPKC (Diaz-Meco *et al.*, 1996), it was of interest to determine whether the activation of IKK is affected in *Par4*⁻/⁻ EFs. Activation of IKK, and the degradation of IκBα induced by TNF-α and IL-1, were not affected by the absence of PAR4 (see supplementary

A

B

Fig. 4 | Mitogen-activated protein kinase signalling in *Par4*⁻/⁻ embryonic fibroblasts. Wild-type and *Par4*⁻/⁻ embryonic fibroblasts were stimulated for varying lengths of time with 10 ng ml⁻¹ of tumour necrosis factor-α (TNF-α) (**A**) or interleukin-1 (IL-1) (**B**), after which cell extracts were prepared and the activity of c-Jun amino-terminal kinase (JNK; determined by level of Ser 63 phosphorylation of Jun) or p38 (determined by level of Thr 71 phosphorylation of activating transcription factor 2 (ATF 2) were analysed in immunoprecipitates as described in Methods.

information online). In addition, the activation of NF-κB by TNF-α or IL-1, as determined by electrophoretic mobility-shift assays, was not significantly altered in *Par4*⁻/⁻ EFs (see supplementary information online). This is consistent with observations suggesting that ζPKC regulates NF-κB activation at the IKK level in some tissues, such as lung, but not in others (Leitges *et al.*, 2001).

Role of PAR4 in the activation of JNK and p38

In addition to activating NF-κB, TNF-α is a potent inducer of the c-Jun amino-terminal kinase (JNK) and p38 kinase cascades. The p38 and JNK pathways have both been implicated in the control of apoptosis and the inflammatory response. Recent evidence has shown that there is cross-talk between these signalling pathways such that inhibition of NF-κB in *IKKβ*⁻/⁻ and *RelA*⁻/⁻ cells is accompanied by a more sustained activation of JNK in response to TNF-α, which contributes to the induction of apoptosis by TNF-α (De Smaele *et al.*, 2001; Tang *et al.*, 2001). We therefore determined whether an absence of PAR4 from EFs leads to alterations in the activation of JNK and p38. Wild-type and *Par4*⁻/⁻ EFs were stimulated with TNF-α for varying durations, after which JNK or p38 were immunoprecipitated and their activities were determined. Interestingly, stimulation of wild-type EFs with TNF-α gave a high level of JNK activation, with JNK activity peaking at 10–20 min and remaining at high levels up to 60 min (Fig. 4A). Although the early stimulation (from five to ten minutes) of JNK by TNF-α was either unaffected or only slightly affected by the absence of PAR4, JNK activity was significantly lower at 20 min in *Par4*⁻/⁻ cells than in wild-type cells, and was completely inhibited
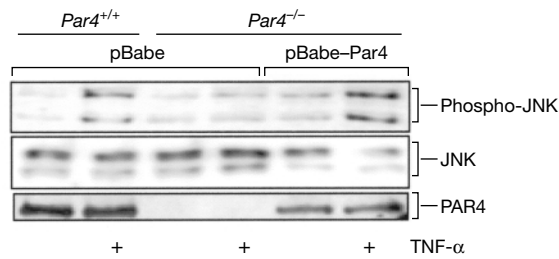
Fig. 5 | Expression of *Par4* restores normal c-Jun amino-terminal kinase signalling in *Par4*-deficient cells. Wild-type cells were transduced with the pBabe retroviral vector and *Par4*-deficient embryonic fibroblasts (EFs) with either pBabe or pBabe–PAR4 retroviral vectors. Cells were then stimulated with tumour necrosis factor-α (TNF-α) at 10 ng ml⁻¹ for 30 min and c-Jun amino-terminal kinase (JNK) activity was determined with an antibody specific for phospho-JNK. Levels of JNK and PAR4 expression were analysed using antibodies against these proteins. Representative data from at least three independent experiments are shown.

in *Par4*⁻/⁻ cells at 45 and 60 min (Fig. 4A). Stimulation of p38 was also sustained in TNF-α-treated cells (Fig. 4A). We also detected a reduction in TNF-α-induced p38 activation in *Par4*⁻/⁻ cells, although there was less of a reduction than that seen for JNK activity in these cells (Fig. 4A). Furthermore, when stimulated with IL-1, a less sustained activation of JNK and p38 was seen in *Par4*⁻/⁻ EFs as compared with that in the wild-type control EFs (Fig. 4B). Immunoblot analyses with anti-JNK and anti-p38 antibodies showed that there was no significant difference in JNK and p38 protein levels between wild-type and *Par4*⁻/⁻ cells (see supplementary information online). When the expression of the JNK upstream-activator, MAPK kinase 4 (MKK4), was analysed, its stimulation in *Par4*⁻/⁻ cells was less sustained than in wild-type EFs (see supplementary information online). Interestingly, from the point of view of the specificity of PAR4 function, the activation of both the p38 and the JNK kinase cascades by osmotic shock was not affected by the absence of PAR4 (see supplementary information online). These results suggest that an absence of PAR4 selectively inhibits the sustained activation of JNK and p38 in response to TNF-α and IL-1. When PAR4 was ectopically expressed in *Par4*⁻/⁻ cells the sustained activation of JNK was restored (Fig. 5).

Enhanced expression of XIAP in *Par4*⁻/⁻ EFs

One possible explanation for the ability of NF-κB to affect the activation of JNK by TNF-α is that the expression of the anti-apoptotic protein X-chromosome-linked inhibitor of apoptosis (XIAP) is reduced in the absence of NF-κB activation in *RelA*⁻/⁻ and *IKKβ*⁻/⁻ cells (Tang et al., 2001). Ectopic expression of XIAP in *RelA*⁻/⁻ cells, but not of other NF-κB-dependent genes, restores the transient mode of JNK activation that is observed in wild-type cells (Tang et al., 2001). Because *Par4*⁻/⁻ cells show enhanced NF-κB-dependent transcription, it is possible that these cells express increased levels of XIAP, which may account for the lower level of sustained JNK activation seen in these cells. To determine whether this hypothesis is correct, wild-type and *Par4*⁻/⁻ EFs were stimulated for varying durations with TNF-α, and the levels of XIAP were determined by immunoblot analysis. The data shown in Fig. 6A show that stimulation of wild-type EFs with TNF-α induces XIAP expression after 45 min, as previously reported.

XIAP levels were constitutively increased in *Par4*⁻/⁻ cells (Fig. 6A). Therefore, the downregulation of JNK in *Par4*⁻/⁻ cells is consistent with alterations in the levels of XIAP. Interestingly, the increased XIAP levels in *Par4*⁻/⁻ cells were detected even when these cells were treated with cycloheximide (Fig. 6B). Thus, the presence of cycloheximide decreases both actin and XIAP levels in wild-type and *Par4*⁻/⁻ cells (Fig. 6B, lanes 2 and 5), but the levels of XIAP, although reduced when compared with those in the untreated cultures (Fig. 6B, lanes 5 and 6, compared with lane 4), remain elevated in *Par4*⁻/⁻ cells (Fig. 6B, lanes 5 and 6) as compared with those in wild-type cells (Fig. 6B, lanes 2 and 3). This may explain why *Par4*⁻/⁻ EFs are more resistant to apoptosis than wild-type cells in response to TNF-α even in the presence of cycloheximide (Fig. 2A and B). It should be noted that the concentrations of cycloheximide used in these experiments only partially inhibit protein synthesis, maintaining the differences between the two cell types in regard to the levels of anti-apoptotic proteins such as XIAP.

PAR4 is not required for activation of ERK

Previous studies have suggested that the aPKCs are essential activators of extracellular-signal-related protein kinase (ERK) (Berra et al., 1995; Schonwasser et al., 1998) and that the overexpression of PAR4 leads to strong inhibition of the ERK pathway (Berra et al., 1997). However, although in B cells from ζ*PKC*⁻/⁻ mice there is a clear reduction in the activation of ERK (Martin et al., 2002), the absence of ζPKC does not affect the activation of ERK in EFs in response to several stimuli (Leitges et al., 2001). Therefore, we analysed whether the absence of PAR4 in *Par4*⁻/⁻ EFs affected ERK activation. In contrast to the stimulation of JNK in these cells, the activation of ERK by
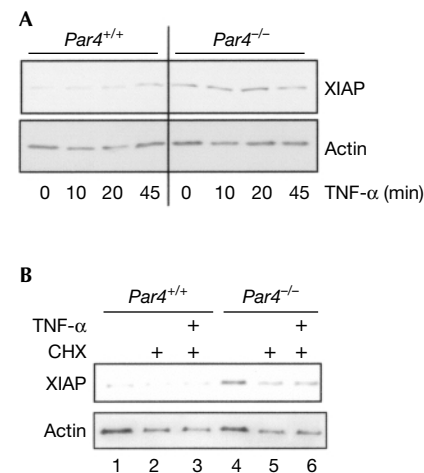
Fig. 6 | X-chromosome-linked inhibitor of apoptosis induction in *Par4*⁻/⁻ embryonic fibroblasts. (**A**) Wild-type and *Par4*-deficient embryonic fibroblasts (EFs) were incubated with tumour necrosis factor-α (TNF-α) at 10 ng ml⁻¹ for varying lengths of time, cell extracts were made, and the levels of X-chromosome-linked inhibitor of apoptosis (XIAP) determined by immunoblotting. (**B**) Wild-type or *Par4*-deficient EFs were incubated with cycloheximide (CHX) at 10 μg ml⁻¹ with or without TNF-α at 10 ng ml⁻¹ for 12 h. Levels of XIAP were determined by immunoblotting. Levels of actin expression are also shown, as a control. Representative data from three independent experiments are shown.
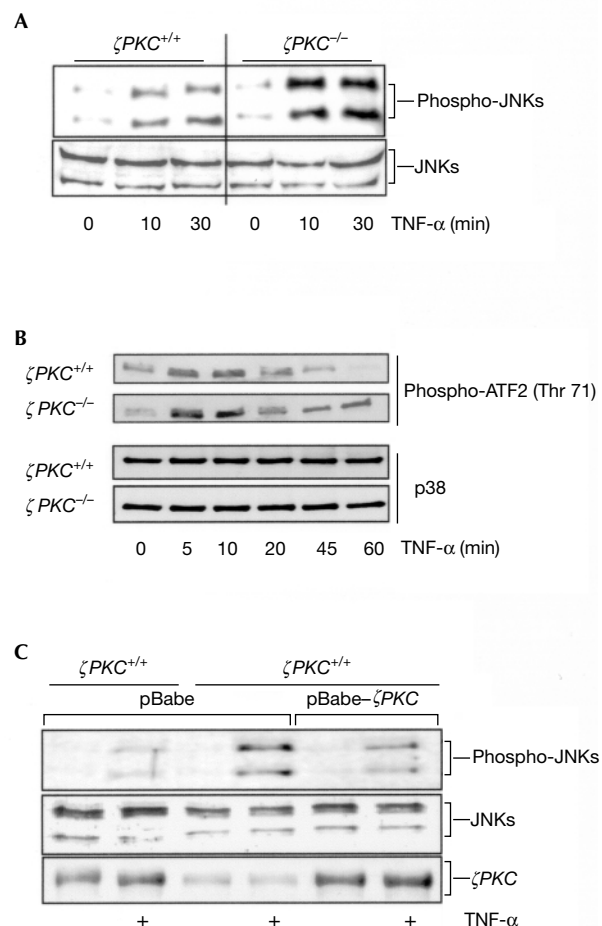
Fig. 7 | Mitogen-activated protein kinase signalling in embryonic fibrobalsts null for the ζ isoform of protein kinase C. Wild-type or ζ protein kinase C (ζPKC)-deficient embryonic fibroblasts (EFs) were incubated with tumour necrosis factor-α (TNF-α) at 10 ng ml⁻¹ for varying durations, cell extracts were prepared and c-Jun amino-terminal kinase (JNK) (**A**) and p38 (**B**) activities were determined as described in Methods. Levels of JNK and p38 are shown as a control. Representative data from at least three independent experiments are shown. (**C**) Wild-type cells were transduced with the retroviral pBabe vector, whereas ζPKC-deficient EFs were transduced with either pBabe or pBabe–ζPKC retroviral vectors. Cells were stimulated with TNF-α at 10 ng ml⁻¹ for 30 min and JNK activity was determined as above. Levels of JNK and ζPKC were determined using appropriate antibodies. Representative data from three independent experiments are shown.

TNF-α, platelet-derived growth-factor (PDGF) or serum was not affected by the absence of PAR4 (see supplementary information online).

Absence of ζPKC leads to enhanced activation of JNK

As PAR4 is an inhibitor of ζPKC and, according to the data presented in this study, is a regulator of the sustained activation of JNK, it is possible that the absence of ζPKC might lead to a hyperactivation of JNK. To address this possibility, we treated either wild-type or ζPKC-deficient primary cultures of EFs with TNF-α for varying durations,

after which JNK activity was determined. The absence of ζPKC correlates with enhanced activation of JNK by TNF-α (Fig. 7A). When the activity of p38 was analysed, it was clear that the loss of ζPKC leads to a more sustained increase in TNF-α-stimulated p38 activity (Fig. 7B). Similar results were obtained in cells activated by IL-1 (data not shown). These findings are consistent with PAR4 being a negative regulator of ζPKC and with the fact that the loss of PAR4 leads to the impairment of the sustained activation of JNK and p38 by TNF-α and IL-1. When ζPKC was ectopically expressed in *ζPKC⁻/⁻* cells, the increase in the sustained activation of JNK (Fig. 7C) and p38 (data not shown) in response to TNF-α was diminished in comparison with the increase detected in wild-type cells. It should be noted that the anti-ζPKC antibody used in this experiment to monitor the expression of transduced ζPKC cross-reacts with λ/ιPKC.

SPECULATION

These results show that PAR4 is an important regulator of apoptosis due to its ability to modulate the aPKCs and NF-κB, which in turn regulate the levels of several anti-apoptotic genes, including XIAP. This is particularly important as XIAP has been reported to control JNK activation, which contributes to the activation of apoptosis (De Smaele *et al.*, 2001; Tang *et al.*, 2001). The inactivation of NF-κB by PAR4 increases JNK activation in response to TNF-α and IL-1 (Reuther-Madrid *et al.*, 2002). Previous studies have suggested that the activation of p38 is not affected (De Smaele *et al.*, 2001; Tang *et al.*, 2001; Reuther-Madrid *et al.*, 2002). However, we show here that the loss of PAR4 affects not only the activation of JNK, but also that of p38. These results suggest that PAR4 and the aPKCs modulate the extent of JNK activation through NF-κB, whereas the regulation of p38 takes place through NF-κB-independent signals. Further studies are needed to determine whether other anti-apoptotic genes that may target p38 are constitutively activated in *Par4⁻/⁻* cells.

Future experiments will provide information about the effect that the absence of PAR4 has on the physiology of whole animals and tissues. PAR4 was first identified as having an important function in prostate cancer cells (Sells *et al.*, 1994). Therefore, we can speculate that loss of the *Par4* gene may induce neoplastic alterations in the prostate in both mice and humans. A link between PAR4 and cancer has already been reported (Barradas *et al.*, 1999; Nalca *et al.*, 1999): tumour transformation leads to a dramatic downregulation of PAR4 protein levels (Barradas *et al.*, 1999), which, consistent with the data reported here, correlates with an increased stimulation of the NF-κB pathway in transformed cells (Diaz-Meco *et al.*, 1999). However, it remains to be shown that downregulation of PAR4 is commonly observed in human tumours or autoimmune diseases.

METHODS

Reagents and antibodies. Reagents were purchased as follows: TNF-α (Promega), cycloheximide (Sigma), and PDGF (Upstate Biotechnology). Polyclonal antibodies anti-PAR4, anti-IKKγ, anti-ERK, anti-p38, anti-IκBα, anti-IKKβ, anti-MKK4 and anti-actin, and monoclonal antibodies anti-phospho-ERK and anti-phospho-JNK (antibody H1) were purchased from Santa Cruz. Monoclonal anti-caspase-3 and anti-XIAP antibodies, and the polyclonal anti-JNK antibody were purchased from Becton Dickinson. Polyclonal anti-phospho-c-Jun (Ser 63), anti-phospho-ATF-2 (Thr 71), anti-phospho-MKK4 (Thr 261) and anti-phospho-PKC (Thr 410) antibodies were from Cell Signaling. All antibodies were used in accordance with the manufacturers' instructions.

Generation of *Par4⁻/⁻* mice. Mice genetically deficient for *Par4* were produced using standard procedures. A genomic DNA segment containing murine *Par4* was identified by screening a commercial library (Down-to-the-Well, Genome Systems; *129Sv* genetic background) by PCR using primers complementary to exon two of *Par4*. The construct that eliminates exons one and two of *Par4* (Fig. 1A) was linearized and electroporated into R1 embryonic stem (ES) cells (*129Sv*). One ES cell clone was used to produce chimaeric male mice, which were crossed with CD1 females, and the progeny from this cross were genotyped by Southern blotting. EFs were isolated from embryos at 13.5 days post-coitum from crosses between *Par4⁺/⁻* mice of mixed genetic background *CD1/129Sv* (a ratio of 3:1).

Western blotting and *in vitro* kinase and electrophoretic mobility-shift assays. Cells were lysed with cell extraction buffer (Cell Signaling). Soluble fractions were immunoprecipitated with antibodies, as indicated in Results and Discussion. The immunoprecipitates were washed extensively, separated by SDS–polyacrylamide gel electrophoresis and transferred to Nitrocellulose-ECL membranes (Amersham Biosciences). The proteins present in the immunoprecipitates were detected by chemiluminescence using an Amersham Biosciences ECL Kit. *In vitro* kinase assays for JNK and p38 were carried out in accordance with the manufacturer's instructions (Cell Signaling). For IKK, activity was determined in anti-IKKγ immunoprecipitates using glutathione *S*-transferase–IκBα1-54 as described (Leitges *et al.*, 2001). Electrophoretic mobility-shift assays were carried out as described (Martin *et al.*, 2002). Cell cultures were transduced with pBabe, pBabe–PAR4 or pBabe–ζPKC retroviral constructs as described (Barradas *et al.*, 1999).

Apoptosis assay. Subconfluent EFs were incubated with TNF-α (10 ng ml⁻¹), cycloheximide (10 μg ml⁻¹) or both for 72 h, after which the percentage of apoptotic cells was determined by TUNEL analysis using the Mebstain Apoptosis Kit II (MBL International).

Luciferase assays. Subconfluent cultures of EFs, either wild-type or *Par4⁻/⁻*, were transfected using Lipofectamine Plus (Invitrogen) with 100 ng of a multimerized κB–luciferase reporter-gene plasmid (described previously in Leitges *et al.*, 2001) and 2 ng of the control reporter pRL–CMV (Promega). In some experiments, cells were co-transfected with pCDNA3–HA–PAR4 (Lallena *et al.*, 1999). After 24 h, cells were stimulated for 6 h with varying concentrations of TNF-α, extracts were prepared and the levels of luciferase activity were determined using the Dual-Luciferase Reporter Assay System (Promega) in accordance with the manufacturer's instructions.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

REFERENCES
Barradas, M., Monjas, A., Diaz-Meco, M.T., Serrano, M. & Moscat, J. (1999) The downregulation of the pro-apoptotic protein Par4 is critical for Ras-induced survival and tumour progression. *EMBO J.*, **18**, 6362–6369.

Berra, E., Diaz-Meco, M.T., Lozano, J., Frutos, S., Municio, M.M., Sanchez, P., Sanz, L. & Moscat, J. (1995) Evidence for a role of MEK and MAPK during signal transduction by protein kinase Cζ. *EMBO J.*, **14**, 6157–6163.

Berra, E., Municio, M., Sanz, L., Frutos, S., Diaz-Meco, M. & Moscat, J. (1997) Positioning atypical protein kinase C isoforms in the UV-induced apoptotic signaling cascade. *Mol. Cell. Biol.*, **17**, 4346–4354.

De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D.U., Jin, R., Jones, J., Cong, R. & Franzoso, G. (2001) Induction of gadd45β by NF-κB downregulates pro-apoptotic JNK signalling. *Nature*, **414**, 308–313.

Diaz-Meco, M.T., Municio, M.M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L. & Moscat, J. (1996) The product of *par4*, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. *Cell*, **86**, 777–786.

Diaz-Meco, M.T., Lallena, M.J., Monjas, A., Frutos, S. & Moscat, J. (1999) Inactivation of the inhibitory κB protein kinase/nuclear factor-κB pathway by Par4 expression potentiates tumour necrosis factor-α-induced apoptosis. *J. Biol. Chem.*, **274**, 19606–19612.

Guo, Q., Fu, W., Xie, J., Luo, H., Sells, S.F., Geddes, J.W., Bondada, V., Rangnekar, V.M. & Mattson, M.P. (1998) Par4 is a mediator of neuronal degeneration associated with the pathogenesis of Alzheimer disease. *Nature Med.*, **4**, 957–962.

Lallena, M.J., Diaz-Meco, M.T., Bren, G., Pay, C.V. & Moscat, J. (1999) Activation of IκB kinase β by protein kinase C isoforms. *Mol. Cell. Biol.*, **19**, 2180–2188.

Leitges, M., Sanz, L., Martin, P., Duran, A., Braun, U., Garcia, J.F., Camacho, F., Diaz-Meco, M.T., Rennert, P.D. & Moscat, J. (2001) Targeted disruption of the ζPKC gene results in the impairment of the NF-κB pathway. *Mol. Cell*, **8**, 771–780.

Martin, P., Duran, A., Minguet, S., Gaspar, M.L., Diaz-Meco, M.T., Rennert, P., Leitges, M. & Moscat, J. (2002) Role of ζPKC in B-cell signaling and function. *EMBO J.*, **21**, 4049–4057.

Moscat, J. & Diaz-Meco, M.T. (2000) The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters. *EMBO Rep.*, **1**, 399–403.

Murray, N.R. & Fields, A.P. (1997) Atypical protein kinase C ι protects human leukemia cells against drug-induced apoptosis. *J. Biol. Chem.*, **272**, 27521–27524.

Nalca, A., Qiu, S.G., El-Guendy, N., Krishnan, S. & Rangnekar, V.M. (1999) Oncogenic Ras sensitizes cells to apoptosis by Par4. *J. Biol. Chem.*, **274**, 29976–29983.

Reuther-Madrid, J.Y., Kashatus, D., Chen, S., Li, X., Westwick, J., Davis, R.J., Earp, H.S., Wang, C-Y. & Baldwin, A.S. Jr (2002) The p65/RelA subunit of NF-κB suppresses the sustained anti-apoptotic activity of Jun kinase induced by tumour necrosis factor. *Mol. Cell. Biol.*, **22**, 8175–8183.

Schonwasser, D.C., Marais, R.M., Marshall, C.J. & Parker, P.J. (1998) Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol. Cell. Biol.*, **18**, 790–798.

Sells, S.F., Wood, D.P. Jr, Joshi-Barve, S.S., Muthukumar, S., Jacob, R.J., Crist, S.A., Humphreys, S. & Rangnekar, V.M. (1994) Commonality of the gene programs induced by effectors of apoptosis in androgen-dependent and -independent prostate cells. *Cell Growth Differ.*, **5**, 457–466.

Sells, S.F. *et al.* (1997) Expression and function of the leucine zipper protein Par4 in apoptosis. *Mol. Cell. Biol.*, **17**, 3823–3832.

Tang, G., Minemoto, Y., Dibling, B., Purcell, N.H., Li, Z., Karin, M. & Lin, A. (2001) Inhibition of JNK activation through NF-κB target genes. *Nature*, **414**, 313–317.

Zhang, Z. & DuBois, R.N. (2000) *Par4*, a proapoptotic gene, is regulated by NSAIDs in human colon carcinoma cells. *Gastroenterology*, **118**, 1012–1017.