EXTRACELLULAR SIGNAL REGULATED KINASE 5: MOLELULAR MECHANISM OF REGULATION AND BIOLOGICAL PROPERTIES

A collection of manuscripts submitted in accordance with the requirements of the University of Manchester for the degree of Doctor in Philosophy by published work

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I would like to dedicate this thesis to

my husband, Dr.Qingqiu Pu,
and daughter, Fay

Abstract

Extracellular signal-regulated protein kinase (ERK5) is a mitogen-activated protein kinase (MAPK) that is activated by dual phosphorylation via an upstream activator MAPK/ERK kinase 5, MEK5. The activity of ERK5 is regulated by a wide range of mitogens and cellular stresses, such as epidermal growth factor (EGF) and sorbitol. Since its cloning in 1995, the efforts of many scientists has led to major progress in understanding the regulation of ERK5 and its function. In particular, genetically modified mice in which the *erk5* gene has been mutated have provided important information regarding the physiological relevance of this signalling pathway during cardiovascular development. Furthermore, the discovery that ERK5 is an important mediator of cell survival has reinforced interest in this signalling pathway. This study is based on the generation of two novel mouse models in which the *mek5* or the *erk5* gene has been deleted. The phenotypic analysis of the *mek5-/-* mice has demonstrated that MEK5 is a non-redundant component of the ERK5/myocyte enhancer factor 2 (MEF2)-dependent cell survival pathway required for normal cardiovascular development. Analysis of *mek5-/-* mouse embryonic fibroblasts showed that the MEK5/ERK5 signalling pathway protects cells from osmotic stress by down-regulating Fas ligand (FasL) expression via a mechanism that implicates protein kinase B (PKB)-dependent inhibition of Forkhead box O3a (Foxo3a) transcriptional activity. In addition to its role in promoting cell survival, ERK5 is required for regulating EGF-induced c-Jun expression, a potential mechanism by which the MEK5/ERK5 cascade contributes to cell proliferation. Mutant mice in which the *erk5* gene is specifically deleted in tissues

will be used in future studies to elucidate the functional significance of ERK5 in development and pathogenesis.

Preface

After obtaining a MB ChB degree from the medical school of Wuhan University, China, and a MD degree from the Medical School of Wits University, South Africa, I made a career movement to the University of Manchester, UK, where I developed an interest in the mitogen-activated protein kinase signalling pathways. In particular, I have been involved in a number of projects investigating the molecular regulation of the extracellular signal-regulated protein kinase 5 and its function during mammalian development and in cell survival. My work is included in six papers listed as follows:

Wang, X., Merritt, A.J., Seyfried, J., Guo, C., Papadakis, E.S., Finegan, K.G., Kayahara, M., Dixon, J., Boot-Handford, R.P., Cartwright, E.J., Mayer, U. and Tournier C. (2005) Targeted deletion of *mek5* causes early embryonic death and defects in the extracellular signal-regulated kinase 5/myocyte enhancer factor 2 cell survival pathway. *Mol Cell Biol.*, **25,** 336-345.

Kayahara, M., **Wang, X.** and Tournier, C. (2005) Selective regulation of *c-jun* gene expression by mitogen-activated protein kinases via the 12-*O*-tetradecanoylphorbol-13-acetate-responsive element and myocyte enhancer factor 2 binding sites. *Mol Cell Biol.*, **25,** 3784-3792. (Kayahara M. and Wang X. equally contributed to the study).

Seyfried, J., **Wang, X.,** Kharebava, G., and Tournier, C. (2005) A novel mitogen-activated protein kinase docking site in the N-terminus of MEK5α organizes the components of the extracellular signal-regulated kinase 5 signalling pathway. *Mol Cell Biol.*, **25,** 9820-9828.

Wang, X., Finegan, KG., Robinson, A., Knowles, L., Khosravi-Far, R., Hinchliff, K.A., Boot-Handford, R.P., and Tournier, C. (2006) Activation of extracellular signal-regulated protein kinase 5 down-regulates FasL upon osmotic stress expression following osmotic stress. *Cell Death Differ.* (in press). (Wang X and Finegan KG equally contributed to the study).

Wang, X., Tournier, C. (2006) Regulation of cellular functions by the ERK5 signalling pathway. *Cell. Signal.,* **18,** 753-760.

Papadakis, E.S., Finegan, K.G., **Wang, X.,** Robinson, A.C., Guo, C., Kayahara, M. and Tournier, C. (2006) The regulation of Bax by JNK is a prerequisite to the mitochondrial-induced apoptotic pathway. *FEBS Lett.,* **580,** 1320-1326.

Moreover, during this period, I have learnt a wide range of techniques stretching from molecular biology to cellular biology. It is worth noting that I have grasped a gene targeting technique which allowed me to generate three transgenic mouse models for dissecting the physiological role of the ERK5 signaling pathway. Finally, I shall state

that the work presented here was supported by grants awarded by the British Basic Sciences Research Council.

Declaration

The work presented here has been entirely conducted in the Faculty of Life Sciences, University of Manchester, whilst the candidate is a member of staff of this university. No proportion of the work has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning. As requested under clause 8b, I would like to state that:

For the first manuscript, my contribution was to generate the *mek5* null mouse model, conduct all *in vivo* analyses and *in vitro* cell proliferation and survival studies. The contributions of other authors were to provide some technical support, and to work on the manuscript in an advisory capacity.

For the second manuscript, whilst I and Midori Kayahara worked equally to the study, my contribution was to generate the *erk5 -/-* mouse embryonic fibroblasts, and determine the regulation of c-Jun transcriptional activity upon different stimuli by the reporter gene expression assay. Midori Kayahara was responsible for the biochemical analysis, including real-time PCR, and protein kinase assays.

For the third manuscript, I created the *erk5LoxP/LoxP* mouse model from which the ERK5 null mouse embryonic fibroblasts were derived. In collaboration with Katherine G Finegan, I discovered that ERK5 signaling pathway promotes cell survival by down-regulating FasL expression. The inputs of other authors were to provide technical help, and to advise on the experiment planning.

Overall, Dr. Cathy Tournier was responsible for supervising all the projects.

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I would like to take this opportunity to express my sincere gratitude to the following people:

My supervisor Dr. Cathy Tournier, she is the one of people who influences my life attitude the most. She encourages me to persist in my research interests and to accomplish the projects by giving me a thorough scientific training and boosting my confidence. Also she sets an example of how to be fully committed to the work.

Professor David Garrod and Dr. Anita Merritt who are my mentors and friends, for helping me along the way with numerous advice, and making my life in and out lab much more happy and meaningful.

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Last but not least, I own a large part of everything I have achieved so far to my whole family.

I - Introduction

1. Overview of MAP kinases

More than 500 protein kinases have been recognized in the human genome. These kinases play crucial roles in regulating cellular processes and participating in signal transmission. Among them, the mitogen-activated protein kinases (MAPKs) are well characterized. They belong to an evolutionary conserved family of proline-directed protein kinases that phosphorylate Ser and Thr residues preceding a Pro residue. MAPKs act through discrete cascades in which each protein kinase sequentially phosphorylate the next. MAPK kinase kinase activates MAPK kinase, which then phosphorylate MAPK. These cascades are carefully orchestrated and regulated. The endpoint of these signaling pathways is that the active MAPKs control, by phosphorylation, the activity of numerous transcription factors and enzymes through regulating binding partners, conformation changes, subcellular localization, and protein stability (Widmann et al., 1999; Yang el al., 2003).

There are at least four MAPK families: extracellular-regulated protein kinases 1 and 2 (ERK1/2), ERK5, c-Jun NH_2-terminal protein kinases (JNKs), and P38 MAPKs (Table 1). MAPK activity is increased following phosphorylation at Thr and Tyr residues within a Thr-Xaa-Tyr (T-X-Y) motif in the activation loop by a MAPK/ERK kinase (MEK or MKK) (Widmann et al. 1999). Xaa corresponds to a Glu residue in ERK, a Pro residue in JNK and a Gly residue in p38 MAPK subfamilies. MAPK activators include MEK1 and MEK2 for ERK1/2, MEK5 for ERK5, MKK4 and MKK7 for JNKs, and MKK3 and MKK6 for p38 MAPKs. MEKs are activated by phosphorylation at Ser and Thr residues by a MEK kinase (MEKK).

Table 1 : Mammalian MAP kinases

MAP Kinase	Other names	Predicted size	Brief description	P site motif	References
ERK1 ERK2	P44 MAPK P42 MAPK	44KD 42KD	Cloned in1990,ERK1 and ERK2 share almost 85% identical sequences. They are expressed ubiquitously	TEY	Boulton et al., 1990 Boulton et al., 1991
ERK5	Big MAPK1 (BMK1)	98KD	identified in 1995, shares 66% similar sequences to ERK1/2. It is expressed ubiquitously	TEY	Lee et al., 1995 Zhou et al., 1995
JNK1 JNK2 JNK3	SAPKγ SAPKα SAPKβ	46KD-55KD	Discovered in1990, cloned in 1994. JNK1,2,3 are more than 85% identical within the core catalytic domains. JNK1,2 are expressed widely, JNK3 is restricted in the brain, heart and testis	TPY	Kyriakis et al., 1994 Dérijard et al., 1994 Gupta et al., 1996
P38α P38β P38γ P38δ	P38, CSBP, SAPK2 P38-2 ERK6, SAPK3 SAPK4	38KD	P38α was identified in 1994, P38β, P38γ and P38δ were discovered between 1996-1997. P38α and P38β are ubiquitously expressed, while P38γ and P38δ are differentially expressed depending on tissue types. P38 isoforms share 60% identical sequences	TGY	Han et al., 1994 Jiang et al., 1996 Jiang et al., 1997 Li et al., 1996

Two main mechanisms have been proposed to ensure specific transmission of the signals from upstream kinases to MAPKs (Van Drogen and Pete, 2002; Whitmarsh and Davis, 1998): i) adaptor/scaffold proteins that assemble the different components of a cascade, and ii) direct physical interactions between the components of a cascade. Both mechanisms may operate in parallel and allow distinct responses of the same MAPK signalling pathways to different stimuli. Gene targeting experiments in mice have provided evidence that MAPK modules are associated with different biological responses (Kuida and Boucher, 2004). For example, the ERK subfamilies are mostly associated with cell proliferation and survival, while JNKs and p38 MAPKs are mainly activated in response to cytokines and extracellular stresses and can mediate apoptosis.

2. Identification and Molecular Regulation of ERK5

2.1. Cloning

Two groups cloned ERK5 in 1995. Dixon and co-workers (Zhou el at., 1995) first identified the ERK5 activator, MEK5. Based on the assumption that MEKs interact with MAPKs, they used MEK5 as bait in a yeast two-hybrid screen and identified ERK5 as a binding partner. Further studies confirmed the binding of ERK5 with MEK5 but not with MEK1 or MEK2 supporting the highly specific nature of the MEK/MAPK interaction. At the same time Ulevitch and co-workers (Lee el at., 1995) identified a protein identical to ERK5 that they called big MAPK 1 (BMK1). The full-length sequence of human ERK5 contains an open reading frame of 2445-base pairs encoding 816 amino acids. With a predicted molecular mass of 98 kDa, ERK5 is more than twice the size of the other MAPKs due to a 396 amino acid C-terminal extension. The N-

terminal conserved MAPK catalytic domain shares 50% homology with ERK1/2. Other analogy between ERK5 and ERK1/2 subfamilies includes the ability of the commonly used MEK1/2 inhibitors, PD98059, U0126, and PD184352, to block the ERK5 pathway (Kamakura et al., 1999; Mody et al., 2001).

Gene analysis reveals that the mouse *erk5* gene is encoded by 6 exons and 5 introns spanning ~ 5.6 kb. Alternative splicing across introns 1 and/or 2 generate three isoforms, a, b, and c (Yan el al., 2001). Compared with ERK5a, ERK5b and ERKc lack 69 and 139 amino acids at their N-termini, respectively. Immunoblot analysis of a mouse embryonic extract showed that ERK5a is expressed to a greater extent than ERK5c and much greater than ERK5b (Yan et al., 2001). Both ERK5b and c are deficient in protein kinase activity and can block ERK5a activation by MEK5 and ERK5-induced transcription via MEF2C (Yan et al., 2001). A premature in-frame stop codon introduced by failure to splice intron 4 gives rise to another mouse splice variant, *erk5-T*, which encodes a truncated protein at the C-terminal end unable to translocate to the nucleus (McCaw el al., 2005). ERK5-T can retain ERK5 in the cytoplasm upon stimulation by forming heterodimers (McCaw et al., 2005). Together, these data suggest that ERK5 splice variants may act as dominant negative mutants of the full-length protein, thereby providing a novel regulatory mechanism of ERK5 activity. Further studies will need to be performed to clarify in vivo the physiological function of alternative splicing of the *erk5* gene in specific tissues under specific conditions.

2.2. Functional domains

The N-terminal domain of ERK5a is important for cytoplasmic targeting (aa 1-77), association with the upstream kinase MEK5 (aa 78-139) and oligomerisation (aa 140-406) (Yan et al., 2001). Unlike ERK1/2, ERK5 oligomerises in both stimulated and unstimulated conditions. The unique and very large C-terminal domain is required for the dynamic nuclear shuttling of ERK5 (Buschbeck et al., 2005). It contains both nuclear localisation and export signals (NLS and NES), and two proline rich (PR1 and PR2) regions that may serve as binding sites for proteins containing Src-homology 3 (SH3) domains (Buschbeck et al., 2005; Yan et al., 2001; Zhou et al., 1995). Interestingly, the PR regions of the mouse and the human proteins differ significantly in their sequence. The identification of specific binding partners that interact with ERK5 may reveal significant differences in signalling mechanisms between human and mouse. The C-terminal tail also has an auto-inhibitory function that influences the activation of ERK5. This is demonstrated by enhanced ERK5 activity following deletions of the C-terminus (Buschbeck et al., 2005; Zhou et al., 1995). These studies suggest a model where the C-terminal tail masks the CD domain preventing the interaction of ERK5 with its substrates. Upon stimulation, ERK5 phosphorylates its C-terminus thereby causing a conformational change that exposes the docking site and the NLS (Buschbeck et al., 2005). This explains the dynamic nuclear shuttling of ERK5. However, the cellular distribution of ERK5 varies largely between different cell types under non-stimulated conditions ranging from an overall diffuse pattern to predominantly nuclear (Buschbeck et al., 2005). Furthermore, Kondoh et al. reported that the N-terminal half of ERK5 bound to the C-terminal half and that binding was

necessary for nuclear export of ERK5. The activating phosphorylation of ERK5 resulted in the dissociation of the binding between the N- and C-terminal halves and thus inhibited nuclear export ERK5, causing its nuclear import. These results reveal a phosphorylation-dependent control mechanism for nucleocytoplasmic shuttling of ERK5 (Kondoh et al., 2006).

2.3. Downstream targets

An important step in understanding the function of a MAPK cascade is to identify its downstream effectors. The transcription factors of the myocyte enhancer factor (MEF) family, MEF2A, C, and D are among the best-characterised substrates of ERK5 (Kato et al., 1997; Kato et al., 2000;Yang et al., 1998). Phosphorylation of MEF2C by ERK5 enhances its transcriptional activity and subsequently leads to increased *c-jun* gene expression (Kato et al., 1997). Consistent with this study, we have recently demonstrated that ERK5 is selectively required for the regulation of c-Jun expression following epidermal growth factor (EGF) stimulation of the cells, but not following UV radiation (Kayahara et al., 2005). While MEF2D is a specific substrate of ERK5, MEF2A and MEF2C activities are controlled by both p38 MAPKs and ERK5 (Kato et al., 1997; Kato et al., 2000; Han et al., 1997; Ornatsky et al., 1999;Yang et al., 1998; Zhao et al., 1999). However, p38 MAPKs fail to compensate for the defect in MEKK2/3-induced transcription via MEF2A caused by *mek5* gene deletion, indicating that the ERK5 signalling pathway is essential for regulating MEF2A activity (Wang et al., 2005). The C-terminal tail of ERK5 contains a MEF2-interacting region (aa 440-501) and a transcriptional activation domain (aa 664-789) essential for co-activation of

MEF2 (Kasler et al., 2000). The functional importance of the tail in ERK5-mediated transcription via MEF2 is consistent with the inability of a C-terminal truncated ERK5 mutant that possesses an active protein kinase, to stimulate MEF2 activity (Yan et al., 2001).

Other direct substrates of ERK5 include Sap1, and c-Myc (English et al., 1998; Kamakura et al., 1999). While the effect of phosphorylation of c-Myc by ERK5 remains unclear, ERK5-dependent phosphorylation of Sap1 enhances transcription via a serum response element that may be responsible for increasing expression of c-Fos (Kamakura et al., 1999; Terasawa et al., 2003). In addition to acting on the *c-fos* promoter, the ERK5 signalling pathway stimulates the transcriptional activity of c-Fos and Fra-1 by a mechanism that implicates a kinase lying downstream of ERK5, which could be p90 ribosomal S6 protein kinase (RSK) (Terasawa et al., 2003). Increased RSK activity may also be implicated in mediating the co-operative effect of ERK5 and ERK1/2 to regulate nuclear factor kappa B (NF-κB) function (Pearson et al., 2001). In contrast to MEF2, Fos does not interact with ERK5 (Terasawa et al., 2003). However, similar to MEF2 regulation, a C-terminal truncated ERK5 mutant is unable to stimulate Fos activity (Terasawa et al., 2003). This is consistent with the requirement of ERK5 to enter the nucleus for maximal Fos activation.

2.4. Dual phosphorylation of ERK5 by MEK5

ERK5 activity is increased in response to growth factors, oxidative stress and hyperosmolarity via the dual phosphorylation of its TEY motif by MEK5 (Mody et al., 2003) (Fig. 1). The phosphorylation of ERK5 by MEK5 may contribute to stabilising

ERK5 in an active conformation promoted by the auto-phosphorylation of its C-terminal tail (Buschbeck et al., 2005; English et al., 1998; Mody et al., 2003). The physiological significance of MEK5 was demonstrated by the analysis of *mek5* gene ablation in mice (Wang et al., 2005). Similar to the *erk5-/-* embryos (Regan et al., 2002; Sohn et al., 2002; Yan et al., 2003), the *mek5-/-* fetuses displayed abnormal cardiac development.

The MEK5 cDNA encodes a 444-amino acid protein, which displays approximately 40% identity to known MEKs (English et al., 1995; Zhou et al., 1995). The alternative splicing of the mRNA gives rise to two isoforms with different N-termini, MEK5α (50 kDa) and MEK5β (40 kDa) (English et al., 1995). The N-terminal extension of MEK5α is implicated in its restricted localization to the particulate fraction while MEK5β is ubiquitously distributed and is primarily cytosolic (English et al., 1995). MEK5β has been proposed to act as a kinase dead dominant negative variant that can suppress ERK5 signalling (Cameron et al., 2004). However, consistent with the ability of a MEK5β transgene to activate ERK5 in vivo (Nicol et al., 2001), we found that MEK5β is an active enzyme (Seyfried et al., 2005). Our results indicate that MEK5α is a stronger activator of ERK5 than MEK5β due to its higher affinity for ERK5. This led us to identify a novel MAPK docking site in the N-terminus of MEK5α that is distinct from the consensus motif identified in other MEKs (Tanoue and Nishida, 2003). It consists of a cluster of acidic residues at position 61 and 63-66 on the rat sequence. This domain is critical for MEK5α to promote maximal ERK5 activation and to increase transcription via MEF2 (Seyfried et al., 2005).

2.5. Upstream kinases

Both MEKK2 and MEKK3, but not MEKK1, are able to activate MEK5 in a stimulus and cell type specific manner (Chao et al., 1999; English et al., 1995; Sun et al., 2001). WNK1 (with no lysine (K) was recently identified as a protein kinase upstream of MEKK2/3 in the ERK5 signalling pathway (Xu et al., 2004). The N-terminal extension of MEK5α contains a phox and Bem1p (PB1) domain that mediates the binding interaction of MEK5α with MEKK2/3 (Nakamura and Johnson, 2003). Blocking the PB1-dependent formation of the MEKK/MEK5 complex prevents MEK5 activation (Nakamura and Johnson, 2003; Seyfried et al., 2005). This appears inconsistent with the ability of MEKK2 to increase MEK5β activity (Seyfried et al., 2005). Indeed, MEK5β that lacks the PB1 domain is unlikely to bind MEKK2. To explain such discrepancy we propose that the N-terminus of MEK5α contains an auto-inhibitory domain that masks the phosphorylation sites of MEK5 by upstream kinases (Seyfried et al., 2005). The interaction of MEK5 with MEKK2 affects the overall conformation of MEK5α so that Ser311 and Thr315 become accessible for phosphorylation.

As both MEKK2 and ERK5 interact with the N-terminal extension of MEK5α, it is possible that MEKK2 and ERK5 compete for binding to MEK5 rather than forming a ternary complex. Based on a similar model proposed to explain the organisation of the JNK signaling pathway (Xia et al., 1998), we hypothesise that MEKK2 and MEK5 form a complex that is dissociated upon activation, so that activated MEK5 becomes free to interact with its substrate ERK5 (Seyfried et al., 2005). Consistent with our data, Nakamura et al. have recently shown that the PB1 region of MEKα is required for

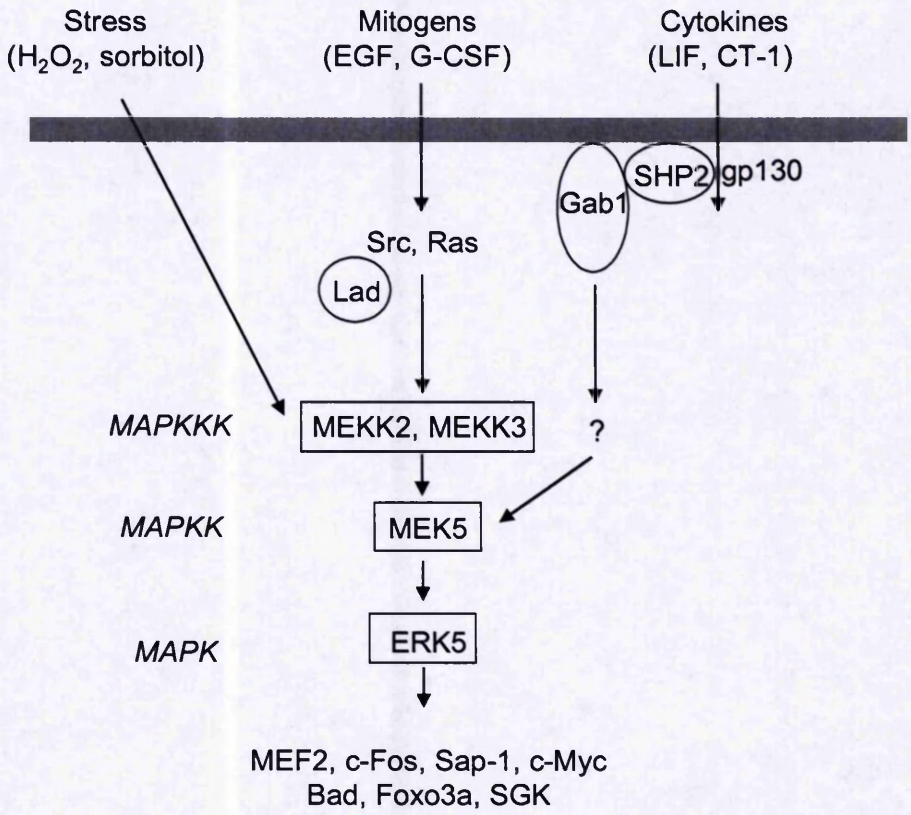
MEKK2 and ERK5 interaction (Nakamura et al., 2006). However, in contrast to our model, their results suggest that the formation of a ternary MEKK2-MEK5-ERK5 complex is critical for ERK5 activation.

2.6. Adaptor/scaffold proteins

Adaptor/scaffold proteins facilitate and specify MAPK activation in response to physiological stimuli by forming multi-enzyme complexes (Whitmarsh and Davis, 1998). For example, a functional interaction between MEKK2 and the Lck-associated adapter (Lad) is required for EGF-induced ERK5 activation via Src (Sun et al., 2003). Lad may be responsible for increasing the binding affinity between MEKK2 and MEK5, and for recruiting the MEKK2/MEK5 complex to the receptor. Grb2-associated binder 1 (Gab1) is another adaptor protein implicated in mediating ERK5 activation in cardiomyocytes in response to leukemia inhibitory factor (LIF) via the gp130 signal complex (Nakaoka et al., 2003). LIF-induced tyrosine phosporylation of Gab1 and protein tyrosine phosphatase SHP2 leads to their association. Gab1/SHP2 interaction is crucial for LIF-induced elongation of cardiomyocytes via ERK5 (Nakaoka et al., 2003). The muscle specific protein kinase A (PKA) anchoring protein mAKAP also contributes to the transduction of the hypertrophic signal via ERK5 in cardiomyocytes (Dodge-Kafka et al., 2005). MEK5 and ERK5 interact with the phosphodiesterase E4D3 (PDE4D3) and the cAMP-dependent exchange factor for the small GTPase Rap1 (Epac1) to form a functional complex with PKA and mAKAP. ERK5-mediated PDE4D3 phosphorylation decreases cAMP catabolism. The subsequent rise in cAMP

concentration activates Epac1 that suppress LIF-induced ERK5 activation via Rap1 (Dodge-Kafka et al., 2005).

Fig1: Schematic mode of regulation of ERK5



3. Biological Functions of ERK5

3.1. ERK5 regulation of neuronal survival

Tamaki and co-workers were the first to implicate ERK5 in the survival response of PC12 cells to oxidative stress (Suzaki et al., 2002). The physiological significance of these results was provided by the demonstration that ERK5 contributes to the survival response of dorsal root ganglion (DRG) neurones to neuronal growth factor (NGF)

(Watson et al., 2001). TrkA receptors present at the surface of the extending axon auto-phosphorylate following the binding of NGF. Phosphorylated TrkA receptors are internalised into a "signalling endosome" that is retrogradely transported from the extending axon to the cell body where it activates ERK5. ERK5 then initiates a phosphorylation cascade resulting in the activation of the transcription factor Ca^{++}/cAMP response element binding protein (CREB) that regulates the transcription of survival and apoptotic genes (Watson et al., 2001). Evidence that ERK5 contributes to mediating the survival of neurones in the central nervous system via the activation of the transcription factor MEF2 has since been reported (Liu et al., 2003; Shalizi et al., 2003).

3.2. ERK5 and cell proliferation

The discovery that serum was a potent inducer of *c-jun* gene transcription via ERK5-induced MEF2C transcriptional activation provided the first evidence that the ERK5 signalling pathway was involved in regulating cell proliferation (Kato et al., 1997). Consistent with this study, mitogens including EGF and granulocyte colony-stimulating factor (G-CSF), were subsequently found to transmit their growth promoting signals via ERK5 (Dong et al., 2001;Kato et al., 1998). However, no marked difference was observed in the ability of *erk5-/-*, *mek5-/-*, and wild type fibroblasts to progress through S phase (Hayashi et al., 2004; Wang et al., 2005). This suggests that the ERK5 signalling pathway may be important for promoting or regulating the proliferation of certain cell types under certain conditions.

The molecular mechanism by which ERK5 mediates its proliferative effect is not clear. It may implicate the activation of SGK, a protein kinase that is closely linked to the G1/S transition of the cell cycle (Buse et al., 1999). The phosphorylation of SGK by ERK5 at serine 78 is required for mediating SGK activation and for promoting the entry of cells into S phase of the cell cycle in response to growth factor (Hayashi et al., 2001). The transcriptional activation of the *cyclin D1* gene, a key cell proliferation checkpoint, the deregulation of which is frequently associated with neoplastic transformation, has also been shown to be regulated by the ERK5 cascade. The expression of a kinase-dead mutant of ERK5 blocks the synthesis of endogenous cyclin D1 protein induced by serum in a number of breast cancer cell lines (Mulloy et al., 2003). Evidence from *jnk-/-* fibroblasts suggests that c-Jun is not involved in this process (Schreiber et al., 1999). However, the requirement of ERK5 in regulating cyclin D1 expression is disputed by the ability of PD184352 to block increased levels of cyclin D1 and cell proliferation without affecting ERK5 activation (Squires et al., 2002).

3.3. ERK5 and Cancer

3.3.1. Activation of ERK5 by oncogenes

Mutant *ras* has been identified in cancers of many different origins, including pancreas (90%), colon (50%), lung (30%), thyroid (50%), bladder (6%), ovarian (15%), breast, skin, liver, kidney, and some leukaemias. Among the signalling pathways suspected to be involved in mediating the oncogenic effect of Ras is the ERK5 cascade. In certain cell types, including PC12, C2C12, and COS7 cells, ERK5 is activated by

Ras (Kamakura et al., 1999; English et al., 1998; English et al., 1999). Furthermore, foci induced by a dominant active mutant of Raf (Raf-BxB), a well characterised downstream effector of Ras, are increased in number by enhancing the activity of the ERK5 pathway and are decreased in number by disruption of MEK5 or ERK5 function (English et al., 1999). This is consistent with the ability of Raf-1 to enhance the activation of ERK5 by Ras (English et al., 1999), although neither MEK5 nor ERK5 activity is increased following over-expression of Raf-BxB (English et al., 1998; English et al., 1999). The requirement of ERK5 in cellular transformation and malignancy needs to be confirmed by testing the effect of *erk5* gene deletion on the ability of oncogenic Ras to induce typical morphological changes, loss of contact inhibition, and anchorage-independent growth of fibroblasts, and tumourigenicity in vivo.

In addition to Ras, many other oncogenes are able to activate ERK5. For example, Cot potently stimulates the activity of the *c-jun* promoter via JNK, p38 MAPK and ERK5 to induce neoplastic transformation in NIH3T3 cells (Chiariello et al., 2000). Src is involved in the formation of a functional Lad/MEKK2/MEK5 complex that regulates MEF2 activity (Sun et al., 2003). ERK5 synergises with ERK1/2 to promote Src-dependent cell proliferation (Abe et al., 1997; Scapoli et al., 2004). In line with this study, concomitant activation of ERK5 and ERK1/2 causes the disruption of the actin cytoskeleton, a fundamental step of Src-dependent cell transformation (Barros and Marshall, 2005). ERK1/2 acts either through suppressing Rho activation or by down-regulating the levels of the Rho effectors, ROCKI and ROCK II, whereas the mechanism by which ERK5 regulates the process is unclear.

3.3.2. ERK5 in tumour cells

Receptor tyrosine kinases of the ErbB family are activated following interaction with members of the EGF family of ligands. Over-expression or mutations of these receptors in cancers is associated with poor prognosis, shorter disease-free intervals, increased risk of metastasis, and resistance to chemotherapy. The analysis of ERK5 in human-derived tumour cell lines has demonstrated a link between the presence of constitutively activated ERK5 and activated forms of ErbB2, ErbB3 and ErbB4 (Esparís-Ogando et al., 2002). The ability of a dominant negative mutant form of ERK5 to decrease the growth rate of breast cancer cells suggests that the ERK5 pathway delivers accelerating-growth signals in human cancer via an ErbB-dependent mechanism (Esparís-Ogaando et al., 2002). The activity of the signal transducer and activator of transcription 3 (STAT3) is regulated by ErbB. Increased MEK5 expression in breast cancer cell lines expressing an activated form of STAT3 (Song et al., 2004) provides a mechanism by which mutations in the *ErbB* genes may indirectly activate ERK5.

MEK5 expression has been assessed in 127 cases of prostate cancer and 20 cases of benign prostatic hypertrophy (Mehta et al., 2003). The results indicate that elevated MEK5 expression correlates with bone metastasis and unfavorable prognosis. One possible explanation implicates the deregulation of matrix metalloproteinase 9 activity associated with MEK5 over-expression which results in the degradation of the extracellular matrix surrounding the cancer cells, thus enabling them to invade (Mehta et al., 2003).

More recently, IL-6 was shown to activate ERK5 in cells isolated from multiple myeloma patients (Carvajal-Vergara et al., 2005). Multiple myeloma is a carcinoma caused by the accumulation of terminally differentiated B cells in the bone marrow. A dominant negative form of ERK5 restricts the proliferation of myeloma cells and sensitises cells to the apoptosis-induced drugs (Carvajal-Vergara et al., 2005).

3.3.3. ERK5 in tumour development

In addition to its contribution to the malignancy of tumour cells, genetic evidence that ERK5 is implicated in tumour-associated angiogenesis was provided by analysing the effect of the deletion of the *erk5* gene in vasculature development of melanoma and carcinoma xenografts (Hayashi et al., 2005). The number of large blood vessels was greatly diminished in tumours growing in the flank region of the *erk5-/-* mice. The results indicate that ERK5 is required for both vascular endothelial growth factor (VEGF)- and fibroblast growth factor (FGF)-induced angiogenesis by a mechanism that implicates the phosphorylation of the ribosomal protein S6 via RSK.

3.4. ERK5 and heart function

3.4.1. Role of ERK5 during cardiovascular development

The targeted deletion of the *erk5* and *mek5* genes in mice has provided genetic evidence for an essential role of the ERK5 signalling pathway during heart development (Regan et al., 2002; Sohn et al., 2002; Wang et al., 2005; Yan et al., 2003). The phenotypes observed in these mice are almost identical. They die around embryonic day 10 (E10) due to cardiovascular defects that include disorganisation of

the trabeculae and underdevelopment of the myocardium. Vasculogenesis and angiogenesis are impaired in both the embryo itself and the extraembryonic tissue including the yolk sac and placenta. However, the analysis of a mouse model featuring a conditional mutation in the *erk5* gene demonstrates that the cardiovascular defect is not a consequence of a placental defect (Hayashi et al., 2004). Similar phenotypic abnormalities displayed by the *mef2-/-*, and *mekk3-/-* embryos identify the MEKK3/MEK5/ERK5/MEF2 cascade as an essential signalling cascade that controls early cardiovascular development (Lin et al., 1997; Yang et al., 2000).

The heart defect observed in conventional ERK5 knockout mutants was reproduced in embryos in which ERK5 was specifically ablated in ECs (Hayashi et al., 2004). The irregular alignment and rounded morphology of the *erk5-/-* ECs surrounding the blood vessels in the developing heart is consistent with an essential role of ERK5 for the maintenance of vascular integrity. The normal development of mice lacking ERK5 in cardiomyocytes confirms that the abnormal development of the heart displayed by the *erk5-/-* embryo is a consequence of abnormal vasculogenesis and angiogenesis (Hayashi et al., 2004). The requirement of ERK5 for the maintenance of vascular integrity is also indispensable in adult mice that display hemorrhages from multiple organs and lethality within 2-4 weeks after the induction of Cre recombinase (Hayashi et al., 2004). The role of ERK5 for the survival of ECs in vitro (Pi et al., 2004) is consistent with the elevated apoptosis of ECs in vivo caused by the loss of ERK5 and decreased MEF2C activity (Hayashi et al., 2004). Abnormal angiogenesis may also be caused by increased expression of VEGF due to deregulation of hypoxia

inducible factor 1α (HIF1α) activity (Sohn et al., 2002). ERK5-induced HIF1α degradation occurs via an ubiquitin-dependent mechanism (Pi et al., 2005).

3.4.2. Role of ERK5 in cardiac hypertrophy

Cardiac hypertrophy involves the alteration of myocyte shape and extracellular matrix that results in wall thickening, followed by chamber dilation and myocardiac dysfunction. This process is initially compensatory for an increased workload, the prolongation of which leads to congestive heart failure, arrhythmia, and sudden death. Hypertrophy-stimulating factors including LIF, and oxidative and osmotic stress, activate ERK5 in cultured cardiomyocytes (Nakaoka et al., 2003; Nicol et al 2001). Over-expression of dominant active MEK5 elicits serial assembly of sarcomeres while dominant negative MEK5 blocks LIF-induced elongation of cardiomycoytes (Nakaoka et al., 2003). In vivo evidence that the ERK5 signalling pathway plays a role in the hypertrophic process is supported by the ability of over-expressed MEK5 to induce eccentric hypertrophy in the heart, which results in heart failure and sudden death (Nicol et al., 2001). This is consistent with increased activation of ERK5 during left ventriclular hypertrophy (Kacimi and Gerdes, 2003). It is worth noting that the deletion of seven amino acids in MEF2A, a well characterised downstream substrate of ERK5 (Kato et al., 1997; Yang et al., 1998), has been shown to be responsible for the onset of coronary artery disease/myocardial infarction (CAD/MI) in patients (Wang et al., 2003). Whether other MEF2 family members are involved and whether ERK5 is the upstream kinase that regulates MEF2A function responsible for the pathogenetical manifestations of CAD/MI remains to be determined.

II - Manuscript 1: Targeted deletion of *mek5* causes early embryonic death and defects in the extracellular signal-regulated kinase 5/myocyte enhancer factor 2 cell survival pathway

In the past 10 years, gene disruption studies have increased our understanding of the physiological function of the MAPK signaling pathways. For example, the analysis of mutant mice in which the *erk5* gene can be conditionally deleted (Hayashi et al., 2004) has revealed that the requirement of ERK5 for the survival of EC is responsible for the cardiovascular defect observed in the *erk5-/-* embryos (Regan et al., 2002; Sohn et al., 2002; Yan et al., 2003). ERK5-deficient embryos die between embryonic day 9.5 and 11.5 with defective blood vessel development, failure of cardiac looping and altered ventricular maturation. Increased expression of vascular endothelial growth factor (VEGF) in the ERK5 knockout mice may contribute to altered angiogenic remodeling and vasculature destabilization (Sohn et al., 2002). MEF2-deficient mice display similar abnormalities (Lin et al., 1997; Lin et al., 1998). The heart of the *mef2c-/-* embryos lacks looping morphogenesis that subsequently leads to absence of the future right ventricle associated with impaired vascular structure.

Key upstream regulatory components of the ERK5 signalling pathway, including MEKK2 and MEKK3 have been examined using a similar approach. The results show that mice deficient in MEKK3 expression display defects in angiogenesis during early development and die around day 11 of gestation (Yang et al., 2000). Small vessels are affected, particularly in the placenta, reflecting a defect in endothelial cell function. In contrast to MEKK3, mice that lack MEKK2 do not display gross morphological defects

apart from an increased proliferation in T cells upon activation (Guo et al., 2002). Together these studies indicate that while MEKK3 is indispensable for normal development, MEKK2 is a redundant protein whose ablation can be compensated by another MAPK kinase kinase.

Overall, the similarity of the phenotypic abnormalities displayed by the *erk5-/-*, *mef2c-/-*, and *mekk3-/-* embryos provided strong genetic evidence that MEKK3, ERK5, and MEF2C operate in a common pathway. Further investigation was required to examine whether MEK5 was the protein kinase responsible for conveying the active signal from MEKK3 to ERK5. The first in vivo study showed that over-expression of a constitutively active mutant form of MEK5 driven by the myosin light chainα promoter resulted in an enlargement of the heart with a progressive dilation and thinning of the ventricular wall by 6 weeks of age (Nicol et al., 2001). Most transgenic animals survived no longer than 12 weeks. This was consistent with in vitro studies indicating that MEK5 regulated the organisation of sarcomeric proteins of the cardiac muscle cells, and was required for vascular smooth muscle proliferation and differentiation of myoblasts into myotubes via ERK5 (Dinev et al., 2001; Zhao et al., 2002).

To elucidate the physiological significance of MEK5, I examined the effect of *mek5* gene ablation in mice. The MEK5 null mouse model was engineered by inserting a LacZ-Neomycin targeting cassette in frame into exon 2 of the *mek5* gene. MEK5 deficient mice died at E10.5 with cardiovascular defects reminiscent of the phenotypic abnormalities observed in the *erk5-/-* embryos. In addition to the heart, the head and the dorsal regions of mutants exhibited a marked decrease in proliferation and an increase in apoptosis compared to the wild type littermates. Furthermore, I also found that the

absence of MEK5 prevented MEKK2 and MEKK3 from increasing the transcriptional activity of MEF2A and MEF2D. Overall, these results provide the first genetic evidence that MEK5 is the sole upstream activator of ERK5, and is a non-redundant component of the ERK5/MEF2 dependent cell survival pathway that is required for early embryonic development.

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Targeted Deletion of *mek5* Causes Early Embryonic Death and Defects in the Extracellular Signal-Regulated Kinase 5/Myocyte Enhancer Factor 2 Cell Survival Pathway

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To elucidate the physiological significance of MEK5 in vivo, we have examined the effect of *mek5* gene elimination in mice. Heterozygous mice appear to be healthy and were fertile. However, *mek5*$^{-/-}$ embryos die at approximately embryonic day 10.5 (E10.5). The phenotype of the *mek5*$^{-/-}$ embryos includes abnormal cardiac development as well as a marked decrease in proliferation and an increase in apoptosis in the heart, head, and dorsal regions of the mutant embryos. The absence of MEK5 does not affect cell cycle progression but sensitizes mouse embryonic fibroblasts (MEFs) to the ability of sorbitol to enhance caspase 3 activity. Further studies with *mek5*$^{-/-}$ MEFs indicate that MEK5 is required for mediating extracellular signal-regulated kinase 5 (ERK5) activation and for the regulation of the transcriptional activity of myocyte enhancer factor 2. Overall, this is the first study to rigorously establish the role of MEK5 in vivo as an activator of ERK5 and as an essential regulator of cell survival that is required for normal embryonic development.

The mitogen-activated protein kinase (MAPK) cascades constitute a complex network of signaling pathways that are involved in the regulation of numerous cell functions (9). They consist of the sequential activation of protein kinases that include MAPKs, MAPK/extracellular signal-regulated kinase (ERK) kinases (MEKs or MKKs), and MEK kinases (MEKKs) (9). MAPKs are activated by dual phosphorylation on threonine (T) and tyrosine (Y) residues within a T-X-Y motif by MEKs. MEKs are activated by MEKKs. Two main mechanisms have been proposed to ensure specific transmission of the signals from upstream kinases to MAPKs (38, 40): (i) scaffold proteins that assemble the different components of a cascade; (ii) physical interactions between the components of a cascade. Both mechanisms may operate in parallel and allow different responses of the same MAPK signaling pathways to different stimuli.

At least four MAPK subfamilies have been identified: ERK1/2, ERK5, c-Jun NH_2-terminal protein kinases (JNKs), and p38 MAPKs. MAPK activators include MEK1 and MEK2 for ERK1/2, MEK5 for ERK5, MKK4 and MKK7 for JNKs, and MKK3 and MKK6 for p38 MAPKs (9). Targeted deletion of the *mapk* and *mek/mkk* genes has contributed substantially to our increased understanding of the physiological role of these pathways in development and pathogenesis. In particular, the recent elimination of the *erk5* gene in mice has provided genetic evidence that ERK5 is required for normal cardiac development (31, 34, 42).

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ERK5, also known as big MAPK, is almost twice the size (815 amino acids) of the other MAPKs (45). Its unique COOH-terminal tail contains a myocyte enhancer factor 2 (MEF2)-interacting domain and a potent transcriptional activation domain (12). The ERK5 catalytic NH_2-terminal domain is 50% identical to ERK2. The activity of a number of transcription factors has been shown to be regulated by ERK5, including MEF2, c-Fos and Fra-1, Sap1, c-Myc, and NF-κB (6, 11, 13, 15, 28, 37). In vitro, the ERK5 signaling pathway has been implicated in MEF2-dependent gene expression during muscle differentiation and neuronal survival (4, 20, 33). The signaling cascade that leads to ERK5 activation is stimulated in response to mitogens and a number of stresses (1, 11, 14, 39, 41).

In vitro protein kinase assays and transfection studies with constitutively activated MEK5 have demonstrated that MEK5 is a potent activator of ERK5 (5, 45). The MEK5 cDNA encodes a 444-amino-acid protein, which displays more than 50% homology with the other known MEKs. Two alternative splice variants encoding two isoforms, MEK5α (50 kDa) and MEK5β (40 kDa), have been identified (5). MEK5β is ubiquitously distributed and primarily cytosolic while MEK5α is expressed mostly in liver and brain and is in the particulate fraction. MEK5 activity is regulated by MEKK2 and MEKK3 (2, 35). Consistent with the abnormal phenotype displayed by the *erk5*$^{-/-}$ mice, transgenic mice overexpressing activated MEK5 in the heart display eccentric cardiac hypertrophy that progresses to dilated cardiomyopathy and sudden death (26).

The in vivo role of MEK5 has not been rigorously established, mainly because of the lack of available pharmacological and genetic reagents that specifically alter MEK5 activity (24). Therefore, we have engineered a novel genetically modified

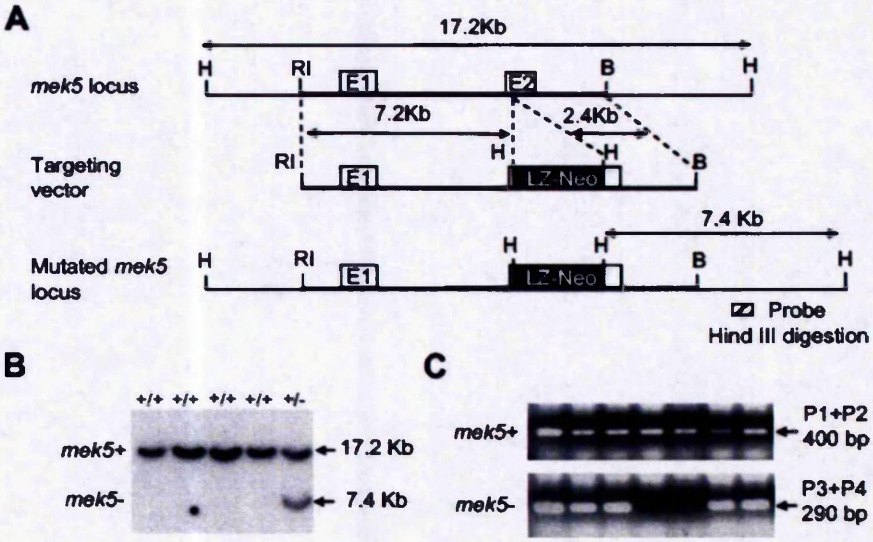

FIG. 1. Strategy for the targeted disruption of the *mek5* gene. (A) The genomic region at the *mek5* locus, the *mek5* targeting vector, and the predicted structure of the mutated *mek5* gene are depicted. Restriction enzyme sites are indicated (B, BamH1; RI, EcoRI; H, HindIII). White boxes are *mek5* exons. The black box is the β-Gal neomycin cassette (LZ-Neo). (B) Southern blotting analysis of HindIII-restricted genomic DNA prepared from ES cell clones indicates the presence of wild-type (+/+) and heterozygous (+/−) genotypes. The blot was probed with a random-primed ^{32}P-labeled mouse MEK5 genomic probe (see hatched box in panel A). (C) Genomic DNAs isolated from mouse tails were amplified by two separate PCRs with primers specific for the *mek5* gene (P1 + P2) and for the LZ-Neo cassette (P3 + P4).

mouse model deficient in MEK5 expression. Our results show that the targeted deletion of the *mek5* gene causes early embryonic death. Embryonic day 10.5 (E10.5) *mek5*$^{-/-}$ embryos display cardiovascular defects suggesting that, like ERK5, MEK5 is required for normal cardiac development. In addition to the heart, the head and dorsal regions of the mutant embryos exhibit a marked decrease in proliferation and an increase in apoptosis compared to wild-type littermates. Consistent with these results, *mek5*$^{-/-}$ fibroblasts are more sensitive than wild-type cells to sorbitol-induced caspase 3 activity. However, no marked difference was observed in the cell cycle progression of the wild-type and mutant cells. Further studies demonstrate that MEK5 is required for mediating ERK5 activation in response to both mitogenic and stress signals. Moreover, the absence of MEK5 expression prevents the MEK kinases MEKK2 and MEKK3 from increasing the transcriptional activity of MEF2A and MEF2D, two well-characterized downstream substrates of ERK5 (15). Altogether, these results provide clear genetic evidence that MEK5 is a nonredundant component of the ERK5/MEF2-dependent cell survival pathway that is required for mediating normal cardiac development.

MATERIALS AND METHODS

Generation of *mek5*$^{-/-}$ mice. The sequence of the *mek5* gene locus was obtained from GenBank (accession no. AC124753). Gene analysis revealed that the *mek5* gene is encoded by at least 16 exons and 15 introns spanning ~120 kb. MEK5 genomic DNA was cloned from a 129/Sv mouse strain-derived genomic RPCI-21 PAC library (United Kingdom HGMP Resource Centre) with a MEK5 cDNA probe. A 9.6-kb EcoRI-BamHI genomic fragment encompassing exons 1 and 2 of the *mek5* gene was subcloned into pBluescript II KS vector (Stratagene). A β-galactosidase (β-Gal) and neomycin resistance (LZ-Neo) cassette containing a stop codon and a polyadenylation termination signal was inserted in frame into exon 2 by using an engineered HindIII restriction site. This gave rise to a targeting vector comprising 7.2-kb EcoRI-HindIII and 2.4-kb HindIII-BamHI fragments of MEK5 homologous sequences at its 5′ and 3′ extremities, respectively (Fig. 1). The resulting plasmid (50 μg) was linearized with NotI and electroporated into R1 embryonic stem (ES) cells (kindly provided by Andras Nagy, Samuel Lunenfeld Research Centre, Mount Sinai Hospital, Toronto, Canada). Neomycin-resistant clones selected with 500 μg of G418 (Invitrogen)/ml were screened by Southern blotting. Homologous recombined ES cell clones were injected into C56BL/6 blastocysts and transferred into pseudopregnant CD1 females to generate chimeras. Resulting high-grade agouti-marked male chimeras were mated with C57BL/6 females for germ line transmission of the mutation. All mice employed for this study were hosted in a pathogen-free facility at the University of Manchester. The animal studies were carried out according to Home Office and Institutional guidelines.

Southern blot analysis. Genomic DNA prepared from ES cells by standard protocols (36) was digested with HindIII, analyzed by electrophoresis, blotted onto a nylon membrane, and hybridized with a radiolabeled 600-bp genomic fragment located outside the 3′ region of the targeting vector (Fig. 1). This probe hybridizes to a 7.4-kb fragment (disrupted allele) and to a 17.2-kb fragment (endogenous *mek5* gene).

Genotype determination of mice and embryos. *mek5*$^{+/-}$ offspring were identified by two separate PCRs on tail DNA. The wild-type allele was amplified with forward (P1, 5′-GCTCATGTTTCTGTG-3′) and reverse (P2, 5′-TGTGCCGT ATGATGATC-3′) primers. The mutant allele was amplified with a neomycin-specific primer set (P3, 5′-CTTGGGTGGAGAGGCTATTATTC-3′; P4, 5′-AG GTGAGATGACAGGAGATC-3′). Genotype determination of embryos was performed by PCR of genomic DNA isolated from the yolk sacs by using a three-primer set (forward primer 1, 5′-ATCAGAATGAGGCTCAGG-3′; forward primer 2, 5′-GCGCATCGCCTTCTATCG-3′; reverse primer, 5′-TGTGC CGTATGATGATC-3′). Fragments (600 and 700 bp) were amplified from the wild-type and disrupted alleles, respectively.

Tissue culture and preparation of lysates. Mouse embryonic fibroblasts (MEFs) obtained from wild type and *mek5*$^{-/-}$ E9.5 embryos were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 10 U of penicillin/ml, 100 mg of streptomycin/ml, and 50 μM 2-mercaptoethanol at 37°C in a humidified atmosphere with 5% CO_2. Transfection assays were performed by using the Lipofectamine method according to the manufacturer's recommendations (Invitrogen).

Proteins were extracted from cells or tissues in Triton lysis buffer (TLB; 20 mM Tris [pH 7.4], 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 10% glycerol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin/ml, 10 μg of aprotinin/ml). Extracts were clarified by

centrifugation (14,000 × *g* for 10 min at 4°C). The concentration of soluble proteins in the supernatants was quantified by the Bradford method (Bio-Rad).

Immunoblot analysis. Cell and tissue extracts (50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% or 8% polyacrylamide gel) and electrophoretically transferred to an Immobilon-P membrane (Millipore, Inc.). The membranes were incubated with 5% nonfat dry milk or 3% bovine serum albumin at 4°C overnight and then probed with polyclonal antibodies to ERK5, tubulin (Sigma), β-Gal (Promega), or MEK5. The antibodies to ERK5 and MEK5 were obtained by immunizing rabbits against the synthetic peptides SGPPPPDPGGLTPQPST and NEQDIRYRDTLGHGN, corresponding to amino acids 680 to 694 and 162 to 176 of the ERK5 and the MEK5 proteins, respectively (Eurogentec). Immune complexes were detected by enhanced chemiluminescence (Pierce) with rabbit anti-mouse immunoglobulin G coupled to horseradish peroxidase as the secondary antibody (Amersham-Pharmacia).

Protein kinase assay. JNK, ERK1/2, and ERK5 protein kinase activity was measured in cell lysates following incubation with glutathione *S*-transferase (GST)–c-Jun and glutathione-Sepharose beads or with polyclonal antibodies to ERK1/2 or ERK5 and protein A-agarose beads for 2 to 3 h at 4°C, respectively. Complexes were washed three times with TLB and twice with kinase buffer (25 mM HEPES [pH 7.4], 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.1% orthovanadate) prior to being incubated at 30°C for 20 min in kinase buffer containing 50 μM [γ-^{32}P]ATP (10 Ci/mmol) and 1 μg of GST-Myc or GST-MEF2C for the ERK1/2 and ERK5 assays, respectively. SB203580 (1 μM; Promega) was added to the ERK5 assay to prevent the phosphorylation of MEF2C by p38 MAPKs. The reactions were terminated by the addition of Laemmli sample buffer. Proteins were resolved by SDS-PAGE (12% polyacrylamide gel) and identified by autoradiography. The incorporation of [^{32}P]phosphate was quantitated by PhosphorImager analysis.

Reporter gene expression assay. The reporter plasmid pG5E1bLuc (32) was transiently cotransfected together with a construct encoding the fusion proteins GAL4-MEF2A, GAL4-MEF2D, and GAL4-cJun (10) with or without expression vectors encoding MEKK2 or MEKK3. A pRL-Tk plasmid encoding *Renilla* luciferase was employed for monitoring transfection efficiency. Aliquots of cell lysates were assayed for firefly and *Renilla* luciferase activities according to the manufacturer's instructions (Promega). GAL4-MEF2A and GAL4-MEF2D and MEKK2 and MEKK3 constructs were kindly provided by Hung-Ying Kao (Case Western University, Cleveland, Ohio) and Christian Widmann (Université de Lausanne, Lausanne, Switzerland), respectively.

Histological and immunohistochemistry analysis. Freshly isolated embryos and placentas were fixed at room temperature either in Bouin's fluid overnight or in 4% paraformaldehyde for 2 h, dehydrated, and embedded in paraffin. Four- to six-micrometer-thick sections were cut. For histological analysis, sections were stained with hematoxylin and eosin (17). For β-Gal staining, sections were analyzed by the ABC peroxidase method (Vector) with a primary polyclonal antibody to β-Gal (1:500 dilution; Promega) and a secondary biotinylated anti-rabbit antibody (1:200 dilution; Vector). For bromodeoxyuridine (BrdU) staining, pregnant female mice were injected with 400 mg of BrdU/kg of body weight and sacrificed 1.5 h later prior to analysis. Immunohistochemistry was performed by the boric acid buffer method (30), with the exception that the rat monoclonal antibody to BrdU was obtained from Immunologicals Direct, Oxford Biotechnology Ltd. For terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining, sections were processed by using an in situ cell death detection kit (POD; Roche) according to the manufacturer's instructions.

Flow cytometry. For cell cycle analysis, 70% confluent cells were labeled with 1 μM BrdU for 30 min, collected by trypsinization, and stained with an anti-BrdU antibody (DAKO) and propidium iodide as previously described (18). For apoptosis assay, 70% confluent MEFs were collected by trypsinization. Caspase 3 activity was measured by using the carboxyfluorescein FLICA apoptosis detection kit (Immunochemistry Technologies) according to the manufacturer's instructions. Analysis was performed on a Becton Dickinson fluorescence-activated cell sorter vantage model with the CellQuest program.

Reverse transcription (RT)-PCR. Total RNA was isolated from pools of E9.5 hearts by using the Trizol reagent as instructed by the manufacturer. RNA concentration and quality were assessed visually by ethidium bromide-agarose gel electrophoresis (1%) under UV illumination and comparison with known amounts of mouse embryonic total RNA (Ambion). cDNA synthesis was carried out in a final volume of 20 μl of first-strand buffer (Invitrogen) containing around 3 μg of total RNA, 20 U of SuperScript II reverse transcriptase (Invitrogen), 0.025 μg of oligo(dT)/μl, and 0.5 mM concentrations of deoxynucleoside triphosphates. The primers used were as follows: *cripto* forward primer, 5′-GCTGTCTGAATGGAGG-3′; *cripto* reverse primer, 5′-AAGGCAGGCGCCAGCTAG-3′; *champ* forward primer, 5′-TCCTTCTGCGAAATGTG-3′; *champ* reverse primer, 5′-GAGAGCCTGGAG TTCAG-3′; β-*actin* forward primer, 5′-CCAACTTGATGTATGAAGGCTTTG-3′; β-*actin* reverse primer, 5′-GCCTGTACACTGACTTGAGACCAAT-3′. The other primers have been described previously (22). PCR cycles were determined under conditions of linearity by sampling the reactions from 20 to 30 cycles for each primer set. PCR products were visualized by ethidium bromide-agarose gel electrophoresis (1.2%) under UV illumination.

TABLE 1. Genotyping analysis of multiple litters of *mek5*$^{+/-}$ intercrosses

Time of gestation	% (no.) of mice with genotype[a]:			Total no. of mice
	+/+	+/−	−/−	
E9.5	23 (77)	51 (174)	26 (88)	339
E10.5	34 (45)	50 (67)	16 (21)	133
E11.5	36 (8)	59 (13)	5 (1)	22
E13.5	42 (8)	58 (11)	0 (0)	19
Adult	35 (131)	65 (241)	0 (0)	372

[a] Percentages were determined by PCR. As determined by observing heart beating, 4 of 21 homozygous (−/−) E10.5 embryos were viable. No *mek5*$^{-/-}$ embryos survived after 11.5 days.

RESULTS

Targeted deletion of *mek5* gene causes early embryonic death. To make the MEK5 mutant mice, a targeting vector was designed to modify the *mek5* gene by homologous recombination in ES cells so that exon 2 was interrupted by a neomycin resistance cassette (Fig. 1A). The linearized construct was electroporated into ES cells. The 180 colonies that survived the standard Geneticin (G418) selection were analyzed for homologous recombination by Southern blotting. An example of 1 positive clone is shown in Fig. 1B. Homologous recombination occurred with a frequency of 10%. Chimeric mice were generated from ES cells possessing the targeted allele. These mice were subsequently bred onto the C57BL/6 background to generate heterozygous *mek5*$^{+/-}$ animals. The genotyping of the mice was performed by two separate PCRs on the genomic DNA extracted from tail snips with specific primers (Fig. 1C).

mek5$^{+/-}$ mice appeared to be healthy and were fertile. Genotyping analysis of multiple litters of *mek5*$^{+/-}$ intercrosses at 3 weeks after birth indicated that no *mek5*$^{-/-}$ mice were born (Table 1). To determine the age at which *mek5*$^{-/-}$ embryos die, we performed a timed mating analysis. Our experiments clearly demonstrated that up to E9.5 *mek5*$^{+/+}$, *mek5*$^{+/-}$, and *mek5*$^{-/-}$ fetuses were present at the expected Mendelian ratios (Table 1). However, the proportion of *mek5*$^{-/-}$ embryos started to decline around E10.5, and none could be found beyond E11.5. E10.5 *mek5*$^{-/-}$ embryos appeared abnormal (Fig. 2A). They were smaller than their wild-type or heterozygous counterparts and displayed retarded development of the head and limbs, and many exhibited dilated pericardial sacs. This abnormal phenotype was strikingly similar to the one described for *erk5*$^{-/-}$ embryos (31, 34, 42). No marked difference in the extent of vascular invasion and in the general architecture of the placenta was observed between E9.5 and E10.5 wild-type and mutant embryos (Fig. 2B and C). We concluded that a placental defect was unlikely to be the cause for the early lethality displayed by the *mek5*$^{-/-}$ embryos. This is consistent with studies that report that the phenotype of the *erk5*$^{-/-}$ embryos is manifest prior to placental abnormality (34,

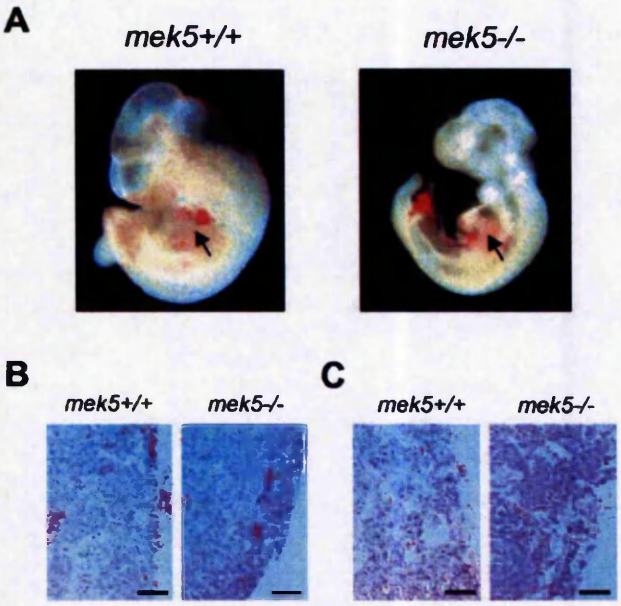

FIG. 2. Analysis of MEK5-deficient embryos. (A) A lateral view of freshly isolated wild-type (*mek5*$^{+/+}$) and homozygous (*mek5*$^{-/-}$) E10.5 embryos taken under a light microscope demonstrates retarded development of the head, limbs, and heart in mutant embryos. Arrows point to the heart regions. (B and C) Hematoxylin and eosin staining of sagittal sections of E9.5 (B) and E10.5 (C) *mek5*$^{+/+}$ and *mek5*$^{-/-}$ placentas. Scale bar, 50 μm.

42). Together, these results indicate that MEK5 is required for normal early embryonic development. The phenotypic similarity with the *erk5*$^{-/-}$ mice suggests that MEK5 is a critical component of the ERK5 signaling pathway.

Tissue-specific expression of MEK5 protein in embryos and adult mice. To shed some light on the embryonic lethality caused by the deletion of the *mek5* gene and thus increase our knowledge of the biological function of the MEK5 signaling pathway, we examined the pattern of MEK5 protein expression in mice. The targeting vector was designed so that the *lacZ* gene present in the neomycin cassette inserted in exon 2 was in frame with the starting codon of the *mek5* gene. This strategy allowed expression of β-Gal in place of MEK5 in cells where the homologous recombination event had occurred (Fig. 3). β-Gal staining of mutant fetuses demonstrated that MEK5 was specifically expressed in the heart and in two areas in the tip of the tail of E9.5 embryos (Fig. 3A). The areas in the tail region seem to correspond to embryonic blood vessels. A control experiment showed no β-Gal staining of wild-type embryos (data not shown). The level and distribution of MEK5 protein expression in adult mice was assessed by immunoblot analysis of tissues extracted from wild type (+/+) and heterozygous (+/−) mice. The membrane was stained with India ink to monitor protein loading. The experiment showed that MEK5 expression followed by β-Gal expression in +/− tissues was ubiquitous, with the highest levels in the heart, skeletal muscle, and brain tissue (Fig. 3B). One protein with a similar pattern of expression and an apparent molecular weight of 50 kDa was detected with the anti-MEK5 antibody in wild-type extracts (data not shown). The anti-MEK5 antibody did not detect the lowest-molecular-mass MEK5β isoform (40 kDa) that has previously been detected in rat tissues (5). The reason is not yet obvious but could be due to the different methods employed to prepare the extracts. Together with the dilated pericardial sacs exhibited by the *mek5*$^{-/-}$ embryos, the specific and high levels of expression of MEK5 in both embryonic and adult hearts led us to investigate the effect of MEK5 deletion on cardiac development.

***mek5*$^{-/-}$ embryos display abnormal development of the heart.** No marked morphological differences were observed between the hearts of wild-type and *mek5*$^{-/-}$ embryos up to

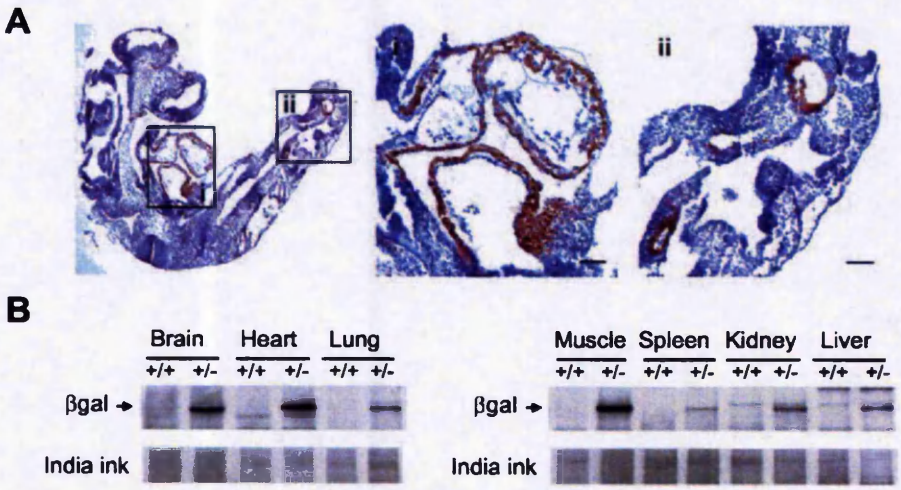

FIG. 3. Expression of MEK5 in embryos and adult tissues. (A) Whole-mount immunohistochemistry to β-Gal in an E9.5 *mek5*$^{-/-}$ embryo demonstrates the specific expression of MEK5 at this early stage of development. Enlarged views of the heart (i) and the tips of the tails (ii) are shown. Scale bars, 50 μm. (B) MEK5 expression is highest in brain, heart, and skeletal muscle tissue. Homozygous (+/+) and heterozygous (+/−) adult mouse tissue extracts (50 μg) were analyzed for β-Gal expression by immunoblot analysis with a specific polyclonal antibody to β-Gal. Protein loading was monitored by India ink staining.

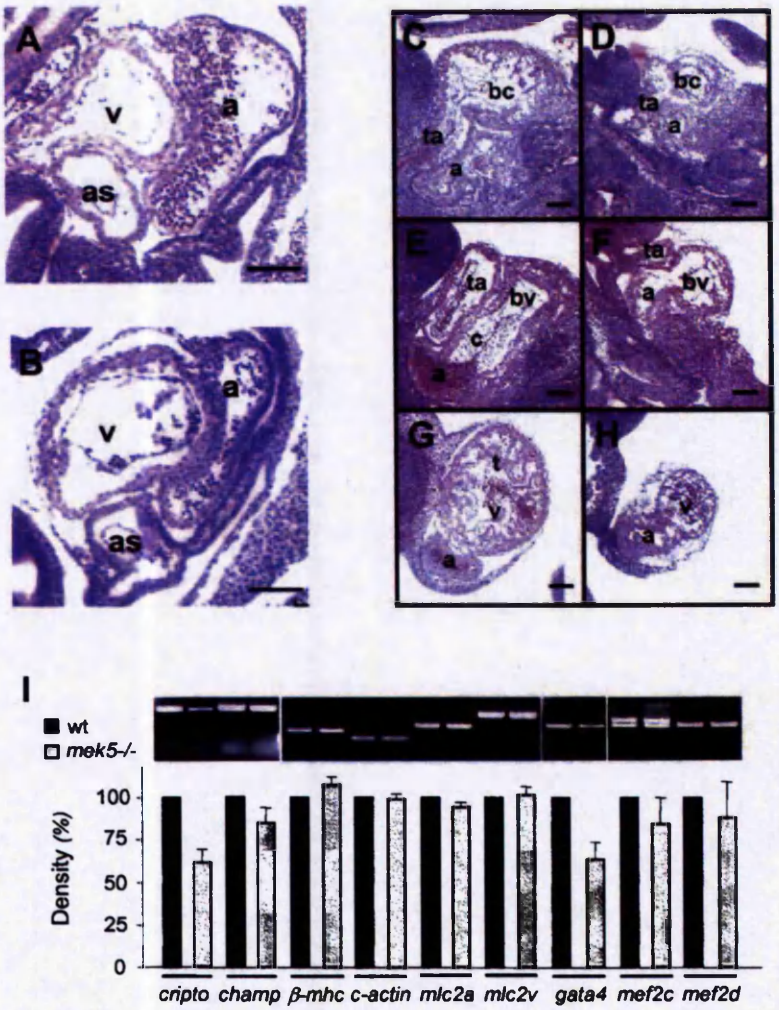

FIG. 4. *mek5*$^{-/-}$ embryos display abnormal development of the heart. Hematoxylin and eosin staining of sagittal sections of E9.5 *mek5*$^{+/+}$ (A) and *mek5*$^{-/-}$ (B) and E10.5 *mek5*$^{+/+}$ (C, E, and G) and *mek5*$^{-/-}$ (D, F, and H) embryos. The spiral septum of the outflow tract displays little or no septal development in *mek5*$^{-/-}$ embryos (D) compared to wild-type embryos (C). The mutant heart displays little evidence of endocardial cushion tissue formation in the atrioventricular canal (compare panels E and F). The trabeculation of both the bulbus cordis and the common ventricular chamber is highly disorganized in *mek5*$^{-/-}$ embryos (D and H) compared to wild-type embryos (C and G). Abbreviations: a, common atrial chamber; as, aortic sac; bc, bulbus cordis; bv, bulbo-ventricular canal; c, cushion tissue; t, trabeculae; ta, truncus arteriosus; v, common ventricular chamber. Scale bars, 10 μm. (I) Semiquantitative RT-PCR analysis of cardiac-related transcripts expressed in wild type (+/+) and mutant (−/−) heart embryos. PCR amplifications were performed with cDNAs synthesized from RNA isolated from E9.5 embryonic hearts. PCR products were quantitated under UV illumination. The results normalized to the levels of amplification of β-actin are expressed as the percentage of expression of the gene in wild-type heart embryos. The data correspond to the means ± standard errors of two independent experiments. Abbreviations are as follows: β-*mhc*, β-*myosin heavy chain*; *c-actin*, *cardiac* α-*actin*; *mlc2*, *myosin light chain 2*.

E9.5 (Fig. 4A and B). In contrast, E10.5 *mek5*$^{-/-}$ embryos displayed striking cardiovascular defects. Histological analysis showed that, at this stage in embryonic development, the hearts of *mek5*$^{-/-}$ embryos were clearly less well developed than those in their wild-type littermates; they showed severely retarded growth, a lack of septal development, and disrupted trabecula formation (Fig. 4C to H). The spiral septum of the outflow tract, which will form the aortic and pulmonary trunks, were clearly developing in wild-type hearts (Fig. 4C) while *mek5*$^{-/-}$ embryos displayed little or no septal development in this region of the heart (Fig. 4D). There was also little evidence of the formation of the endocardial cushion tissue in the atrioventricular canal compared to the well-defined endocardial cushions in this region in wild-type littermates (Fig. 4E and F). By E10.5, trabeculation of both the bulbus cordis and the common ventricular chamber was well organized in wild-type mice (Fig. 4C and G) but highly disorganized in *mek5*$^{-/-}$ mice (Fig. 4D and H).

To identify the molecular mechanism by which MEK5 regulates cardiac development, we compared the levels of expression of a number of cardiac structural transcripts including the MEF2 target genes *cripto*, *champ*, *cardiac* α-*actin* (*c-actin*), and *myosin light chain 2* (*mlc2*) in wild-type and mutant E9.5 hearts (19, 21). The expression of the transcription factors GATA4

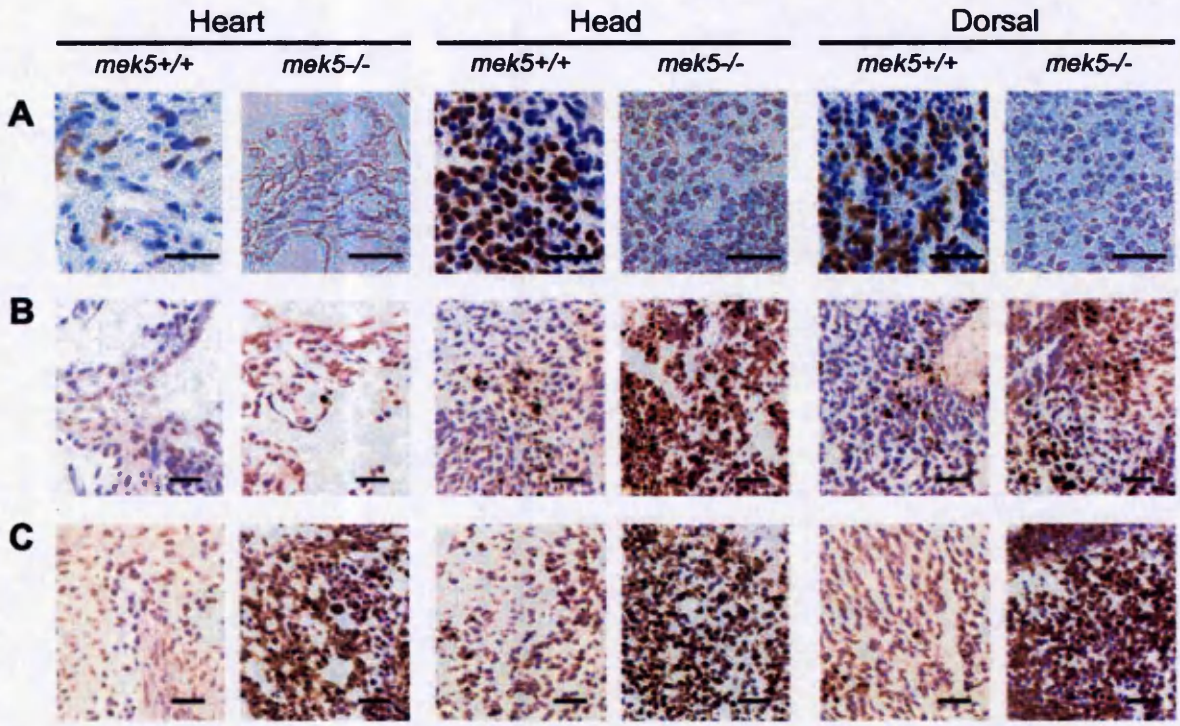

FIG. 5. *mek5*$^{-/-}$ embryos exhibit defects in cell proliferation and cell death. Sections of the heart, head, and dorsal regions stained with BrdU (A) reveal decreased cell proliferation in homozygous (*mek5*$^{-/-}$) compared to wild-type (*mek5*$^{+/+}$) E10.5 embryos. TUNEL staining indicates increased apoptosis in the heart, head, and dorsal regions of the *mek5*$^{-/-}$ E9.5 (B) and E10.5 (C) embryos. Scale bars, 25 μm.

and MEF2C that are required for normal heart development was also investigated (19, 25). Consistent with the *erk5*$^{-/-}$ (31) phenotype, RT-PCR analysis revealed a decrease of *cripto* (40%) and, to a lesser extent, of *champ* (15%) expression in the *mek5*$^{-/-}$ hearts compared with the *mek5*$^{+/+}$ hearts (Fig. 4I). Absence of MEK5 expression also significantly inhibited (35%) the levels of the *gata4* transcript. In contrast, MEK5 was not required for normal expression of the β-*myosin heavy chain* (β-*mhc*), *c-actin*, *mlc2a*, *mlc2v*, *mef2c*, or *mef2d* (Fig. 4I).

These results indicate that MEK5 is required for normal cardiac development. Similar cardiovascular defects displayed by the *erk5*$^{-/-}$ mice suggest that the physiological role of MEK5 is mediated by its ability to regulate ERK5 activity. The functional consequence of the defect in *cripto*, *champ*, and *gata4* expression in the *mek5*$^{-/-}$ embryos remains to be identified.

Effect of MEK5 deletion on cell growth and cell survival. Consistent with its role in stimulating early gene expression through the regulation of MEF2 and AP-1 activity, ERK5 has been shown to contribute to cell proliferation and the survival response of neurons in the brain (16, 20, 33, 39). We examined whether MEK5 was required for mediating these biological processes in vivo. Freshly collected E9.5 and E10.5 embryos were processed for immunohistochemistry to detect cell proliferation by BrdU incorporation (Fig. 5A) or DNA fragmentation, one of the hallmarks of late stage apoptosis, by TUNEL staining (Fig. 5B and C). The heart, head, and dorsal regions of the mutant E10.5 embryos exhibited a marked decrease in proliferation and an increase in apoptosis compared to their wild-type littermates (Fig. 5A and C). Increased apoptosis in *mek5*$^{-/-}$ embryos was observed as early as E9.5, suggesting that the proliferation defect may be a consequence of abnormal cell survival in the mutant fetuses (Fig. 5B). To further test this hypothesis, we examined the effect of MEK5 deletion on the ability of MEFs to progress through the cell cycle and to undergo apoptosis (Fig. 6).

Immunoblot analysis using an anti-MEK5 antibody confirmed the absence of MEK5 expression in homozygous MEFs (Fig. 6A). The level of ERK5 expression was not significantly affected by the targeted deletion of the *mek5* gene. The results showed that following 48 h of starvation, the readdition of 10% serum for 24 h stimulated the reentry of *mek5*$^{-/-}$ MEFs into the cell cycle similar to the wild-type cells (Fig. 6B). In contrast, *mek5*$^{-/-}$ MEFs exhibited increased levels of sorbitol-induced caspase 3 activity compared to wild-type cells (Fig. 6C). The protective role of MEK5 against the toxic effect of sorbitol was specific, since the absence of MEK5 expression did not significantly affect the ability of UV radiation to activate caspase 3 (Fig. 6C). UV does not activate ERK5 in MEFs (data not shown). These data confirm our hypothesis that MEK5 is not essential for normal cell cycle progression but is a critical component of the survival signaling pathways in mitotic cells.

MEK5 is required for mediating ERK5 activation and MEF2-dependent transcription. In vitro protein kinase assays and transfection studies with a constitutively activated mutant of MEK5 have demonstrated that MEK5 is a potent activator of ERK5 (45). However, the in vivo role of MEK5 in the ERK5

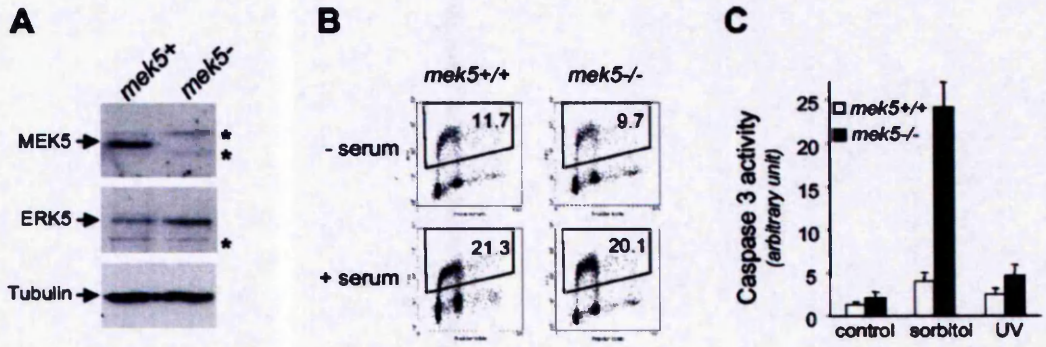

FIG. 6. MEK5 is implicated in cell survival. (A) MEF extracts (50 μg) were analyzed for MEK5 and ERK5 expression by immunoblot analysis with specific polyclonal anti-MEK5 and anti-ERK5 antibodies. The detection of tubulin expression was performed to monitor protein loading. Asterisks indicate nonspecific bands. (B) Seventy percent confluent MEFs were serum starved for 48 h prior to being stimulated with 10% fetal bovine serum for 24 h. Cell proliferation was assessed by fluorescence-activated cell sorter analysis to follow BrdU incorporation. The percentage of fibroblasts present in the S phase of the cell cycle is indicated. The figure shows data representative of the results from three independent experiments. − serum, without serum; + serum, with serum. (C) Wild-type and *mek5*$^{-/-}$ fibroblasts were incubated for 6 h with 500 mM sorbitol or exposed to UV radiation (60 J/m^2) followed by incubation for 16 h. Caspase 3 activity was monitored by using the carboxyfluorescein FLICA apoptosis detection kit. The data, expressed as units of fluorescence, correspond to the means ± standard errors of the results from two independent experiments.

signaling pathway needs to be further established, as additional ERK5 activators may exist. To determine the physiological significance of MEK5 as an ERK5 activator, we examined the effect of *mek5* gene elimination on ERK5 activation in MEFs in response to mitogenic and stress stimuli (Fig. 7). Epidermal growth factor (EGF) and sorbitol treatment caused a marked increase in ERK5 protein kinase activity in wild-type but not in *mek5*$^{-/-}$ MEFs (Fig. 7A and B). A control experiment showed that the residual phosphorylation of GST-MEF2C in knockout *mek5* fibroblast extracts was independent of the presence of the anti-ERK5 antibody in the protein kinase assay. Consistent with the absence of ERK5 activation in the *mek5*$^{-/-}$ MEFs, immunoblot analysis of the immune complexes demonstrated that MEK5 was required for mediating ERK5 phosphorylation. Wild-type extracts displayed retarded migration of ERK5 following SDS-PAGE analysis, and this was absent from homozygous knockout *mek5* extracts (Fig. 7A and B). Control experiments showed similar activation of ERK1/2 (Fig. 7C) and JNK (Fig. 7D) in *mek5*$^{-/-}$ compared to wild-type MEFs in response to EGF and sorbitol treatment, respectively.

To confirm the requirement of MEK5 in regulating ERK5 protein kinase activity, we investigated the effect of MEK5 deletion on the transcriptional regulation of MEF2, which is a well-characterized substrate of ERK5 (13, 15). Fibroblasts were cotransfected with the reporter plasmid pG5E1bLuc together with constructs encoding GAL4, GAL4-MEF2A, or GAL4-MEF2D, with or without expression vectors encoding MEKK2 or MEKK3. A control experiment was performed with GAL4-cJun, since cJun is not a direct downstream substrate of ERK5. MEKK2- and MEKK3-induced MEF2A, MEF2D, and cJun transcriptional activity was determined by the luciferase reporter assay. The results demonstrated that the absence of MEK5 protein expression prevented both MEKK2 and MEKK3 from increasing the transcriptional activity of MEF2 (Fig. 7E). In contrast, no marked differences in the activation of cJun were detected under these conditions. Consistent with in vitro evidence that MEK5 is upstream of ERK5, these data provide clear genetic evidence that MEK5 is the only ERK5 activator in cells.

DISCUSSION

The lack of available pharmacological and genetic reagents that specifically alter MEK5 activity has prevented further progress into the understanding of the role of MEK5 in vivo. We have addressed this issue by engineering a novel genetically modified mouse model deficient in MEK5 expression.

The *mek5*$^{-/-}$ embryos exhibited heart defects, including the lack of septal development and disrupted trabecula formation. Similar phenotypic abnormalities displayed by the *erk5*$^{-/-}$, *mef2c*$^{-/-}$, and *mekk3*$^{-/-}$ embryos identify MEK5 as an essential physiological component of the MEKK3/ERK5/MEF2 cascade (19, 31, 34, 42, 43). In vitro, the ERK5 signaling pathway has been shown to protect endothelial cells (EC) from apoptosis by phosphorylating Bad (29). Consistent with this study, the analysis of mutant mice in which the *erk5* gene can be conditionally deleted suggests that the requirement of ERK5 for the survival of EC is responsible for the cardiovascular defect observed in *erk5*$^{-/-}$ and *mek5*$^{-/-}$ embryos (8). Therefore, decreased *cripto*, *champ*, and *gata4* expression associated with impaired differentiation of cardiomyoblasts may be a consequence rather than a cause for the abnormal development of the *mek5*$^{-/-}$ heart.

In addition to protecting EC, ERK5 has been shown to promote neuronal survival (20, 33, 39). Blocking ERK5 activation by expressing dominant-negative MEK5 increased the death of neurons that were supported only by neurotrophin stimulation of distal axons (39). Based on these studies, we hypothesized that, similar to the heart defect, the retarded development of the brain of *mek5*$^{-/-}$ embryos (Fig. 2A) was caused by increased cellular apoptosis. MEK5, like ERK5, is expressed in the embryonic brain but at a level barely detectable by β-Gal staining (20, 33, 39) (Fig. 3A). Our results confirmed that E9.5 and E10.5 *mek5*$^{-/-}$ embryos displayed a

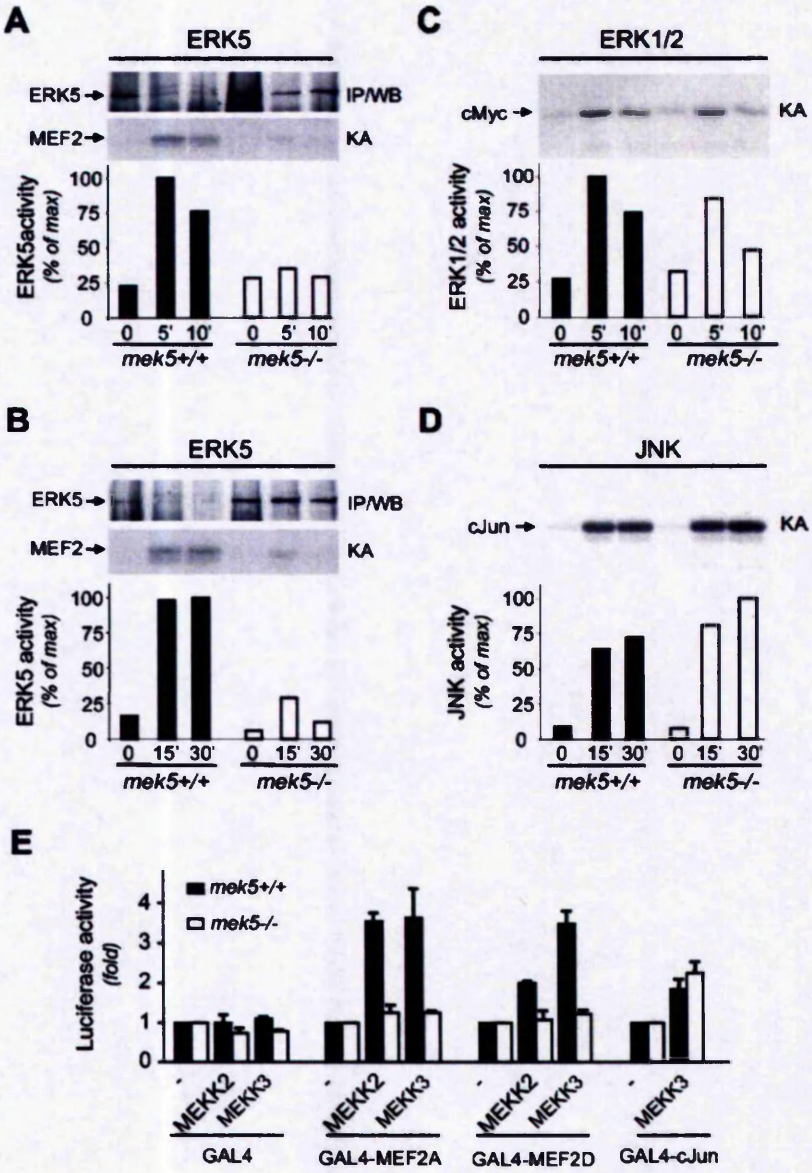

FIG. 7. Disruption of the *mek5* gene prevents ERK5 activation and the transcriptional regulation of MEF2 factors. Wild type (+/+) and homozygous knockout (−/−) *mek5* MEFs were treated with EGF (50 ng/ml) (A, C) or sorbitol (300 mM) (B, D) for the times indicated (in minutes). Endogenous ERK5 (A, B), ERK1/2 (C), and JNK (D) activity was measured by protein kinase assay (KA) in the presence of [γ-^{32}P]ATP. The radioactivity incorporated into GST-MEF2C, GST-cMyc, or GST-cJun was quantitated after SDS-PAGE by PhosphorImager analysis. The presence of ERK5 in the immune complexes was detected by immunoblot analysis (IP/WB). Data representative of the results from three independent experiments are shown. (E) Fibroblasts were transiently transfected as described in Materials and Methods. MEF2 and cJun transcriptional activity was measured by the dual-luciferase reporter assay system. Firefly luciferase activity was normalized to that of *Renilla* luciferase and expressed as change relative to the control (−). The data correspond to the means ± standard errors of three independent experiments performed in duplicate.

marked increase of cell death in the brain compared to their wild-type littermates (Fig. 5B and C). Consistent with this study, we showed that MEK5 was essential for mediating the caspase-dependent apoptotic response of mitotic cells (Fig. 6C). Analysis of a genetically modified mouse model in which the *mek5* gene can be conditionally deleted is necessary to further address in vivo the contribution of the MEK5 survival pathway for the normal development of the brain.

In contrast to apoptosis, our results showed no marked difference in the ability of *mek5*$^{-/-}$ compared to wild-type fibroblasts to progress through the S phase (Fig. 6B). This suggests that the cell proliferation defect observed in different areas of the *mek5*$^{-/-}$ embryos is a secondary effect of increased apoptosis in the mutant embryos (Fig. 5 and 6). However, it is important to emphasize that, although our study indicates that MEK5 is not a general regulator of the cell cycle, it does not

rule out the possibility that the MEK5/ERK5 signaling pathway may be important for promoting or regulating the proliferation of certain cell types, such as cancer cells. Indeed, our work is based on primary cultures of fibroblasts, but studies with immortalized cell lines have provided evidence for a role of the ERK5 signaling pathway in mediating mitogen-induced cell cycle progression (16). This conclusion supports the idea that MEK5 may be implicated in mediating the transforming effect of oncogenes such as Ras, Src, Erb2, and Cot. Consistent with a role of MEK5 in oncogenic signaling, it is reported that foci induced by a dominant active mutant of Raf (Raf-BxB) are decreased in number by disruption of MEK5 function (7). More recently, MEK5 overexpression has been demonstrated to be associated with metastatic prostate cancer (23).

The control of gene expression is one mechanism by which the ERK5 signaling pathway regulates cell function. For example, ERK5-dependent regulation of the transcriptional activity of MEF2 has been implicated in the promotion of smooth muscle cell differentiation and neuronal survival (4, 20, 33, 39). The MEF2 family belongs to the group of MADS (MCM1, agamous, deficiens, serum response factor) box transcription factors. Their transcriptional activity is regulated by phosphorylation by various protein kinases. These include the ERK5 and p38 MAPKs that phosphorylate both MEF2A and MEF2C (13, 15, 27, 44). In contrast, MEF2D is a specific substrate of ERK5 (44). Our results confirmed that MEK5 was required for regulating the transcriptional activity of MEF2D (Fig. 7E). They also demonstrated that, although both ERK5 and p38 MAPK regulate MEF2A activity, expression of p38 MAPK was not sufficient to compensate for the inability of the MEK kinases MEKK2 and MEKK3 to increase MEF2A activity in *mek5*$^{-/-}$ fibroblasts. MEKK2 and MEKK3 have both been shown to increase p38 MAPK activity in cells (3). Together, these data indicate that the requirement of the ERK5 and p38 MAPK signaling pathways in the regulation of gene expression via MEF2 factors may be cell type specific. The functional consequence of MEF2 regulation by the MEK5/ERK5 cascade during myogenesis and brain development is yet to be determined.

Overall, our results provide the first genetic evidence that MEK5 is a critical component of the ERK5/MEF2 signaling pathway that is required for mediating normal heart development. Disruption of the *mek5* gene causes increased cell death in embryos and fibroblasts, indicating that MEK5 is required for the regulation of cell survival.

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III - Manuscript 2: Selective regulation of *c-jun* gene expression by mitogen-activated protein kinases via the 12-*O*-Tetradecanoylphorbol-13-Acetate-responsive element and myocyte enhancer factor 2 binding sites

The transcription factors of the myocyte enhancer factor (MEF) family, MEF2A, C and D are among the best-characterised substrates of ERK5 (Kato et al., 1997; Kato et al., 2000; Yang et al., 1998). The phosphorylation of MEF2C by ERK5 in response to serum enhances its transcriptional activity, and subsequently leads to increased *c-jun* gene expression (Kato et al., 1997). c-Jun is a critical component of the transcription factors AP-1 that consist of homo- or hetero-dimers of basic region-leucine zipper (bZIP) proteins that belong to the Jun, Fos, ATF, and Maf sub-families (Angel et al.,1987; Angel and Karin, 1991). bZIP dimers recognise either 12-*O*-tetradecanoylphorbol-13-acetate (TPA)- or cAMP-response elements (TRE or CRE, respectively) in the promoter regions of numerous genes, including *c-jun* indicating that c-Jun regulates its own expression (Angel et al., 1988). In addition, the *c-jun* promoter region contains several MEF2 sites that preferentially bind MEF2A and MEF2D (Han et al., 1992; Han et al., 1995; Ornatsky et al., 1996).

The transcriptional activities of c-Jun, ATFs, and MEF2 are regulated upon phosphorylation by a wide range of protein kinases, including MAP kinases. For example, JNK is required for the phosphorylation of c-Jun at Ser63 and Ser73 (Morton et al., 2003; Pulverer et al., 1991), while JNK, p38 and ERK1/2 phosphorylate ATF2 which heterodimerises with c-Jun to interact with TRE in the *c-jun* promoter (Gupta et

al., 1995; Morton et al., 2004; Ouwens et al., 2002; Raingeau et al., 1996). MEF2A and MEF2C activities are controlled by both p38 MAPK and ERK5, whereas MEF2D is a specific substrate of ERK5 (Kato et al., 2000; Ornatsky et al., 1999; Zhao et al., 1999). However these studies are based on various cell lines, and thus are not directly comparable.

In the present study, we examined the role of ERK5 in the regulation of c-Jun expression. Consistent with previous results (Kato et al., 1997; Marinissen et al., 1999), our study demonstrated that c-Jun was a downstream target of ERK5 in response to EGF stimulation but not UV treatment. Our results provided clear evidence that the regulation of c-Jun expression by UV involved both JNK and p38, while JNK, ERK1/2, and ERK5 were required to mediate the effect of EGF. In addition, we showed that p38 negatively affects EGF-dependent regulation of c-Jun by its ability to inhibit JNK activity.

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Selective Regulation of c-*jun* Gene Expression by Mitogen-Activated Protein Kinases via the 12-*O*-Tetradecanoylphorbol-13-Acetate-Responsive Element and Myocyte Enhancer Factor 2 Binding Sites

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To further understand how the mitogen-activated protein kinase (MAPK) signaling pathways regulate AP-1 activity, we have elucidated the physiological role of these cascades in the regulation of c-*jun* gene expression. c-Jun is a crucial component of AP-1 complexes and has been shown in vitro to be a point of integration of numerous signals that can differentially affect its expression as well as its transcriptional activity. Our strategy was based on the use of (i) genetically modified fibroblasts deficient in components of the MAPK cascades and (ii) pharmacological reagents. The results demonstrate that c-Jun NH_2-terminal protein kinase (JNK) is essential for a basal level of c-Jun expression and for c-Jun phosphorylation in response to stress. In addition to JNK, p38 MAPK or ERK1/2 and ERK5 are required for mediating UV radiation- or epidermal growth factor (EGF)-induced c-Jun expression, respectively. Further studies indicate that p38 MAPK inhibits the activation of JNK in response to EGF, causing a down-regulation of c-Jun. Overall, these data provide important insights into the mechanisms that ultimately determine the function of c-Jun as a regulator of cell fate.

c-Jun is a critical component of the AP-1 transcription factors that consist of homo- or heterodimers of basic region-leucine zipper proteins that belong to the Jun, Fos, ATF, and Maf subfamilies (17). Basic region-leucine zipper dimers recognize either 12-*O*-tetradecanoylphorbol-13-acetate response elements (TRE) or cyclic AMP response elements in the promoter regions of numerous genes. The variety of dimeric complexes and the dual roles of AP-1 as a transcriptional activator and repressor of genes may explain how c-Jun regulates so many different, and sometimes opposing, cellular processes (37). For example, consistent with its role in cell proliferation, c-Jun is induced by the transient expression of oncogenes and is required for the transformation of fibroblasts by activated Ha-Ras (3, 16, 36). Conversely, genetically modified cells have provided evidence that c-Jun is required for mediating the apoptotic response of neurons to stress (5, 32, 37). The role of c-Jun in regulating apoptosis of fibroblasts following UV radiation (UV) remains controversial (5, 42).

As c-Jun is a typical immediate early gene, the induction of c-Jun expression following the stimulation of cells is rapid, large, and transient. Regulation occurs at two levels: (i) the stabilization of the protein via a reduction in c-Jun ubiquitination and degradation (10, 27, 40) and (ii) the control of gene transcription (2). The c-*jun* promoter region contains several regulatory elements, including TRE and myocyte enhancer factor 2 (MEF2) binding sites (12, 13). The TRE binds dimers of c-Jun and ATF factors, indicating that c-Jun regulates its own expression (2). MEF2A and MEF2D seem to be the predominant factors that bind to the MEF2 site (13, 29). The transcriptional activities of c-Jun, ATFs, and MEF2 are regulated upon phosphorylation by various protein kinases, including the mitogen-activated protein kinases (MAPK), which have been implicated in vitro in the transcriptional regulation of c-*jun* (18, 23).

At least four MAPK subfamilies have been identified: extracellular-regulated protein kinases 1 and 2 (ERK1/2), ERK5, c-Jun NH_2-terminal protein kinase (JNK), and p38 MAPK. ERK1/2 and JNK are capable of phosphorylating c-Jun (25, 33). JNK, p38 MAPK, and ERK1/2 phosphorylate ATF2 (11, 26, 31, 34). However, c-Jun and ATF2 activities appear to be regulated in vivo, primarily by JNK (25, 26). MEF2A activity is controlled by both p38 MAPK and ERK5, whereas MEF2D is a specific substrate of ERK5 (19, 30, 43).

Together, these studies clearly establish the regulation of c-Jun as a point of integration of numerous signals transduced by MAPK pathways. However, the data are based on the use of different cell lines and are not directly comparable. Here, we have performed a comprehensive analysis to unravel the respective roles of each of the MAPKs in regulating c-*jun* gene expression in response to apoptotic stress (UV) and after mitogenic stimulation (epidermal growth factor [EGF]). Our results provide clear evidence that the regulation of c-Jun expression by UV involves both JNK and p38 MAPK, while JNK, ERK1/2, and ERK5 are required to mediate the effect of EGF. In addition, p38 MAPK negatively affects EGF-dependent regulation of c-Jun by its ability to inhibit JNK activity.

MATERIALS AND METHODS

Tissue culture and preparation of lysates. Mouse embryonic fibroblasts (MEFs) were established from wild-type, *jnk1*$^{-/-}$ *jnk2*$^{-/-}$ (*jnk*$^{-/-}$), or *erk5*$^{-/-}$ embryonic day 13 mouse embryos (39; Xin Wang, unpublished data). The cells were placed in 1% serum for 24 h prior to being treated with UV (60 J/m^2) or

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EGF (50 ng/ml; Sigma). Where appropriate, the cells were pretreated for 30 min to 1 h with inhibitors as follows: SP600125, 25 μM (Calbiochem); SB203580, 10 μM (Promega); and PD184352, 2 μM. Agonists and inhibitors were added directly to the cell culture medium. Transfection of fibroblasts was carried out using the calcium phosphate method.

Proteins were extracted from cells in triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 10% glycerol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Extracts were clarified by centrifugation (14,000 × *g* for 10 min at 4°C). The concentration of soluble proteins in the supernatants was quantified by the Bradford method (Bio-Rad).

Immunoblot analysis. Cell and tissue extracts (50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide gel) and electrophoretically transferred to an Immobilon-P membrane (Millipore, Inc.). The membranes were incubated with 5% nonfat dry milk or 3% bovine serum albumin at 4°C overnight and then probed with polyclonal antibodies to c-Jun (Cell Signaling), MAPKAPK2 (Cell Signaling), ERK5 (41), ERK1/2 (Santa Cruz), JNK (Santa Cruz), or p38 MAPK (Santa Cruz). Immune complexes were detected by enhanced chemiluminescence (Amersham Biosciences), with rabbit or mouse immunoglobulin G coupled to horseradish peroxidase as the secondary antibody (Amersham Biosciences). Equal protein loading was monitored by detecting the levels of tubulin (Sigma) expression in the cell extracts.

Protein kinase assays. JNK, p38 MAPK, ERK1/2, and ERK5 protein kinase activities were measured in cell lysates in the presence of [γ-^{32}P]ATP following precipitation with glutathione *S*-transferase (GST)–c-Jun (34) or polyclonal antibodies to p38 MAPK (34), ERK1/2 (Santa Cruz), or ERK5 (41), respectively. GST-ATF2 (34), GST–c-Myc (1), and GST-MEF2C (28) were used as substrates for p38 MAPK, ERK1/2, and ERK5, respectively. The radioactivity incorporated into recombinant proteins was quantitated after sodium dodecyl sulfate-polyacrylamide gel electrophoresis by PhosphorImager analysis.

RNA isolation. Total RNA was isolated from cells by using the Trizol reagent (Invitrogen) as instructed by the manufacturer. RNA was treated with DNase by using the DNA-free kit (Ambion) as instructed by the manufacturer. RNA concentrations and quality were assessed visually by ethidium bromide/agarose gel electrophoresis under UV illumination and comparison with known amounts of mouse embryonic total RNA.

Real-time quantitative PCR. cDNA synthesis was carried out in a final volume of 20 μl of first-strand buffer containing 3 μg of total RNA, 20 U SuperScript II reverse transcriptase (Invitrogen), 0.025 μg/μl oligo(dT) (Promega), and 0.5 mM deoxynucleoside triphosphate (Roche). Primers were designed using the Primer Express software from the published mouse c-*jun* and β-*actin* sequences (GenBank accession numbers J04115 and NM-007393, respectively). These were as follows: for c-*jun*, forward primer, 5′-AGCAGGGACCCATGGAAGTT-3′, and reverse primer, 5′-AAAGATGACCTTTGCTTGTGCAT-3′; and for β-*actin*, forward primer, 5′-CCAACTTGATGTATGAAGGCTTTG-3′, and reverse primer, 5′-GCCTGTACACTGACTTGAGACCAATT-3′. These primers were to generate amplicons of 92 bp and 91 bp, respectively. Real-time quantitative PCRs were performed using the SYBR green I core kit (Eurogentec). PCR products were detected in the ABI-PRISM 7700 or 7000 sequence detection system (Applied Biosystems). Results were analyzed using the $2^{-\Delta\Delta G}$ methods (22). The level of expression of c-*jun* mRNA was normalized to β-*actin* mRNA.

Reporter gene expression assay. The reporter plasmid TRE-Luc (35) or MEF2 Luc (13) was transiently cotransfected with or without expression vectors encoding JNK (9), ERK5, or MEKK3 (7). A pRL-Tk plasmid encoding *Renilla* luciferase was employed for monitoring transfection efficiency. Aliquots of cell lysates were assayed for firefly and *Renilla* luciferase activities according to the manufacturer's instructions (Promega).

RESULTS

JNK and p38 MAPK are both required for mediating UV-induced c-Jun expression. We have previously provided genetic evidence for an essential role of JNK in mediating the apoptotic response of MEFs to genotoxic stress, including UV (39). To clarify the role of c-Jun in JNK-dependent cell death, we have tested the effect of deleting *jnk* genes on UV-dependent regulation of c-Jun expression (Fig. 1). UV transiently increased the level of c-*jun* mRNA, with a maximum (sixfold) reached after 1 h in wild-type cells (Fig. 1A). The absence of JNK correlated with a drastic (around 90%) decrease in the basal level of c-*jun* but did not prevent UV from promoting a significant induction of the transcript (16-fold after 1 h) with kinetics similar to that observed in wild-type cells (Fig. 1A). A similar decrease in c-*jun* mRNA was observed following the treatment of the wild-type cells with SP600125, a well-characterized inhibitor of JNK (6), but not with SB203580 or PD184352, that specifically block p38 MAPK activity (21) and ERK1/2 activation (24), respectively (Fig. 1B; see Fig. 2 and 6).

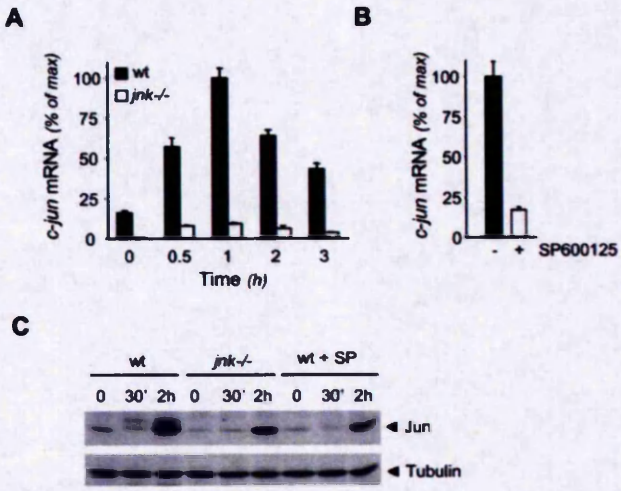

FIG. 1. JNK is required for mediating the effect of UV on c-Jun expression. Wild-type (wt) and *jnk*$^{-/-}$ fibroblasts without treatment or pretreated with SP600125 (25 μM) were UV radiated (60 J/m^2) and incubated for the times indicated. (A and B) Total RNA was extracted, and the amounts of c-*jun* mRNA were measured by quantitative PCR. Results are expressed as percentages of the maximums ± standard errors of duplicate samples. (C) MEF extracts were analyzed for c-Jun expression by immunoblot analysis using a specific polyclonal anti-c-Jun antibody. The detection of tubulin expression was performed to monitor protein loading. The figure is representative of three independent experiments.

Immunoblot analysis of the cell extracts confirmed that JNK was required for the basal level of c-Jun protein but was not essential for its up-regulation in response to UV (Fig. 1C). With a 90% decrease in the amount of mRNA (Fig. 1A), the *jnk*$^{-/-}$ cells displayed around three times less c-Jun protein than the wild-type cells. Despite the low level detected under basal conditions, a significant increase in c-Jun expression was observed in UV-treated *jnk*$^{-/-}$ cell extracts. In contrast, the absence of JNK completely abolished the electrophoretic mobility shift of c-Jun typical of the protein being phosphorylated in wild-type MEFs 30 min after stimulation. Pretreatment of the wild-type cells with SP600125 recapitulated the abnormal phenotype caused by *jnk* gene deletion (Fig. 1C). Consistent with a previous study (25), these results provide further genetic and pharmacological evidences that JNK is required for c-Jun phosphorylation in response to stress.

Since the induction of AP-1 by genotoxic stress is also mediated by the p38 MAPK cascade (17), we tested the effect of the p38 MAPK inhibitor SB203580 on the regulation of c-Jun by UV (Fig. 2). The ability of the compound to block p38 MAPK but not JNK activity is demonstrated by its specific inhibitory effect on UV-induced MAPKAPK2 but not c-Jun phosphorylation (Fig. 2C). Real-time PCR quantification and

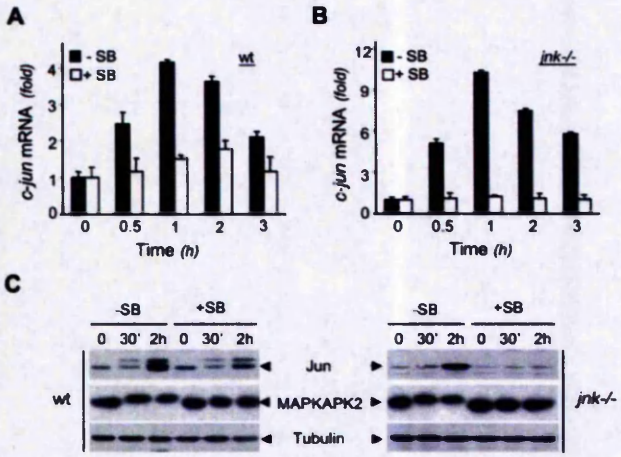

FIG. 2. p38 MAPK contributes to the regulation of c-Jun expression in response to UV. Wild-type (wt) (A and C) and *jnk*$^{-/-}$ (B and C) MEFs without treatment (−) or pretreated (+) with SB203580 (SB; 10 μM) were UV radiated (60 J/m^2) and incubated for the times indicated. (A and B) Total RNA was extracted, and the amounts of c-*jun* mRNA were measured by quantitative PCR. Results are expressed as increases relative to the mRNA extracted from unstimulated fibroblasts ± standard errors of duplicate samples. (C) MEF extracts were analyzed for c-Jun and MAPKAPK2 expression by immunoblot analysis using specific polyclonal anti-c-Jun and anti-MAPKAPK2 antibodies. The detection of tubulin expression was performed to monitor protein loading. The figure is representative of three independent experiments.

immunoblot analysis showed that SB203580 induced a partial inhibition (around 60%) of c-Jun expression in response to UV in wild-type cells (Fig. 2A and C) and a total inhibition in *jnk*$^{-/-}$ fibroblasts (Fig. 2B and C).

Altogether, the data show that JNK is essential for the basal expression of c-Jun and for c-Jun phosphorylation in response to UV. There is around 10 times less c-*jun* mRNA, corresponding to around 3 times less c-Jun protein in *jnk*$^{-/-}$ cells than in wild-type cells (Fig. 1). However, the up-regulation of c-Jun in response to UV stimulation requires both JNK and p38 MAPK (Fig. 2).

JNK and p38 MAPK have opposite effects on EGF-dependent regulation of c-Jun expression. Similar to c-*jun*$^{-/-}$ MEFs, *jnk*$^{-/-}$ fibroblasts display a severe proliferation defect (16, 36, 39). This led us to investigate the role of JNK in regulating c-Jun expression in response to EGF. Compared to UV, EGF induced a more transient and less potent activation of JNK in fibroblasts, reaching a maximum at 5 min and returning to the basal level 30 min after stimulation (Fig. 3A and data not shown). However, similar to UV, EGF induced a marked and transient increase in c-*jun* mRNA in wild-type cells, reaching a maximum (5.5-fold) after 30 min of stimulation (Fig. 3B). With a low basal level, increased expression of c-*jun* in *jnk*$^{-/-}$ fibroblasts following EGF treatment was potent (11-fold after 1 h) and sustained (Fig. 3B). The sustained effect of EGF in *jnk*$^{-/-}$ cells compared to wild-type cells suggests that JNK controls a negative feedback loop resulting in a transient rather than prolonged increase in c-*jun* expression. Consistent with these data, EGF up-regulated the expression of c-Jun protein in *jnk*$^{-/-}$ cells to a level similar to that observed in wild-type cells 2 h following treatment (Fig. 3C).

Since EGF was able to induce a transient increase in p38 MAPK activity in fibroblasts (Fig. 4A), we tested whether the p38 MAPK cascade was responsible for the JNK-independent regulation of c-Jun expression in response to EGF. Surprisingly, the pretreatment of the wild-type MEFs with SB203580 markedly potentiated and delayed the effect of EGF on c-Jun expression (Fig. 4B and D). The level of c-*jun* mRNA in wild-type cells exposed to both SB203580 and EGF was approximately fourfold higher than that in cells exposed only to EGF. This phenomenon was reported previously and did not implicate the destabilization of the transcript by p38 MAPK (14). In contrast, SB203580 did not have any effect on increased c-Jun expression in EGF-treated *jnk*$^{-/-}$ fibroblasts (Fig. 4C and D), indicating that p38 MAPK-induced down-regulation of c-Jun is JNK dependent. Consistent with this hypothesis, fibroblasts

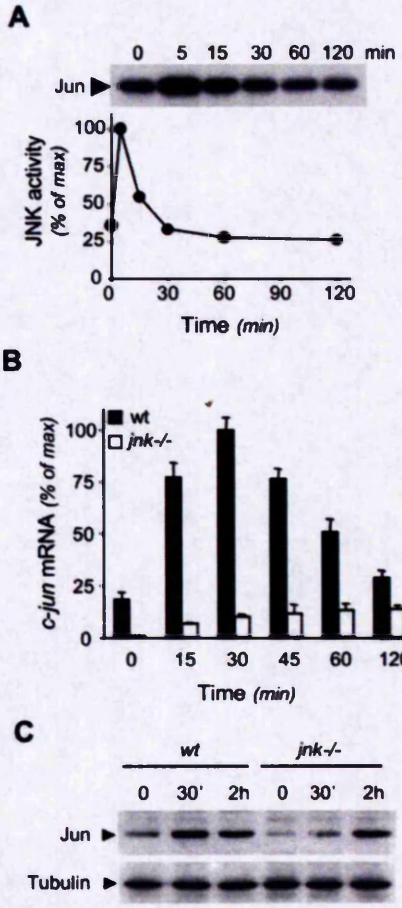

FIG. 3. JNK is required for mediating the effect of EGF on c-Jun expression. Wild-type (wt) and *jnk*$^{-/-}$ fibroblasts were stimulated with EGF (50 ng/ml) for the times indicated. (A) Endogenous JNK activity was measured by protein kinase assay. (B) Total RNA was extracted, and the amounts of c-*jun* mRNA were measured by quantitative PCR. Results are expressed as percentages of the maximums ± standard errors of duplicate samples. (C) MEF extracts were analyzed for c-Jun expression by immunoblot analysis using a specific polyclonal anti-c-Jun antibody. The detection of tubulin expression was performed to monitor protein loading. The figure is representative of three independent experiments.

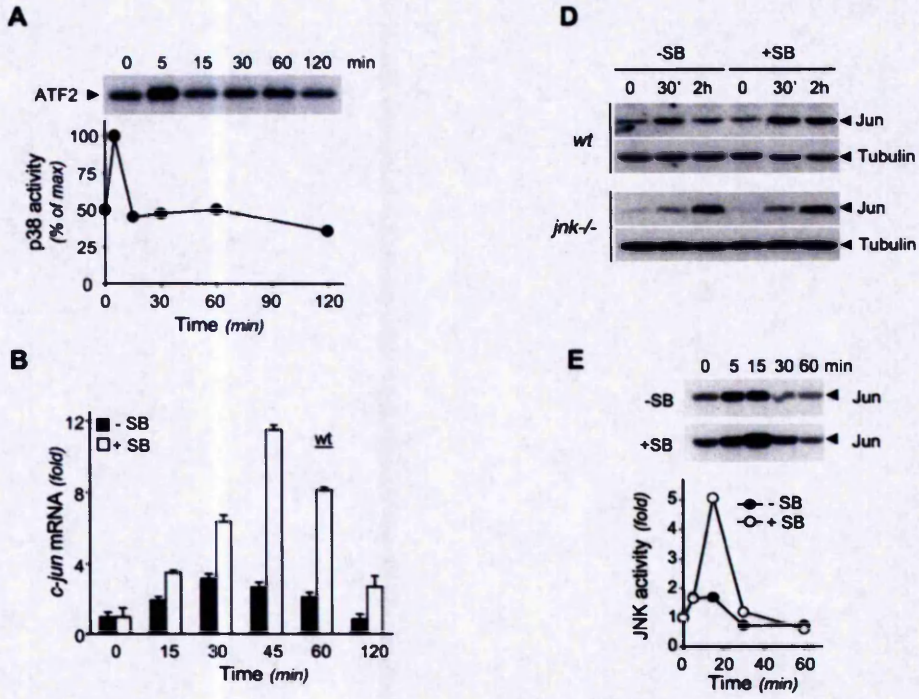

FIG. 4. p38 MAPK antagonizes the effect of JNK on the transcriptional regulation of c-*jun* following EGF treatment. Wild-type (wt) (A, B, D, and E) and $jnk^{-/-}$ (C and D) MEFs without treatment (−) or pretreated (+) with SB203580 (SB; 10 μM) were incubated with EGF (50 ng/ml) for the times indicated. The activities of p38 MAPK (A) or JNK (E) were measured by protein kinase assay. (B and C) Total RNA was extracted, and the amounts of c-*jun* mRNA were measured by quantitative PCR. Results are expressed as increases relative to the mRNA extracted from unstimulated fibroblasts ± standard errors of duplicate samples. (D) MEF extracts were analyzed for c-Jun expression by immunoblot analysis using a specific polyclonal anti-c-Jun antibody. The detection of tubulin expression was performed to monitor protein loading. The figure is representative of three independent experiments.

pretreated with SB203580 displayed higher JNK activity following EGF treatment (Fig. 4E). Based on these results, we concluded that p38 MAPK represses the EGF-dependent increase in c-Jun expression by inhibiting JNK activity. Next, we investigated whether ERKs were involved, in addition to JNK, in controlling c-*jun* gene expression in response to a mitogenic signal.

Inhibition of ERK activities in fibroblasts. The ability of PD98059 to block the activation of both ERK1/2 and ERK5 (24) has led to controversial data about the specific role of ERK1/2 and ERK5 in regulating cell function. To circumvent this problem, we employed PD184352, a more specific inhibitor of the ERK1/2 signaling pathway. Consistent with a previous study (24), PD184352 blocked EGF-dependent stimulation of ERK1/2 (Fig. 5A). At this concentration (2 μM), the drug did not inhibit ERK5 activity but consistently delayed the peak of activation of ERK5 by EGF (Fig. 5B).

Since no specific ERK5 inhibitor exists, we used a novel model of *erk5*-null fibroblasts (Xin Wang, unpublished). Disruption of the *erk5* gene did not alter the expression of ERK1/2, JNK, or p38 MAPK (Fig. 5C). Furthermore, EGF treatment caused a marked increase in ERK5 activity in wild-type but not homozygous knockout *erk5* fibroblasts (Fig. 5D). In contrast, no marked difference was observed in the abilities of EGF to stimulate ERK1/2 and JNK activities in wild-type and $erk5^{-/-}$ MEFs (Fig. 5E and data not shown). These data established that $erk5^{-/-}$ MEFs possess no ERK5 and represent a useful model for studies of the ERK5 signaling pathway.

Both ERK1/2 and ERK5 contribute to mediating EGF-induced c-Jun expression. Inhibition of ERK1/2 activation impaired the early increase in c-*jun* mRNA by EGF and subsequently delayed the induction of the protein in the wild-type fibroblasts (Fig. 6A and C). The ability of PD184352 to interfere with EGF signaling was more pronounced in $jnk^{-/-}$ MEFs for which the drug inhibited the response of the cells to the treatment throughout the time course of induction (Fig. 6B and C). These results indicate that ERK1/2 is required for the early increase in c-Jun expression by EGF but can contribute to the late phase of induction in cells that do not express JNK.

The absence of ERK5 significantly impaired the ability of EGF to increase c-*jun* mRNA expression (Fig. 6D and F). After 15 min of treatment, the level of induction in $erk5^{-/-}$

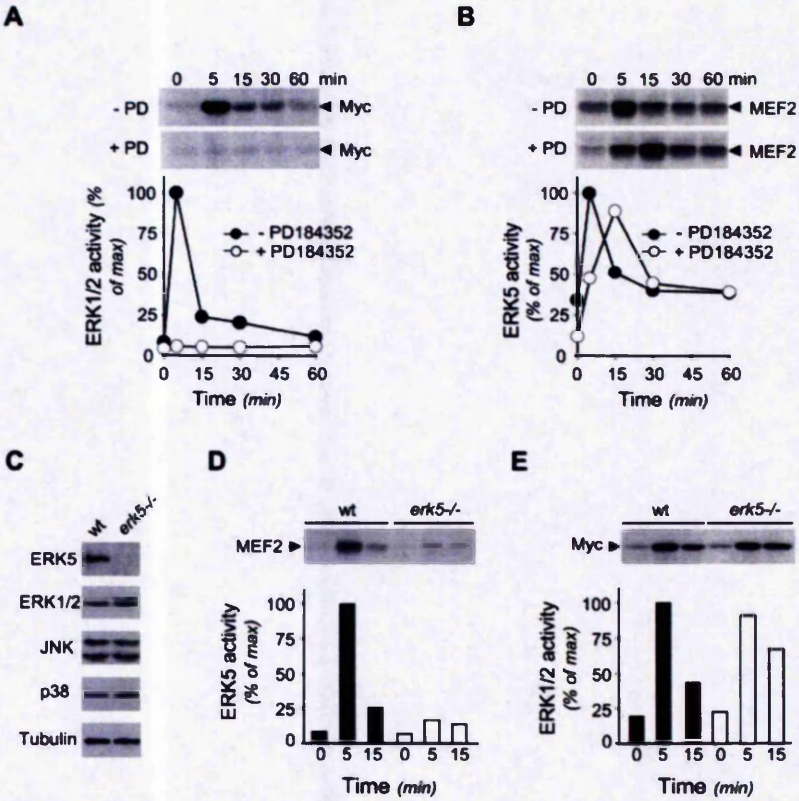

FIG. 5. Inhibition of ERK1/2 and ERK5 in fibroblasts. MEFs without treatment (−) or pretreated (+) with PD184352 (PD; 2 μM) were incubated with EGF (50 ng/ml) for the times indicated. The activities of ERK1/2 (A and E) and ERK5 (B and D) were measured by protein kinase assay. (C) Wild-type (wt) and *erk5*$^{-/-}$ MEF extracts were analyzed for ERK5, ERK1/2, JNK, and p38 MAPK expression by immunoblot analysis using specific polyclonal antibodies. The detection of tubulin expression was performed to monitor protein loading. The figure is representative of two independent experiments.

MEFs was half of what was observed in wild-type cells. In contrast to JNK, ERK5 did not affect the basal level of the transcript. Consistently, the induction of c-Jun protein expression by EGF was lower in *erk5*$^{-/-}$ MEFs than in wild-type MEFs (Fig. 6E). The inhibition of JNK or ERK1/2 further blocked the residual increase in c-*jun* mRNA in the ERK5-deficient fibroblasts treated with EGF (Fig. 6F and data not shown). Consistent with the JNK-dependent negative feedback loop, the pretreatment of the *erk5*$^{-/-}$ MEFs with SP600125 resulted in a prolonged rather than transient increase in c-*jun* expression in response to EGF stimulation (Fig. 6F). Control experiments showed no effect of PD184352 or of the *erk5* gene deletion on UV-dependent regulation of c-Jun (Fig. 6C and E).

Altogether, these results indicate that both ERK1/2 and ERK5 are required for the regulation of c-Jun expression by EGF. The ERK1/2 signaling pathway appears to be redundant to the JNK cascade for the late induction of c-Jun.

Molecular mechanism of the transcriptional regulation of c-*jun* by MAPKs. Next, we investigated the molecular mechanism by which MAPKs regulate the transcription of c-*jun*. Fibroblasts were transfected with the luciferase reporter plasmids containing the TRE or the MEF2 binding site present in the promoter of the c-*jun* gene together with constructs encoding JNK, ERK5, or MEKK3. MEKK3-induced transcriptional activity was determined by measuring luciferase activity.

MEKK3-induced activation of the TRE-Luc was enhanced following the reintroduction of JNK in the *jnk*$^{-/-}$ MEFs. This indicated that the absence of JNK impaired the ability of MEKK3 to activate transcription via AP-1 (Fig. 7A). Consistent with our in vivo data (Fig. 1 and 3), these results confirm that JNK is required but not essential for the transcriptional regulation of c-Jun. In contrast, the absence of ERK5 completely prevented MEKK3 from increasing transcriptional activity via the MEF2 binding site (Fig. 7B). Overexpression of ERK5 in the *erk5*$^{-/-}$ MEFs rescued the defect. The essential role of the ERK5 signaling pathway in regulating MEF2 activity in fibroblasts was previously observed by the inability of the p38 MAPK cascade to compensate for the deletion of the *mek5* gene (41). Control experiments showed that MEKK3-induced transcriptional activation via MEF2 or TRE was not affected by the deletion of the *jnk* or *erk5* gene, respectively (Fig. 7A and B). To investigate the roles of p38 MAPK and ERK1/2, wild-type MEFs were pretreated with SB203580 and PD184352 12 h prior to measurement of the luciferase activity, respectively (Fig. 7C). Both compounds inhibited, by around 45%, the ability of MEKK3 to activate transcription via TRE. In contrast, MEKK3-induced transcription via the MEF2 binding

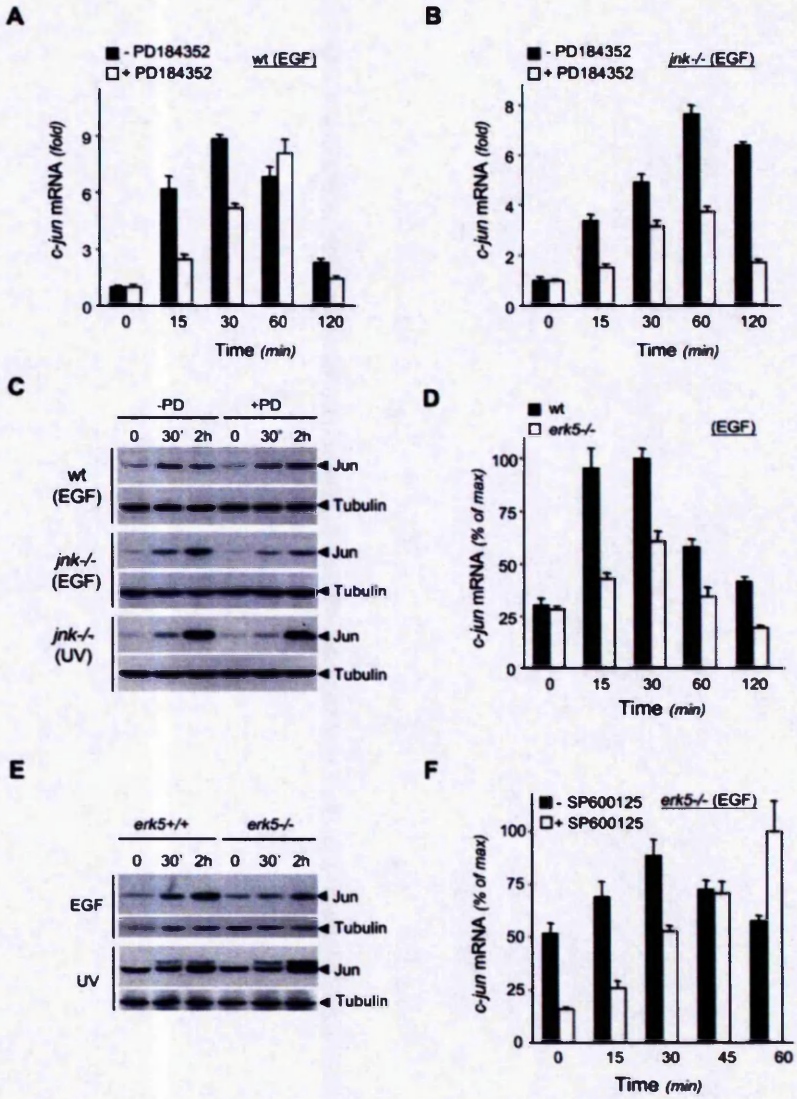

FIG. 6. ERK1/2 and ERK5 are implicated in the regulation of c-Jun expression in response to EGF. Wild-type (wt), *jnk*$^{-/-}$, and *erk5*$^{-/-}$ MEFs without treatment (−) or pretreated (+) with PD184352 (2 μM) or SP600125 (25 μM) were stimulated with EGF (50 ng/ml) or UV radiated (60 J/m^2) for the times indicated. (A, B, D, and F) Total RNA was extracted, and the amounts of c-*jun* mRNA were measured by quantitative PCR. Results are expressed as increases relative to the mRNA extracted from unstimulated fibroblasts (A and B) or as percentages of the maximums (D and F) ± standard errors of duplicate samples. (C and E) MEF extracts were analyzed for c-Jun expression by immunoblot analysis using a specific polyclonal anti-c-Jun antibody. The detection of tubulin expression was performed to monitor protein loading. The figure is representative of three independent experiments.

site was specifically blocked (80% decrease) following the inhibition of p38 MAPK activity but not ERK1/2 activation.

Altogether, these results indicate that, in vitro, the promoter of the c-*jun* gene can be controlled by JNK, p38 MAPK, and ERK1/2 via the TRE and by p38 MAPK and ERK5 via the MEF2 binding site.

DISCUSSION

The plethora of physiological stimuli and environmental insults that regulate c-Jun function has made c-Jun a useful model to study the complexity of gene regulation via AP-1 (17). Here, we have performed a comprehensive study to elucidate how MAPK cascades control gene expression via the transcriptional regulation of c-*jun*. Our results demonstrate that the effect of MAPK on c-Jun expression is mediated (i) by JNK and p38 MAPK in response to UV and (ii) by JNK, ERK1/2, and ERK5 following EGF stimulation. The contribution of ERKs or p38 MAPK, together with JNK, to the transcriptional regulation of the c-*jun* gene may reflect the distinct function of c-Jun in the regulation of cell fate.

The small amounts of c-*jun* present in the *jnk*$^{-/-}$ MEFs compared to those in the wild-type cells indicate that JNK controls the basal level of the transcript (Fig. 1 and 3). JNK has previously been shown to stabilize interleukin 2 mRNA

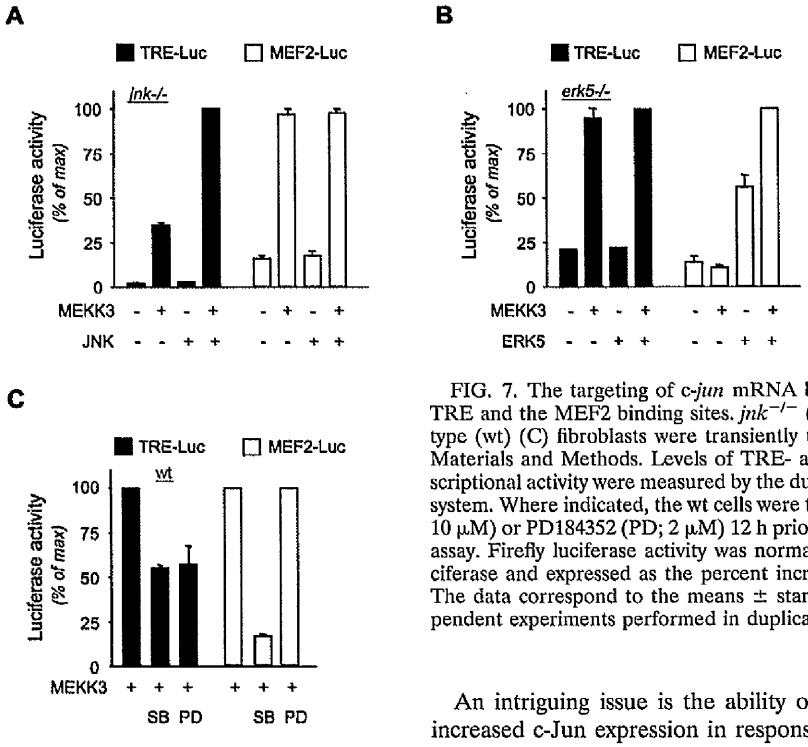

FIG. 7. The targeting of c-*jun* mRNA by MAPK cascades via the TRE and the MEF2 binding sites. $jnk^{-/-}$ (A), $erk5^{-/-}$ (B), and wild-type (wt) (C) fibroblasts were transiently transfected as described in Materials and Methods. Levels of TRE- and MEF2-dependent transcriptional activity were measured by the dual-luciferase reporter assay system. Where indicated, the wt cells were treated with SB203580 (SB; 10 μM) or PD184352 (PD; 2 μM) 12 h prior to the performance of the assay. Firefly luciferase activity was normalized to that of *Renilla* luciferase and expressed as the percent increase over the control (−). The data correspond to the means ± standard errors of three independent experiments performed in duplicate.

through a *cis* element encompassing the 5′ untranslated region and the beginning of the coding region (8). Based on this study, we examined whether JNK increased the stability of c-*jun* by comparing its half-life in unstimulated wild-type and $jnk^{-/-}$ MEFs. Consistent with the presence of an adenosine- or uridine-rich element characterized by multiple copies of the pentanucleotide AUUUA in the 3′ untranslated region of c-*jun*, we confirmed that c-*jun* is a rapidly decaying mRNA with a half-life of 20 min and that this was not significantly affected by *jnk* deletion (data not shown). These results indicate that JNK does not affect the stability of the c-*jun* transcript. Thus, the low level of c-Jun expressed in *jnk*-null MEFs is most likely caused by a transcriptional defect.

In addition to regulating c-Jun expression, JNK is essential for mediating the phosphorylation of c-Jun in response to UV (Fig. 1C). Consistent with this result, a recent study based on the use of phospho-specific antibodies to different phosphorylated forms of c-Jun has shown that JNK is the MAPK required for stress-induced phosphorylation of c-Jun on Ser63 and Ser73 residues (25). MEFs and cortical neurons in which the c-*jun* gene was replaced by a mutant c-*jun* allele with Ser63 and Ser73 mutated to Ala displayed a stress-induced apoptotic defect (5). In light of our previous results (39), these studies suggest that JNK-dependent c-Jun phosphorylation is required for mediating apoptosis. Death genes whose expression is controlled by the JNK/c-Jun signaling pathway have already been identified (37). However, c-Jun function does not always depend on JNK activation. For example, a role for c-Jun in the regulation of G_1 progression has been shown to be independent of its phosphorylation (42). Similarly, JNK-dependent phosphorylation of Ser63 and Ser73 is not essential for c-Jun to cooperate with Ha-Ras in cellular transformation (15, 20).

An intriguing issue is the ability of p38 MAPK to inhibit increased c-Jun expression in response to EGF (Fig. 4). The absence of this inhibitory effect in the JNK-deficient cells indicates that the p38 MAPK-induced down-regulation of c-Jun is JNK dependent. Consistent with this hypothesis, protein kinase assays show that p38 MAPK blocks EGF-dependent activation of JNK. Negative regulation of JNK by p38 MAPK in response to EGF has previously been reported to be medi-

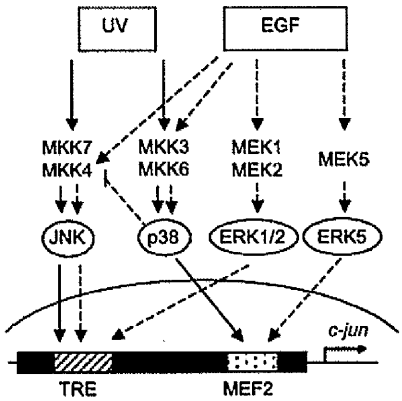

FIG. 8. Model of the regulation of the promoter of the c-*jun* gene by MAPKs. MAPK activators include MEK1 and MEK2 for ERK1/2, MEK5 for ERK5, MKK4 and MKK7 for JNK, and MKK3 and MKK6 for p38 MAPK. UV activates JNK and p38 MAPK, leading to the subsequent increase in c-*jun* expression via the TRE and the MEF2 binding sites, respectively. In contrast, EGF-induced c-*jun* expression is mediated via TRE by JNK and ERK1/2 and via MEF2 by ERK5. The negative regulation of c-*jun* by p38 MAPK in response to EGF treatment is mediated by the ability of p38 MAPK to inhibit JNK activity, most probably via a phosphatase-mediated dephosphorylation of MKK4. UV- and EGF-dependent signaling pathways are represented by solid and broken lines, respectively.

ated via the inactivation of the MAPK kinase MKK4 by the protein phosphatase-2A (4). MKK4 is a dual-specificity protein kinase that functions as a selective activator of JNK in response to extracellular stimuli (38). Altogether, these studies suggest that p38 MAPK represses the EGF-dependent increase in c-Jun expression by inhibiting JNK activity via the specific dephosphorylation of MKK4.

Regulatory elements found in the promoter region of the c-*jun* gene include a TRE and a MEF2 site. Luciferase reporter assays demonstrate that transcriptional regulation via the TRE can be mediated by JNK, p38 MAPK, and ERK1/2 (Fig. 7A and C). This is consistent with the ability of JNK and ERK1/2 to phosphorylate c-Jun (9, 33), while ATF2 is a substrate for JNK, ERK1/2, and p38 MAPK (11, 31, 34). However, in vivo data indicate that JNK and ERK1/2 are the physiological MAPKs involved in mediating the phosphorylation of c-Jun and ATF2 (25, 26). Therefore, the p38 MAPK is likely mediating its effect via its ability to regulate MEF2 activity (Fig. 7C) (30, 43). Consistent with our previous study (41), we show that p38 MAPK is required and ERK5 is essential for the stimulation of MEF2 activity following the overexpression of MEKK3 (Fig. 7B). In light of this knowledge, this study provide for the first time physiological evidence that the transcriptional regulation of the c-*jun* gene is mediated by JNK and ERK1/2 via the TRE and by p38 MAPK and ERK5 via the MEF2 binding site (Fig. 8).

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IV - Manuscript 3: Activation of extracellular signal-regulated protein kinase 5 down-regulates FasL upon Osmotic Stress

Genetic evidence for a crucial role of the ERK5 signalling pathway in mediating cell survival was provided by the analysis of the MEK5- and ERK5-deficient mice which exhibit increased apoptosis in the developing head (Wang et al., 2005; Yan et al., 2003), and a collapse of vasculature due to endothelia cell apoptosis (Regan et al., 2002; Sohn et al., 2002). In vitro studies showed that the phosphorylation of Bad by ERK5 independent of PKB, RSK and PKA, was a potential mechanism by which ERK5 protects endothelial cells from shear stress (Pi et al., 2004). In parallel to this study, I investigated the mechanism by which ERK5 regulates cell survival of fibroblasts in response to osmotic stress.

Our data showed that increased FasL expression was responsible for enhanced cell death in the *mek5-/-* and the *erk5-/-* fibroblasts following sorbitol treatment. A number of response elements have been identified in the promoter of the *fasL* gene including binding sites for the transcription factors c-Jun and Forkhead transcription factor box O3a (Foxo3a, formerly called FKHRL1) (Faris et al., 1998; Kasibhatla et al., 1998; Kolbus et al., 2000; le-Niculescu et al., 1999). We found that c-Jun mRNA and protein expression was down-regulated following sorbitol treatment of the cells (unpublished data). Furthermore we could not detect any electrophoretic mobility shift typical of the protein being phosphorylated. We concluded that c-Jun was unlikely to be involved in the transcriptional regulation of the *fasL* gene under osmotic stress conditions. This prompted us to test the hypothesis that the requirement of the ERK5

signalling pathway to inhibit Foxo3a activity was responsible for the up-regulation of FasL expression in cells deficient in ERK5 or MEK5 expression. Foxo3a is a pro-apoptotic transcription factor. Its phosphorylation by protein kinase B (PKB, also known as Akt) at Thr32 promotes its association with 14-3-3, and thus, its sequestration in the cytoplasm (Brunet et al., 1999). PKB is a well-characterised key mediator of signal transduction implicated in protecting cells against death. Its activation is mediated by dual phosphorylation at Thr308 and Ser473 (Alessi et al., 1996). Phosphorylation at Thr308 is mediated by the 3-phosphoinositide (PtIns)-dependent protein kinase-1 (PDK1) via a PtIns 3 kinase (PI3K)-dependent mechanism (Alessi et al., 1997), while Ser473 is a target for the mammalian target of rapamycin protein kinase, mTor (Sarbassov et al., 2005).

Immunoblot analysis demonstrated that the deletion of the *erk5* or the *mek5* genes decreases both the basal level and sorbitol-induced phosphorylation of Foxo3a at Thr32. Consistent with enhanced Foxo3a transcriptional activity exhibited by *mek5-/-* and *erk5-/-* fibroblasts, I found that absence of MEK5 or ERK5 facilitated the binding of Foxo3a to the promoter of the *fasl* gene. In addition, PKB activity was decreased in *mek5-/-* and *erk5-/-* compared to the wild type MEFs. In contrast no difference was observed in the ability of sorbitol to increase PI3K activity. Based on these results, I concluded that the ERK5 signaling pathway promoted cell survival by down-regulating FasL expression via a mechanism that implicates PKB-dependent inhibition of Foxo3a downstream of PI3K.

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Activation of extracellular signal-regulated protein kinase 5 downregulates FasL upon osmotic stress

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Abstract

Extracellular signal-regulated protein kinase (ERK) 5 is a mitogen-activated protein kinase (MAPK) that is activated by dual phosphorylation via a unique MAPK/ERK kinase 5, MEK5. The physiological importance of this signaling cascade is underscored by the early embryonic death caused by the targeted deletion of the *erk5* or the *mek5* genes in mice. Here, we have found that ERK5 is required for mediating the survival of fibroblasts under basal conditions and in response to sorbitol treatment. Increased Fas ligand (FasL) expression acts as a positive feedback loop to enhance apoptosis of ERK5- or MEK5-deficient cells under conditions of osmotic stress. Compared to wild-type cells, *erk5*−/− and *mek5*−/− fibroblasts treated with sorbitol display a reduced protein kinase B (PKB) activity associated with increased Forkhead box O3a (Foxo3a) activity. Based on these results, we conclude that the ERK5 signaling pathway promotes cell survival by downregulating FasL expression via a mechanism that implicates PKB-dependent inhibition of Foxo3a downstream of phosphoinositide 3 kinase.
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Keywords: ERK5; FasL; MEK5; PKB; Bid; Bim

Abbreviations: ERK, extracellular signal-regulated protein kinase; FasL, Fas ligand; Foxo3a, Forkhead box O3a; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor 2; MEFs, mouse embryonic fibroblasts; MEK, MAPK/ERK kinase; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI3K, phosphoinositide 3 kinase; PKB, protein kinase B; mTor, mammalian target of rapamycin protein kinase

Introduction

The mitogen-activated protein kinases (MAPK) constitute a family of serine/threonine protein kinases involved in the regulation of many cellular functions, including proliferation, survival, and apoptosis.[1] There are at least four subfamilies positioned at the end of distinct signaling cascades that include a MAPK kinase and a MAPK kinase kinase.[1] Although these pathways transmit signals independently of each other, cross talk occurs between the different modules thereby leading to a greater level of complexity that allows the fine-tuning of responses to extracellular stimuli. MAPK modules have been associated with different biological responses. For example, the extracellular signal-regulated protein kinase (ERK) subfamilies 1, 2, and 5 are mostly associated with cell proliferation and survival, wereas c-Jun N-terminus protein kinase (JNK) and p38 MAPK are mainly activated in response to cytokines and extracellular stresses and mediate apoptosis.

ERK5 is more than twice the size of the other MAPKs owing to a very large C-terminal domain.[2,3] Its activity is stimulated in response to growth factors and hyperosmolarity via the MAPK/ERK kinase, MEK5.[4] Little is known about the downstream targets of ERK5. The best-characterized substrates are the transcription factors of the myocyte enhancer factor (MEF) family.[5,6] Phosphorylation of MEF2C by ERK5 enhances its transcriptional activity and subsequently increases expression of the AP-1 family member, c-Jun.[5] Consistent with this study, we have recently demonstrated that ERK5 is selectively required for the regulation of c-Jun expression following EGF stimulation of the cells but not by UV radiation.[7]

Clues as to the physiological role of the ERK5 signaling pathway have recently been provided by the targeted deletion of the *erk5* and *mek5* genes in mice.[4,8] The analysis of mutant mice in which the *erk5* gene can be conditionally deleted revealed that the requirement of ERK5 for the survival of endothelial cells is responsible for the cardiovascular defect observed in *erk5*−/− and *mek5*−/− embryos.[9] The ERK5 signaling pathway protects endothelial cells from apoptosis by phosphorylating and inhibiting the Bcl-2 family member Bad.[10] Evidence that ERK5 contributes to mediating neuronal survival in response to growth factors has also been reported.[11]

The Ser/Thr protein kinase B (PKB, also known as Akt) is another key mediator of signal transduction implicated in protecting cells against death. Activation of PKB is mediated by dual phosphorylation at Thr308 and Ser473.[12] Phosphorylation at Thr308 is mediated by the 3-phosphoinositide (PtIns)-dependent protein kinase-1 (PDK1) via a PtIns 3 kinase (PI3K)-dependent mechanism,[13] whereas Ser473 is a target for the mammalian target of rapamycin protein kinase, mTor.[14] The survival function of PKB can be explained by its ability to inhibit the activity of proapoptotic proteins including Bad[15] and the Forkhead transcription factor box O3a (Foxo3a formerly called FKHRL1).[16] In both cases, phosphorylation provides a mechanism to sequester the proteins in the cytoplasm thereby blocking their apoptotic effect.

Here we have investigated how the ERK5 signaling pathway regulates the survival response of fibroblasts to osmotic stress. Our data indicate that the ERK5 cascade suppresses FasL expression by inhibiting its transcription via Foxo3a. Further experiments demonstrate that PKB activation following sorbitol stimulation is impaired in the absence of ERK5 or MEK5. Overall, this study establishes for the first time a functional cross talk between the ERK5 and the PKB signaling pathways to prevent enhancement of cell death via increased FasL expression.

Results

Deletion of the *erk5* gene causes apoptosis

The role of ERK5 in cell survival was examined by testing the effect of *erk5* gene deletion in homozygous *erk5loxP* mouse embryonic fibroblasts (MEFs) infected with an adenovirus encoding Cre (AdCre). The strategy employed to create the *erk5loxP* mutant mice is provided as Supplementary information (Figure S1). Immunoblot analysis of the cell lysates 72 h postinfection demonstrated the gradual loss of the ERK5 protein following increased Cre expression (Figure 1a). Future experiments were performed with adenoviruses at 100 multiplicity of infection (MOI) to induce the complete deletion of the *erk5* gene. A time course analysis of infection showed that caspase 3 activity was significantly increased in *erk5loxP/loxP* but not in wild-type MEFs infected with AdCre (Figure 1b). The slight effect of AdCre in the *erk5+/loxP* MEFs indicates a low level of cell death associated with heterozygous deletion of the *erk5* gene. Hereinafter, the *erk5loxP/loxP* fibroblasts were immortalized and referred to as *erk5*+/+ or *erk5*−/− MEFs depending on whether they were infected with a control virus encoding green fluorescence protein (GFP) or Cre, respectively.

Immunoblot analysis confirmed the requirement of ERK5 to suppress the activation of caspase 3 under basal conditions, and also in response to sorbitol, a potent inducer of apoptosis and a strong activator of ERK5 (Figure 1c). A reduction of the inactive p32 proenzyme together with the appearance of the active p17 product was detected 3 h earlier in *erk5loxP/loxP* MEFs infected with Cre (−/−) than in *erk5loxP/loxP* MEFs infected with GFP (+/+). More strikingly, sorbitol induced the proteolytic cleavage of the proapoptotic Bcl-2 family member Bid to generate the death-promoting fragment tBid, only in absence of ERK5 (Figure 1c). The level of tBid was maximal after 1 h stimulation consistent with a contribution of Bid upstream of caspase 3.

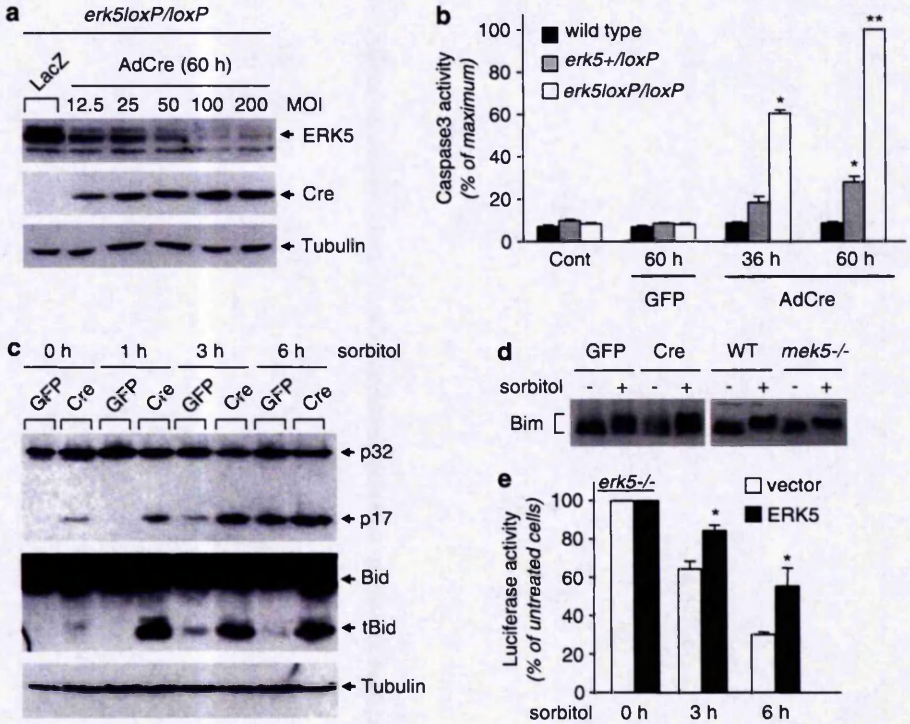

Figure 1 *erk5* gene deletion sensitizes fibroblasts to apoptosis. MEFs were not infected (Cont), or infected with a control virus (lacZ or GFP) or with AdCre befor being incubated with sorbitol where indicated. (**a**, **c**, **d**) Extracts (50 μg) were analyzed for ERK5, Cre, caspase 3, Bid, and Bim expression by immunoblot analysis using specific antibodies. The detection of tubulin expression was performed to monitor protein loading. Similar results were obtained in two to three independent experiments. (**b**) Caspase 3 activity was measured by caspase assay. The data correspond to the mean ± S.E. of three independent experiments performed in duplicate. *P*-values relative to wild-type sample are indicated by asterisks: $^{*}P<0.05$; $^{**}P\leqslant 0.001$. (**e**) *erk5*−/− fibroblasts were cotransfected with an empty vector (vector) or a vector encoding wild-type ERK5, and luciferase (pCMV-luc) to monitor cell viability. At 36 h after transfection, the cells were treated with sorbitol for the indicated times before being lysed and the luciferase activity was measured. The values are normalized to the protein content. The data expressed as % of treated versus untreated cells correspond to the mean ± S.E. of three independent experiments. $^{*}P<0.0001$ indicates significant increase in survival of cells transfected with ERK5

Bim is another BH3-only protein of the Bcl-2 family implicated in the intrinsic cell death signaling pathway. It becomes hyperphosphorylated upon apoptotic stress and dissociates from the microtubule-associated dynein motor complex where it is normally sequestered.[17] Deletion of the *erk5* gene under basal conditions or incubation of the fibroblasts with sorbitol caused distinct electrophoretic mobility shifts indicative of Bim being phosphorylated (Figure 1d). Unlike Bid cleavage, no significant difference was observed in the ability of sorbitol to phosphorylate Bim in wild-type and *erk5*–/– or *mek5*–/– MEFs.

Cell viability assay that employs a luciferase plasmid showed that ectopic expression of ERK5 was able to protect fibroblasts against sorbitol toxicity (Figure 1e). *erk5*–/– MEFs transfected with a vector encoding ERK5 displayed 15 and 45% decrease in luciferase activity, as compared to 35 and 70% when the cells were transfected with an empty vector, following 3 and 6 h sorbitol treatment, respectively. This agrees with our previous data showing that *mek5*–/– MEFs were more sensitive than the wild-type cells to the toxic effect of sorbitol.[4] Similar to the phenotypic defect observed in the *erk5*–/– fibroblasts, enhanced cell death displayed by MEFs lacking MEK5 correlated with increased caspase 3 and Bid cleavage (Supplementary Figure S2).

Together, these studies suggest that activation of the proapoptotic activity of Bim and Bid may be responsible for the abnormal cell death phenotype displayed by the *erk5*–/– MEFs under basal conditions and in response to stress, respectively.

ERK5 protects cells by inhibiting FasL expression

Cleavage of Bid plays a major role in mediating the apoptotic response of cells to factors acting via death receptors, including FasL. As FasL has previously been shown to contribute to stress-induced apoptosis, we tested the hypothesis that an increase in FasL expression was responsible for sorbitol-induced Bid cleavage. Real-time PCR analysis showed that sorbitol enhanced the levels of *fasL* mRNA in the *erk5*–/– and *mek5*–/– MEFs compared to the wild-type (+/+) cells (Figure 2a and b). Consistently, around 60% FasL-positive ERK5- or MEK5-deficient MEFs were detected upon sorbitol stimulation compared to 13–20% positive cells of the corresponding wild-type genotype (Figure 2c and d).

The importance of FasL to mediate the apoptotic effect of sorbitol was demonstrated by the ability of a neutralizing anti-FasL antibody to reduce the activation of caspase 3 (Figure 2e and f). The greatest inhibition (around 75%) was detected in the *mek5*–/– MEFs where caspase 3 activation was decreased to a similar level as in the wild-type cells (Figure 2f). The lower level of inhibition (around 45%) observed in the *erk5*–/– MEFs is explained by the inability of the antibody to block Fas-independent caspase 3 activation caused by *erk5* gene deletion (Figure 2e). Consistently, absence of ERK5 had no effect on FasL expression under basal conditions (time 0) (Figure 2a and c).

Together, these experiments indicate that FasL produced in response to sorbitol acts in a positive feedback loop to enhance the death of ERK5- or MEK5-deficient cells under conditions of osmotic stress.

ERK5 is required to inhibit Foxo3 activity

A number of response elements have been identified in the promoter of the *fasL* gene including binding sites for the transcription factors c-Jun and Foxo3a.[16,18,19] We found that c-Jun mRNA and protein expression was downregulated following sorbitol treatment of the cells (unpublished data). Furthermore, we could not detect any electrophoretic mobility shift typical of the protein being phosphorylated. We concluded that c-Jun was unlikely to be involved in the transcriptional regulation of the *fasL* gene under osmotic stress conditions. This prompted us to test the hypothesis that the requirement of the ERK5 signaling pathway to inhibit Foxo3a activity was responsible for the upregulation of FasL expression in cells deficient in ERK5 or MEK5 expression. Real-time PCR analysis showed similar levels of *foxo3a* mRNA in wild-type, *erk5*–/–, and *mek5*–/– MEFs (Figure 3a). Immunoblot analysis demonstrated that the deletion of the *erk5* or the *mek5* genes decreases both the basal level and sorbitol-induced phosphorylation of Foxo3a at Thr32 (Figure 3b). The phosphorylation of Foxo3a at Thr32 leads to its association with 14-3-3, and thus, its sequestration in the cytoplasm.[16]

The ability of the ERK5 signaling pathway to control Foxo3a activity was examined by luciferase reporter assay (Figure 3c and d). Wild-type fibroblasts were cotransfected with a luciferase reporter plasmid containing three Foxo3a-binding sites from the FasL promoter (FHRE-Luc, 16), together with an empty plasmid (vector) or a plasmid encoding wild-type (WT) or triple mutant (TM) Foxo3a in which Thr32, Ser253, and Ser315 were converted to Ala residues.[16] Foxo3a TM is constitutively expressed in the nucleus and acts as a dominant active mutant. A pRL-Tk plasmid encoding *Renilla* luciferase was employed for monitoring transfection efficiency. The results showed that sorbitol increased FHRE-Luc activity in cells overexpressing Foxo3a WT to a similar level as overexpressed Foxo3a TM (Figure 3c). Unlike in wild-type cells, sorbitol was capable of stimulating endogenous Foxo3a activity in ERK5- or MEK5-deficient fibroblasts transfected with FHRE-Luc (Figure 3d). The wild-type phenotype was restored following the reintroduction of ERK5 or MEK5 in *erk5*–/– or *mek5*–/– fibroblasts, respectively (Figure 3d). The requirement of the ERK5 signaling pathway to suppress Foxo3a-dependent transcription of the *fasl* gene was further demonstrated by chromatin immunoprecipitation (ChIP) assay (Figure 3e). The experiment showed that sorbitol induced the binding of Foxo3a to the promoter of the *fasL* gene in the *erk5*–/– or *mek5*–/– MEFs 3 h earlier than in the wild-type cells.

Together, these studies suggest that the ERK5 signaling pathway blocked sorbitol-induced transcription via Foxo3a by preventing the nuclear translocation of Foxo3a.

Cross talk between the ERK5 and the PKB signaling pathways

Evidence suggested that recombinant Foxo3a was not a substrate of ERK5 *in vitro* (unpublished data). As PKB-dependent phosphorylation of Foxo3a at Thr32 inhibits FasL expression,[16] we examined the possibility that ERK5 was

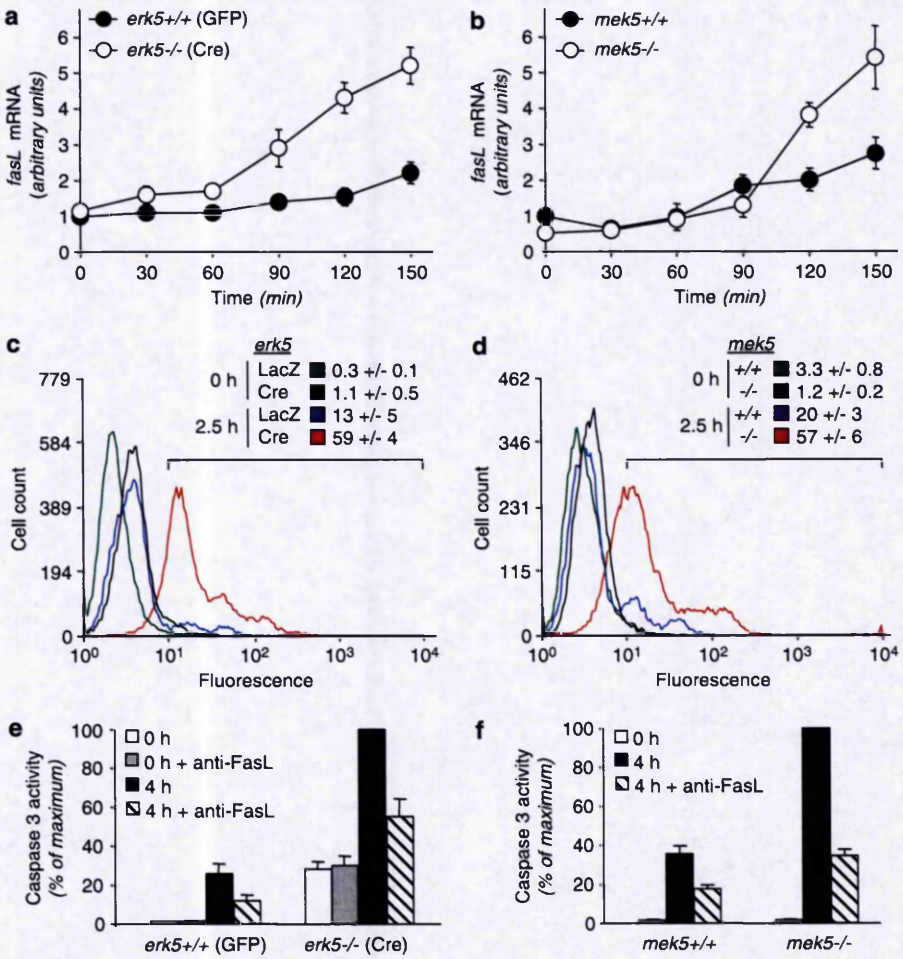

Figure 2 Enhanced sensitivity of ERK5- or MEK5-deficient fibroblasts to sorbitol is caused by increased FasL expression. MEFs were stimulated with sorbitol for the indicated times. (**a**, **b**) Total RNA was extracted and the amounts of *fasL* transcript were measured by quantitative PCR. The data correspond to the mean ± S.E. of three independent experiments performed in duplicate. (**c**, **d**) FasL expression was assessed by flow cytometry. The percent of FasL positive cells ± S.E. of three independent experiments is indicated. (**e**, **f**) Where indicated, the cells were incubated in the presence of a neutralizing anti-FasL antibody. Caspase 3 activity was measured by caspase assay. The data correspond to the mean ± S.D. of duplicate samples. Similar results were obtained in two independent experiments

required for sorbitol-induced PKB activation. The results showed that the deletion of the *erk5* or the *mek5* genes prevented sorbitol from activating PKB (Figure 4a and b). Immunoblot analysis using phospho-specific antibodies demonstrated that absence of MEK5 or ERK5 affected the ability of sorbitol to increase the phosphorylation of PKB at Thr308 and Ser473 (Figure 4c). Consistently, overexpression of a dominant active mutant of MEK5 (MEK5DA) in wild-type fibroblasts expressing Ha-tagged PKB caused a 2.5-fold increased in PKB activity compared to five-fold following sorbitol treatment (Figure 4d). The requirement of the ERK5 signaling pathway in regulating PKB activity was specific in response to stress as we found no difference in the ability of IGF1 to activate PKB in wild-type and *mek5−/−* MEFs (Supplementary Figure S3).

PDK1 is the protein kinase that phosphorylates PKB at Thr308 via a PI3K-dependent mechanism.[13] We found that MEK5 or sorbitol-induced PKB activation was blocked by the pretreatment of the cells with wortmannin or LY294002, two well-characterized PI3K inhibitors (Figures 4d and 5a). The requirement of PI3K in regulating the apoptotic response of MEFs under conditions of osmotic stress was demonstrated by the ability of wortmannin to increase the levels of *fasL* mRNA in wild-type cells treated with sorbitol (Figure 5b). Consistently, wortmannin enhanced the number of FasL-positive cells following sorbitol treatment (Figure 5c). Sorbitol-increased PI3K activity was not affected by the deletion of the *erk5* of *mek5* genes (Figure 5d–g), indicating that the ERK5 signaling pathway promotes cell survival by downregulating FasL expression via a mechanism downstream of PI3K.

Discussion

This study is the first that investigates the molecular mechanism by which the ERK5 signaling pathway promotes

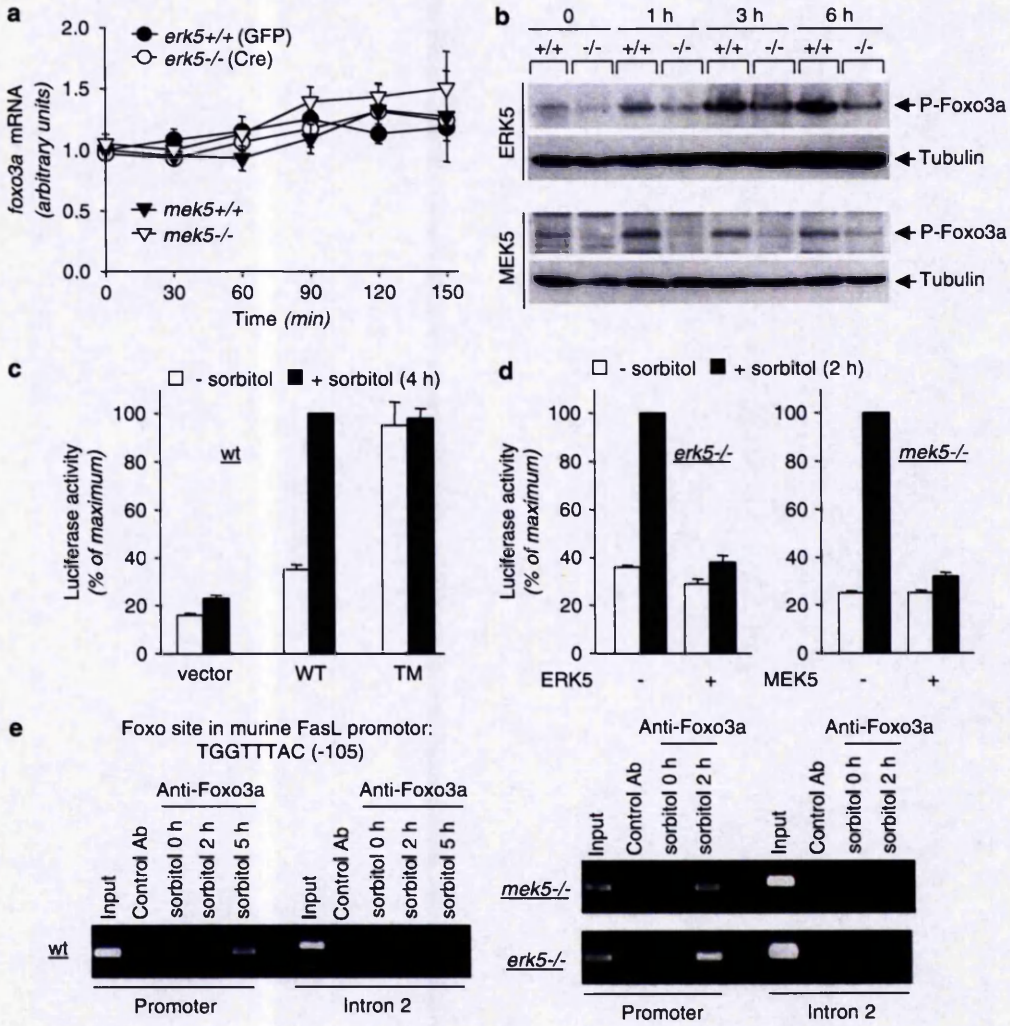

Figure 3 The ERK5 pathway is required to inhibit sorbitol-induced transcription via Foxo3a. MEFs were treated with sorbitol for the indicated times. (**a**) Total RNA was extracted and the amounts of *foxo3a* transcript were measured by quantitative PCR. The data correspond to the mean ± S.E. of three independent experiments performed in duplicate. (**b**) Extracts (50 μg) were analyzed for phosphorylation of Foxo3a at Thr32 by immunoblot analysis using a specific antibody (P-Foxo3a). The detection of tubulin expression was performed to monitor protein loading. Similar results were obtained in two independent experiments. (**c**) Wild-type fibroblasts were transiently cotransfected with the reporter plasmid FHRE-luc together with an empty vector (vector) or a vector encoding wild-type (WT) or dominant active mutant (TM) Foxo3a. (**d**) *erk5−/−* and *mek5−/−* fibroblasts were transiently cotransfected with the reporter plasmid FHRE-luc with (+) or without (−) an expression vector encoding ERK5 or MEK5. Foxo3a-dependent transcriptional activity was measured by the Dual-Luciferase reporter assay system (**c**, **d**). The data correspond to the mean ± S.E. of three independent experiments performed in duplicate. (**e**) Chromatin immunoprecipitation was performed using a Foxo3a-specific or IgG control antibody, followed by PCR-based amplification with primers corresponding to the promoter region (−283/−63) and intron 2 (+2,439/+2,720) of the mouse *fasl* gene. The sequence of the putative Foxo-binding site present in the promoter region at position −105 is indicated. Similar results were obtained in two independent experiments

the survival of mitotic cells (Figure 6). We found that, under basal conditions, the loss of ERK5 expression correlates with increased death of MEFs. This contradicts previous studies that reported no noxious effect associated with *erk5* gene deletion in fibroblasts.[7,9,20] The explanation for such discrepancy may lie in the origin of the cells. Hayashi *et al.*[9] extracted their fibroblasts from adult mice, whereas we have used embryonic cells. The other two studies have used ERK5-null MEFs that, unlike conditionally mutated cells that display a functional *erk5* gene, may have been selected for compensatory survival mechanisms during the immortalization process.[7,20] Similar modifications are likely to be found in the immortalized *mek5−/−* MEFs that did not exhibit any sign of cell death under basal conditions although displaying a low level of Foxo3a phosphorylation (Figure 3b). Alternatively, ERK5 expression may be more important than ERK5 activation in keeping alive proliferating cells. This is consistent with the demonstration that the intrinsic basal ERK5 activity is essential and sufficient to mediate survival of Bcr/Abl-expressing leukemia cells.[21]

In line with this idea, we found that the ERK5 cascade promotes cell survival under basal conditions and in response

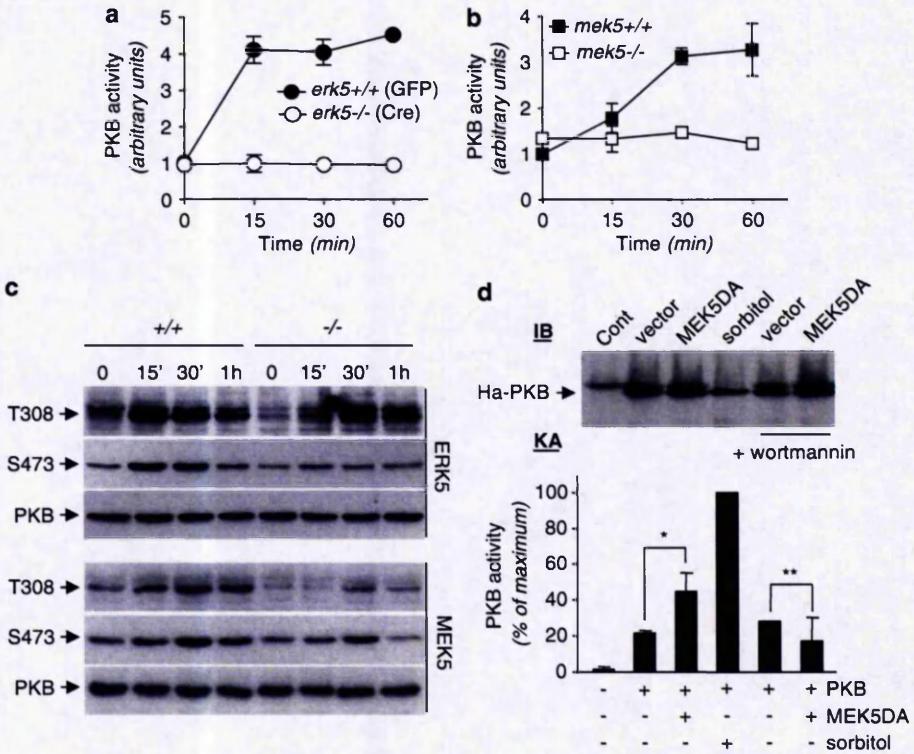

Figure 4 Regulation of PKB activity by the ERK5 signaling pathway. MEFs were incubated with sorbitol for the indicated times. (**a**, **b**) The activity of PKB was measured by protein kinase assay. The data correspond to the mean ± S.E. of three independent experiments. (**c**) Extracts (50 μg) of wild-type (+/+) and homozygous ERK5- or MEK5-null (−/−) fibroblasts were analyzed for PKB expression and phosphorylation at Thr308 and Ser473 by immunoblot analysis using specific antibodies. Similar results were obtained in two to three independent experiments. (**d**) Wild-type fibroblasts were cotransfected with an expression vector encoding Ha epitope-tagged PKB with an empty vector (−) or a vector encoding dominant active MEK5 (+). Where indicated, the cells were stimulated with sorbitol for 30 min or pretreated with wortmannin. The expression of PKB in cell lysates was examined by immunoblot analysis (IB) using an anti-Ha antibody. PKB activity was measured by protein kinase assays (KA). Lysate of untransfected cells was used as control (Cont). The data correspond to the mean ± SE of two independent experiments. *P*-values relative to basal PKB activity are indicated by asterisks: $^{*}P<0.01$; $^{**}P>0.5$

to stress via different mechanisms. Increased FasL expression via upregulation of Foxo3a activity is responsible for enhanced cell death of the *mek5*−/− and *erk5*−/− MEFs treated with sorbitol. The regulation of PKB by the ERK5 pathway constitutes a likely mechanism by which ERK5 inhibits Foxo3a activity (Figure 4). This is consistent with the ability of wortmannin (Figure 5b and c) and of a dominant negative mutant of PKB[22] to upregulate FasL in cells incubated with sorbitol. In contrast, the low basal Foxo3a phosphorylation associated with the loss of ERK5 did not correlate with increased *fasL* transcript (Figure 2a). Consistently, the anti-FasL antibody was unable to reduce activation of caspase 3 caused by *erk5* gene deletion (Figure 2e). Downstream targets of Foxo3a that may contribute to increasing cell death following the loss of ERK5 include Bim.[23] However, no difference in Bim expression was observed between *erk5*−/−, *mek5*−/−, and wild-type cells treated or not with sorbitol (Figure 1e). The contribution of Foxo3a-induced Bim expression may be more relevant to the apoptotic response of hematopoietic cells and neurons deprived of trophic support.[19,23] In contrast, the deletion of the *erk5* gene in fibroblasts correlated with increased phosphorylation of Bim (Figure 1e). The requirement of ERK5 to inhibit Bim phosphorylation, thereby suppressing its proapoptotic activity,[17] provides a possible mechanism by which ERK5 promotes cell survival under basal conditions.

The activation of PKB has been shown to modulate the death response of cells to a number of apoptotic signals.[24] However, its function as a survival factor under osmotic stress conditions remains controversial as PKB has both been shown to be activated[25] and inactivated[26] by sorbitol treatment. Although cell-type differences may explain such discrepancy, the ability of sorbitol to increase PKB activity in fibroblasts has not been consistently observed.[25,27] This could be explained by the fact that sorbitol is a weak activator of PKB making this effect difficult to detect. Our results clearly show that PKB activity is increased by sorbitol in fibroblasts (Figure 4). Overall, our data support the idea that PKB activation is functionally important to act as a brake on the apoptotic process. The specific requirement of the ERK5 signaling pathway in regulating PKB activity in response to sorbitol but not IGF1 (Supplementary Figure S3) underscores the existence of distinct mechanisms implicated in mediating survival signals in response to stress stimuli and growth factors.

Although MEK5DA-induced PKB activation was blocked by the pretreatment of the cells with wortmannin (Figure 4D), we

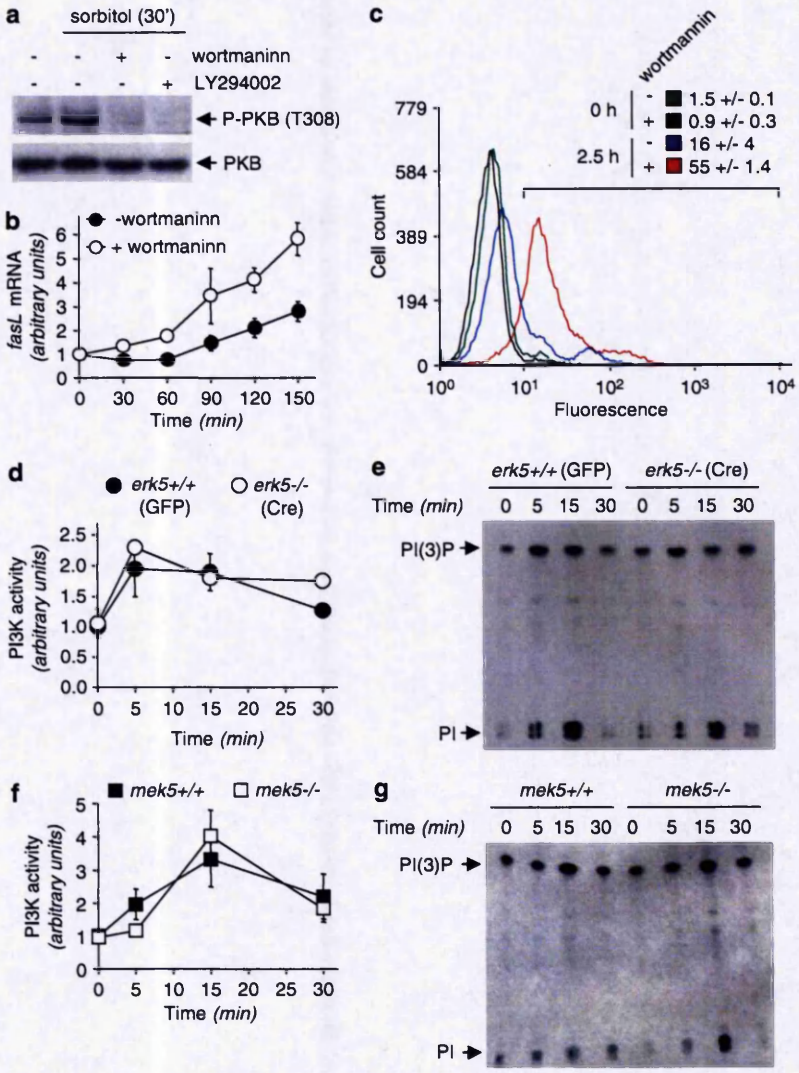

Figure 5 The ERK5 pathway does not affect sorbitol-induced PI3K activation. (**a**, **b**, **c**) Wild-type MEFs were pretreated (+) or not (−) with wortmannin or LY294002 before being incubated with sorbitol for the indicated times. Extracts (50 μg) were analyzed for PKB expression and phosphorylation at Thr308 by immunoblot analysis using specific antibodies (**a**). The figure is representative of two independent experiments. Total RNA was extracted and the amounts of *fasL* transcript were measured by quantitative PCR (**b**). The data correspond to the mean ± SE of three independent experiments performed in duplicate. FasL expression was assessed by flow cytometry (**c**). The percent of FasL-positive cells ± S.E. of three independent experiments is indicated. (**d**, **e**, **f**, **g**) MEFs were incubated with sorbitol for the indicated times. The activity of PI3K was measured by kinase assay. The radioactivity incorporated into PI(3)P was quantitated. The data correspond to the mean ± S.E. of two to three independent experiments

found that sorbitol activated PI3K activity with no marked difference between wild-type and *erk5*−/− or *mek5*−/− MEFs (Figure 5d–g). These data indicate that whereas active PI3K is required for optimal PKB activation, MEK5/ERK5 stimulate PKB activity by a mechanism downstream of PI3K. We have examined the ability of sorbitol to activate PDK1. The high basal level of PDK1 activity detected by protein kinase assay has prevented us from determining whether ERK5 acts upstream of PDK1 or between PDK1 and PKB. Alternatively, decreased PKB activity associated with the loss of the ERK5 signaling pathway may be caused by increased protein phosphatase 2A (PP2A) activity. PP2A has been implicated in inhibiting PKB by promoting its dephosphorylation in response to sorbitol.[26]

Overall, absence of ERK5 or MEK5 expression changes the kinetics of cell death in response to sorbitol. The *erk5*−/− and *mek5*−/− MEFs die quicker than their wild-type counterparts suggesting that the ERK5 cascade delays rather than inhibits the apoptotic process. JNK is most likely responsible for triggering cell death by promoting cytochrome *c* release.[28] In addition to stimulating the intrinsic mitochondrial apoptotic cascade, JNK has been shown to increase FasL expression by a mechanism that implicates the phosphorylation of 14-3-3 and the subsequent nuclear translocation of Foxo3a.[29,30,31]

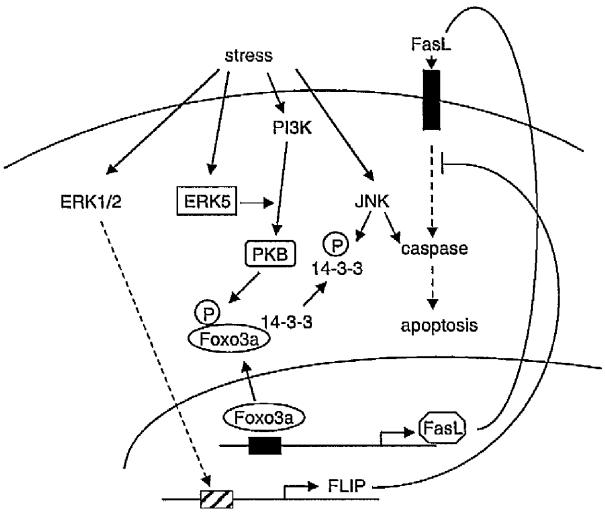

Figure 6 Regulation of cell survival by the ERK5 cascade. ERK5 contributes to inducing the phosphorylation of Foxo3a by PKB via a mechanism downstream of PI3K. Phospho-Foxo3a is sequestered in the cytoplasm via its association with 14-3-3. Under osmotic stress conditions, the nonphosphorylated form of Foxo3a translocates to the nucleus where it increases *fasL* gene transcription. The nuclear translocation of Foxo3a is also triggered by JNK-dependent phosphorylation of 14-3-3. FasL-mediated caspase activation constitutes a positive feedback loop that enhances osmotic stress induced-intrinsic mitochondrial apoptotic cascade via JNK. ERK1/2 suppress sensitivity towards FasL-mediated apoptosis by increasing FLIP expression. The exact mechanism by which ERK5 regulates PKB remains to be identified

The antagonistic effects of JNK and ERK5 on Foxo3a-mediated transcription exemplify their opposite roles in the regulation of cell death and cell survival (Figure 6). Based on these studies, we propose that the survival function of the ERK5/MEK5 cascade may have a more prominent protecting effect on stresses that trigger cell death via a JNK-independent mechanism. In this situation, the level of JNK activity will be low and the ERK5 signaling pathway will be dominant over JNK.

Unlike JNK and similar to ERK5, ERK1/2 promote cell survival.[32] Its ability to increase the expression of the FLICE-like inhibitory protein FLIP suppresses sensitivity towards FasL-mediated apoptosis by preventing caspase 8 activation independently of PKB signaling.[33] The relative importance of ERK1/2 and ERK5 in mediating cell survival is likely to be stimulus- and cell type-specific. For example, whereas ERK1/2 and ERK5 are both required for mediating neuronal survival in response to NGF,[34] ERK5 is critical for the survival of endothelial cells during development.[8,9] Future *in vivo* studies based on mutant mice in which the *erk5* gene can be conditionally deleted will be crucial to establish the physiological importance of ERK5 in regulating trancriptional survival mechanisms via Foxo3a.

Materials and Methods

Tissue culture and preparation of lysates

MEFs obtained from wild-type, *erk5+/loxP*, *erk5loxP/loxP*, and *mek5−/−* embryos were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen), as previously described.[4] The cells were immortalized by successive passages at confluence. Transfection assays were performed using the calcium phosphate method.[35] Sorbitol at 500 mM was used to maximally activate ERK5[4] and to promote death of fibroblasts (Figure 1c and Supplementary Figure S). Time points within 1–2 h stimulation were chosen to study the mechanism upstream of caspase 3, which is maximally activated by sorbitol after 3 h (Figure 1c). Where indicated, the cells were pretreated for 30 min to 1 h with inhibitors as follows: wortmannin (50 nM, Calbiochem), LY294002 (50 μM; Calbiochem). Agonist and inhibitors were added directly to the cell culture medium.

Proteins were extracted from cells in triton lysis buffer (TLB: 20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton-X 100, 25 mM β glycerophosphate, 10% glycerol, 1 mM orthovanadate, 1 mM phenylsulphonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml aprotinin). Extracts were clarified by centrifugation (14 000 × *g* for 10 min at 4°C). The concentration of soluble proteins in the supernatants was quantified by the Bradford method (Bio-Rad).

Viral Infections

The adenoviruses were amplified in 293 cells, the viral solution was purified on $CsCl_2$ gradients, and viral infectivity was determined on NIH3T3 cells. Fibroblasts were infected with recombinant adenovirus at 100 MOI, unless indicated otherwise, for 2 h in serum-free media, and thereafter an equal volume of DMEM containing 4% FBS was added. At 12 h after infection, the virus was removed, and the cells were cultured in media containing 2% FBS for a further 12 h unless indicated otherwise. Mock-infected MEFs used as control were placed for up to 60 h in 2% FBS without displaying any sign of apoptosis.

Immunoblot Analysis

Extracts (50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12, 10, or 8% polyacrylamide gel) and electrophoretically transferred to an Immobilon-P membrane (Millipore, Inc.). The membranes were incubated with 3% nonfat dry milk at 4°C for 30 min and then probed overnight with antibodies to ERK5 (Upstate Biotechnology), tubulin (Sigma), Cre (Chemicon), caspase 3 (Cell Signaling), JNK (Santa Cruz), phospho-JNK (New England Biolabs), Bid (R&D Systems), Bim (Calbiochem), phospho-Foxo3a (Upstate Biotechnology), PKB (Cell Signaling), phospho-PKB (Cell Signaling), and Ha (Covance). Immunecomplexes were detected by enhanced chemiluminescence with anti-mouse or anti-rabbit immunoglobulin G coupled to horseradish peroxidase as the secondary antibody (Amersham-Pharmacia).

Kinase assays

PKB activity was measured in cell lysates following precipitation with a polyclonal antibody to PKB (Upstate Biotechnology) or to Ha (Covance). The kinase reaction was performed at 30°C for 30 min in 25 mM HEPES pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM DTT, 0.1 mM orthovanadate containing 50 μM [γ-^{32}P]ATP (10 Ci/mmol), and the peptide RPRAATF (Upstate Biotechnology) at 0.5 mM. The reaction was stopped and spotted onto 2 cm^2 P81 filter papers. The radioactivity bound to the filters was quantitated by liquid scintillation counting. PI3K activity was measured in cell lysates following precipitation with a polyclonal antibody to PI3K (Upstate Biotechnology) as previously described.[36] Briefly, the kinase reaction was performed at 30°C for

10 min in 50 mM Tris pH 7.4, 80 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA) containing 3 μg phosphatidylinositol (PI), 10 μg phosphatidylserine, and 50 μM [γ-^{32}P]ATP (10 Ci/mmol). After the reaction was stopped, the lipids were extracted, dried, resuspended in chloroform, and spotted on to a silica gel-coated glass tlc plate (Merck). Plates were resolved and exposed to autoradiography film. The radioactivity was quantitated by PhosphorImager analysis.

Reporter gene expression assay

The reporter plasmid FHRE-luc[16] was transiently cotransfected with or without expression vectors encoding wild-type (WT) or triple mutant (TM) Foxo3a,[16] ERK5, or MEK5. A pRL-Tk plasmid encoding *Renilla* luciferase was employed for monitoring transfection efficiency. Aliquots of cell lysates were assayed for firefly and *Renilla* luciferase activities according to the manufacturer's instructions (Promega). Cell viability was quantified by luciferase activity following transfection with the pCMV luciferase plasmid.[37]

Caspase assay

Cell extracts (20 μg) were lysed in 10 mM HEPES pH 7.5, 150 mM NaCl, 2 mM EDTA containing 0.5% NP40. Extracts (20 μg) were incubated with 200 μM DEVD-AMC caspase 3-specific fluorogenic substrate for 1 h. Cleavage of the substrate was measured by spectrofluorometer.

Flow cytometry

MEFs were trypsinized and incubated for 1 h at 4°C in phosphate-buffered salin (PBS) containing 5% FBS and 10 μg/ml primary antibody against FasL (MFL3, BD Pharmingen). After three washes in PBS, cells were incubated for 30 min at 4°C in PBS containing 5% FBS and 10 μg/ml fluorescein isothiocyanate (FITC)-conjugated goat anti-Armenian hamster IgG (Jackson ImmunoResearch Laboratories). Following three washes in PBS, cells were analyzed by FACScan (DAKO CYAN) with Summit 3.3 software at 488 nm excitation and the emission was measured with a 530/40 bandpass filter. Cells stained with FITC-conjugated goat anti-Armenian hamster IgG only served as internal control.

ChIP assay

Samples were prepared using ChIP-IT Enzymatic kit (Active Motif) and anti-Foxo3a antibody (Upstate Biotechnology). DNA samples recovered from DNA–Protein immunocomplexes were amplified by PCR using the following primer sets:[38] promoter region containing FasL-binding site (−283/−63) 5′-GATTCAACTCCCTATGCTCAGATGTGGAG-3′ and 5′-TCCAATTGGCGTCTCTGTGCTAACTGAGAAG-3′; Intron2 region, served as negative control (+2439/+2720) 5′-CAAAGGACCAGAGCCCAGGATACAC-3′ and 5′-GTTGCGATTTTGATACTTTTGTGATGG-3′.

Real-time PCR

Total RNA was isolated using the Trizol™ reagent and cDNA synthesis was carried out as previously described.[7] Real-time quantitative PCRs were performed using the SYBR Green I Core Kit (Eurogentec). Primers used were: forward primer, 5′-AGCCCCTAAACCACAAGGTC-3′ and reverse primer, 5′-TGAATACTGCCCCCAGGTAG-3′ for *fasL*; forward primer, 5′-CTTGTCAAATTCTGTCAGCAACA-3′ and reverse primer 5′-AGGTTTGCACTAGCTGAATACA-3′ for *foxo3a*; and forward primer, 5′-CCAACTTGATGTATGAAGGCTTTG-3′ and reverse primer 5′-AATTGGTCTCAAGTCAGTGTACAGGC-3′ for *β-actin* to generate amplicons of 135, 150, and 91 bp, respectively. PCR products were detected in the ABI-PRISM 7700 sequence detection systems (Applied Biosystems). Results were analyzed using the $2^{-\Delta\Delta G}$ methods.[39] The level of expression of mRNA was normalized to *β-actin* mRNA.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)

Supplement information for the manuscript 3

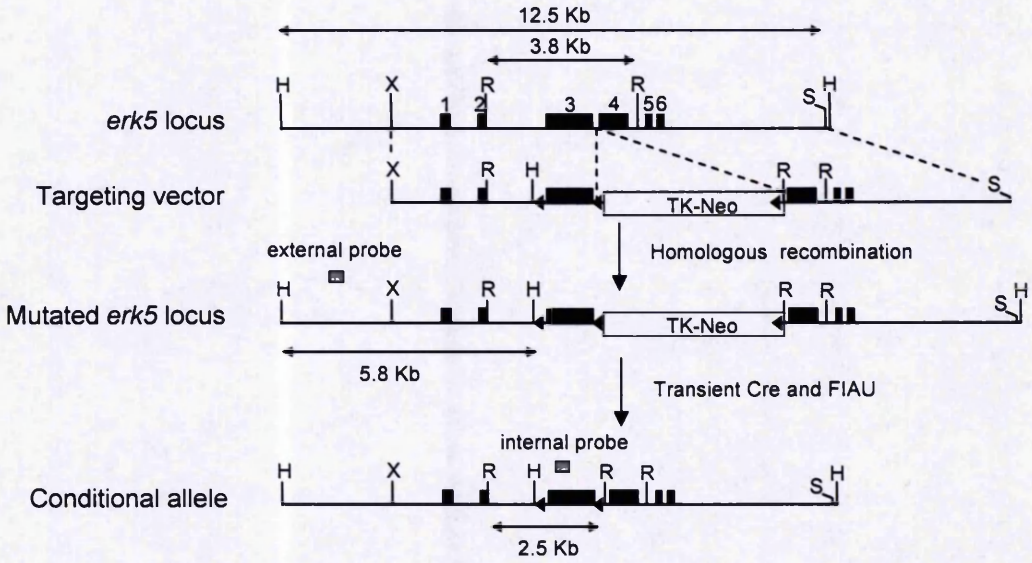
Figure S1 Strategy for the mutation of the *erk5* gene. (**A**) The genomic region at the *erk5* locus, the *erk5* targeting vector and the predicted structure of the mutated *erk5* gene are depicted. Restriction enzyme sites are indicated (H, HindIII; R, EcoR1; S, SpeI; X, XhoI). Black boxes are *erk5* exons. The white box is the thymidine kinase neomycin cassette (TK-Neo). (**B**) Southern blotting analysis of HindIII (1st targeting) and EcoR1 (2nd targeting) restricted genomic DNA prepared from ES cell clones indicates the presence of wild type (*erk5*+) and mutant (*erk5loxP/TK-Neo* and *erk5loxP*) alleles. The blots were probed with random-primed ^{32}P-labeled mouse ERK5 genomic probes (see grey box in panel A). (**C**) Genomic DNA isolated from mouse tails were amplified by PCRs with primers specific for the *erk5* gene.

Figure S2 The *mek5-/-* fibroblasts display enhanced sensitivity to sorbitol. Wild type (+/+) and homozygous *mek5* deleted (-/-) MEFs were untreated (0) or treated with sorbitol (500 mM) for the indicated times. (**A**) Cell survival was measured by MTT assay. The data represent the mean optical density expressed as % of untreated fibroblasts ± SE of triplicate samples. The figure is representative of three independent experiments. (**B**) DNA was extracted and analyzed by gel electrophoresis. DNA laddering is seen in the *mek5-/-* but not in the wild type cells. (**C**) Extracts (50 μg) were analyzed for caspase 3 and Bid expression by immunoblot analysis using specific antibodies.

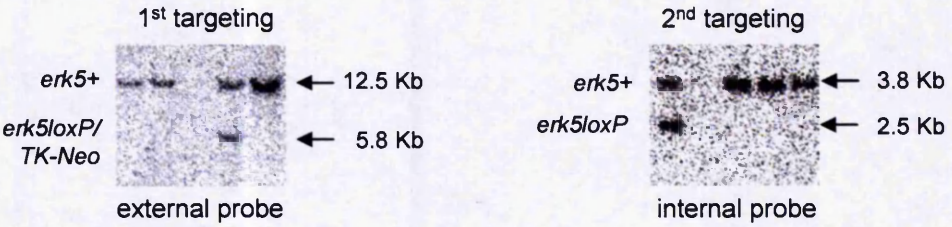
Figure S3 The ERK5 pathway is not required for mediating IGF1-induced PKB activation Wild type (*mek5*+/+) and homozygous null (*mek5-/-*) MEFs were incubated with IGF1 (40 ng/ml) for the indicated times. Extracts (50 μg) were analyzed for PKB expression and phosphorylation at Thr308 and Ser473 by immunoblot analysis using specific antibodies.

S1

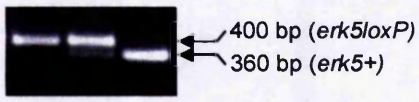
A


12.5 Kb
3.8 Kb
erk5 locus
H
X
R
1
2
3
4
5
6
S
Targeting vector
TK-Neo
external probe
Homologous recombination
Mutated erk5 locus
5.8 Kb
Transient Cre and FIAU
internal probe
Conditional allele
2.5 Kb

B

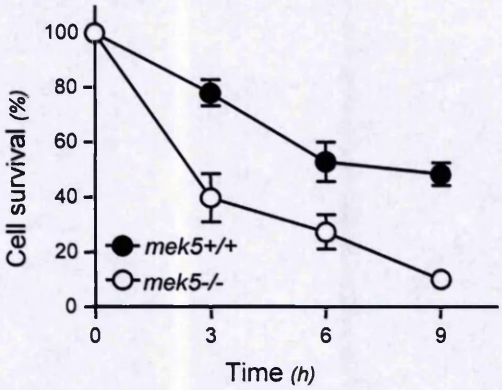

1st targeting
erk5+
erk5loxP/
TK-Neo
12.5 Kb
5.8 Kb
external probe
2nd targeting
erk5+
erk5loxP
3.8 Kb
2.5 Kb
internal probe

C

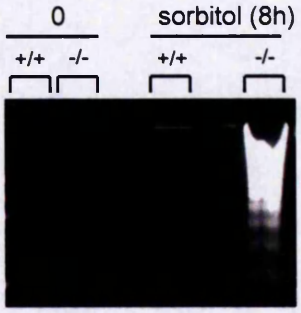

400 bp (erk5loxP)
360 bp (erk5+)

S2

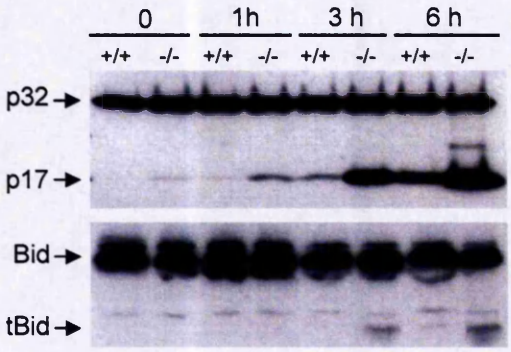
A


100
80
60
40
20
0
Cell survival (%)
mek5+/+
mek5-/-
0
3
6
9
Time (h)

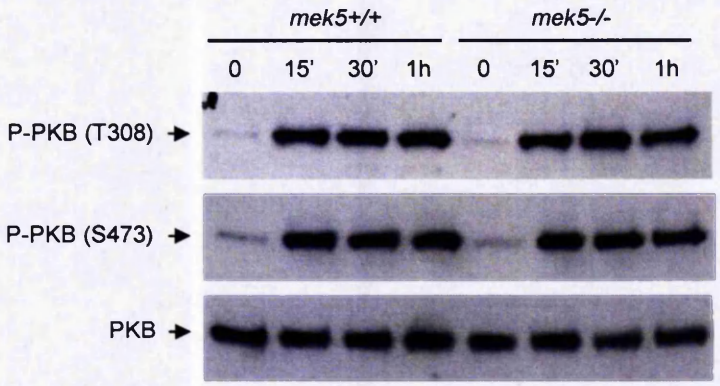
B


0
sorbitol (8h)
+/+
-/-
+/+
-/-

C


0
1h
3 h
6 h
+/+
-/-
+/+
-/-
+/+
-/-
+/+
-/-
p32
p17
Bid
tBid

S3



V - Discussion

My studies were based on the use of the *mek5-/-* and *erk5-/-* fibroblasts and mice, which were generated by gene targeting technology following homologous recombination in embryonic stem (ES) cells (Wang et al., 2005; Kayahara et al., 2005; Wang et al., 2006). The first report of germline transmission of a mutation engineered in ES cells occurred at the HPRT locus (Thompson et al., 1989). Since then, gene targeting technology has led to the production of a variety of mouse models to assess the phenotypic consequences of specific genetic mutations in many loci. In addition to conventional targeting techniques, the conditional inactivation of gene expression using the Cre/LoxP system allows the temporal- and/or tissue-specific ablation of gene expression (Brocard et al., 1997; Jonkers et al., 1997). This strategy involves the generation of mutant mice in which the gene of interest is flanked by LoxP sites. These sites do not interfere with the normal expression of the gene but constitute a binding domain for the DNA recombinase Cre which excises the DNA fragment between the LoxP sites. Thus, the deletion of the LoxP gene to a particular cell lineage at a certain time during development can be triggered in vivo by crossing the mutant LoxP mice with inducible and/or tissue-specific Cre expressing transgenic animals. The Cre/LoxP system circumvents the problem of early embryonic lethality associated with the genetic modification and permits a more accurate understanding of the physiological function of signalling pathways during development and pathogenesis. For example, the analysis of mutant mice in which the *erk5* gene could be specifically deleted in endothelial cells has demonstrated that abnormal development of the heart exhibited by

the ERK5 null embryos was caused by enhanced cell death associated with absence of ERK5 (Regan et al., 2002; Sohn et al., 2002; Yan et al., 2003; Hayashi et al., 2004).

Advantages of using a system in which gene deletion can be induced is further illustrated in my study on cell survival using immortalised *erk5/LoxP* fibroblasts (Wang et al., 2006). The steady expression of ERK5 throughout the immortalisation process has prevented the selection of cells from displaying compensatory survival mechanisms, and showed for the first time that ERK5 expression may be more important than ERK5 activation in keeping alive proliferating cells. Furthermore, control experiments using a recombinant virus expressing GFP instead of Cre can be performed in an identical cellular background. Using this cells model I found that sorbitol-induced phosphorylation of Foxo3a was hampered in the absence of ERK5 or MEK5 (Wang et al., 2006). Further experiments indicated that ERK5-dependent inhibition of Foxo3a activity via PKB was required for suppressing Fas ligand (FasL) expression. This is consistent with a previous study where suppression of PKB signalling was reported to up-regulate FasL (Suhara et al., 2002). In parallel to my study, Pi et al have demonstrated that the survival response of ECs to shear stress is mediated by a mechanism which implicates the phosphorylation of Bad at Ser136 and Ser112, via ERK5, but independent of PKB (Pi et al., 2004).

The ERK5/LoxP mice have also been used to prepare ERK5-deficient sympathetic neurones (unpublished data). Consistent with previous reports, we found that ERK5 is required for mediating neuronal survival following NGF withdrawal (Watson et al, 2001). These results raise the question of the *in vivo* role of ERK5 in the nervous system. Although *erk5* and *mek5* null mice displayed severe growth retardation

in the head region (Wang et al., 2005; Yan et al., 2003), mice in which the *erk5* gene has been specifically deleted in the brain develop normally (Hayashi and Lee, 2004; our unpublished results). This result contrasts with the reduced head structure in *Xenopus* associated with knock down of MEK5 or ERK5 expression using antisense morpholino oligonucleotides (Nishimoto et al., 2005). The defect is not caused by increased apoptosis but arises from the inhibition of neural differentiation associated with the specific suppression of neural markers. Differences in phenotypic abnormalities between mice and *Xenopus* may be explained by the presence of redundant signalling cascades in higher eukaryotes that compensate for the loss of the ERK5 pathway. Under physiological conditions, it remains highly likely that ERK5 participates in the normal development of the brain.

In addition to being an important contributor of cell survival, ERK5 is a component of the growth promoting signals of mitogens, such as epidermal growth factor (EGF) (Wang and Tournier, 2006). However, the molecular mechanisms by which ERK5-mediated cell proliferation and oncogenic processes have yet to be identified. We have recently demonstrated that ERK5 is selectively required for the regulation of *c-jun* mRNA and protein expression following EGF stimulation of the cells, but not in response to UV radiation (Kayaha et al., 2005). Consistently, I found that the *erk5-/-* and *mek5-/-* fibroblasts proliferate normally but display a specific defect in the ability of EGF to prevent a G1/S arrest following serum starvation (my unpublished data). Increased *c-jun* gene expression via ERK5 constitutes a likely mechanism by which ERK5 delivers growth signals, including increasing cyclin D1 expression (Mulloy et al., 2003). Future work using DNA array technology will explore

this hypothesis by identifying novel genes whose expression is modulated by ERK5 via c-Jun.

My next challenge will be to assess the functional significance of the ERK5 signalling pathway in pathological processes, such as heart diseases, brain injury, neurodegenerative diseases, and cancers using mutant mice in which the *erk5* gene is specifically deleted in heart, brain, and skin. These results combined with the analysis of mutant mice deficient in MEKK2/3, MEK5, or MEF2C expression will establish the molecular mechanism by which the ERK5 signalling pathway regulates cell function *in vivo*.

VI - References

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VII – Appendices:

Appendix I:

Wang, X., Tournier, C. (2006) Regulation of cellular functions by the ERK5 signalling pathway. *Cell. Signal.,* **18,** 753-760.

Appendix II:

Seyfried, J., **Wang, X.,** Kharebava, G., and Tournier, C. (2005) A novel mitogen-activated protein kinase docking site in the N-terminus of MEK5α organizes the components of the extracellular signal-regulated kinase 5 signalling pathway. *Mol Cell Biol.,* **25,** 9820-9828.

Appendix III:

Papadakis, E.S., Finegan, K.G., **Wang, X.,** Robinson, A.C., Guo, C., Kayahara, M. and Tournier, C. (2006) The regulation of Bax by JNK is a prerequisite to the mitochondrial-induced apoptotic pathway. *FEBS Lett.,* **580,** 1320-1326.

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Review

Regulation of cellular functions by the ERK5 signalling pathway

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Abstract

Extracellular-regulated protein kinase 5 (ERK5) is a mitogen-activated protein kinase (MAPK) regulated by a wide range of mitogens and cellular stresses. Since its cloning in 1995, the lack of biological tools, including antibodies and specific inhibitors, have made it one of the least studied MAPK subfamilies. The discovery that ERK5 was an important contributor to cell survival mechanisms has increased interest in this signalling pathway. The ability of inhibitors of the classical MAPK (ERK1/2) cascade to block ERK5 activation suggested that ERK5 might regulate some cellular functions originally attributed to ERK1/2. For example, ERK5 is suspected to mediate the effects of numerous oncogenes. A link between abnormal levels of ERK5 expression and cancers was established by the analysis of human tumours. Recently, the targeted deletions of the *erk5* and the *mek5* genes in mice have provided genetic evidence that the ERK5 cascade is a non-redundant signalling pathway essential for normal cardiovascular development. The analysis of genetically modified mice in which the *erk5* gene can be specifically deleted in certain tissues is shedding light into the physiological function of the ERK5 pathway during development and pathogenesis.

Keywords: MAPK; ERK5; BMK1; MEK5; MEKK; Signal transduction; Proliferation; Survival; Transformation; Heart

Contents

Abbreviations: AP-1, activator protein 1; CREB, Ca^{++}/cAMP-responsive element binding protein; E, embryonic day; ECs, endothelial cells; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; FasL, Fas ligand; FGF, fibroblast growth factor; Foxo3a, Forkhead box O3a; G-CSF, granulocyte colony-stimulating factor; Gab1, Grb2-associated binder 1; HIF1α, hypoxia inducible factor 1α; Lad, Lck-associated adapter; LIF, leukemia inhibitory factor; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor 2; MEK, MAPK/ERK kinase; MEKK, MEK kinase; NES, nuclear export signal; NF-κB, nuclear factor kappa B; NGF, nerve growth factor; NLS, nuclear localisation signal; PB1, phox and Bem1p; PKB, protein kinase B; RSK, p90 ribosomal S6 protein kinase; SGK, serum- and glucocorticoid-regulated protein kinase; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor; WNK1, with no lysine (K).

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1. Introduction

The mitogen-activated protein kinases (MAPKs) are crucial components of signalling cascades that regulate numerous physiological processes during development and pathogenesis [1,2]. At least four subfamilies have been identified: extracellular-regulated protein kinases 1 and 2 (ERK1/2), ERK5, c-Jun NH_2-terminal protein kinases (JNKs), and p38 MAPKs. They belong to an evolutionary conserved family of proline-directed protein kinases that phosphorylate Ser and Thr residues preceding a Pro residue. The specificity is determined by docking domains within substrates [3]. These include the D domain that consists of a conserved cluster of positively charged amino acids surrounded by hydrophobic amino acids. The D domain is recognised by amino acid sequences in the C-termini of the MAPKs identified as the common docking (CD) domain. By tethering the substrate to the MAPK, docking interactions contribute to the efficiency of the kinase reaction. MAPKs control by phosphorylation the activity of numerous transcription factors and enzymes, through regulating binding partners, conformational changes, subcellular localisation, and protein stability [1,4].

MAPK activity is increased following phosphorylation at Thr and Tyr residues within a Thr-X-Tyr (T-X-Y) motif in the activation loop by a MAPK/ERK kinase (MEK or MKK) [1]. X corresponds to a Glu residue in ERK, a Pro residue in JNK and a Gly residue in p38 MAPK subfamilies. MAPK activators include MEK1 and MEK2 for ERK1/2, MEK5 for ERK5, MKK4 and MKK7 for JNKs, and MKK3 and MKK6 for p38 MAPKs. MEKs are activated by phosphorylation at Ser and Thr residues by a MEK kinase (MEKK). Two main mechanisms have been proposed to ensure specific transmission of the signals from upstream kinases to MAPKs [5,6]: (i) adaptor/scaffold proteins that assemble the different components of a cascade, and (ii) direct physical interactions between the components of a cascade. Both mechanisms may operate in parallel and allow distinct responses of the same MAPK signalling pathways to different stimuli. Gene targeting experiments in mice have provided evidence that MAPK modules are associated with different biological responses [7]. For example, the ERK subfamilies are mostly associated with cell proliferation and survival, while JNKs and p38 MAPKs are mainly activated in response to cytokines and extracellular stresses and can mediate apoptosis.

2. Identification of ERK5

2.1. Cloning

Two groups cloned ERK5 in 1995. Dixon and co-workers [8] first identified the ERK5 activator, MEK5. Based on the assumption that MEKs interact with MAPKs, they used MEK5 as bait in a yeast two-hybrid screen and identified ERK5 as a binding partner. Further studies confirmed the binding of ERK5 with MEK5 but not with MEK1 or MEK2 supporting the highly specific nature of the MEK/MAPK interaction. At the same time Lee et al. [9] identified a protein identical to ERK5 that they called big MAPK 1 (BMK1). The full-length sequence of human ERK5 contains an open reading frame of 2445-base pairs encoding 816 amino acids. With a predicted molecular mass of 98 kDa, ERK5 is more than twice the size of the other MAPKs due to a 396 amino acid C-terminal extension. The N-terminal conserved MAPK catalytic domain shares 50% homology with ERK1/2. Further analogy between ERK5 and ERK1/2 subfamilies is illustrated by the ability of the commonly used MEK1/2 inhibitors, PD98059, U0126, and PD184352, to block the ERK5 pathway [10,11].

Gene analysis reveals that the mouse *erk5* gene is encoded by 6 exons and 5 introns spanning ~5.6 kb. Alternative splicing across introns 1 and/or 2 generate three isoforms, a, b, and c [12]. Compared with ERK5a, ERK5b and c lack 69 and 139 amino acids at their N-termini, respectively. Immunoblot analysis of a mouse embryonic extract showed that ERK5a is expressed to a greater extent than ERK5c and much greater than ERK5b [12]. Both ERK5b and c are deficient in protein kinase activity and can block ERK5a activation by MEK5 and ERK5-induced transcription via MEF2C [12]. A premature in-frame stop codon introduced by failure to splice intron 4 gives rise to another mouse splice variant, *erk5-T*, which encodes a truncated protein at the C-terminal end unable to translocate to the nucleus [13]. ERK5-T can retain ERK5 in the cytoplasm upon stimulation by forming heterodimers [13]. Together, these data suggest that ERK5 splice variants may act as dominant negative mutants of the full-length protein, thereby providing a novel regulatory mechanism of ERK5 activity. Further studies will need to be performed to clarify in vivo the physiological function of alternative splicing of the *erk5* gene in specific tissues under specific conditions.

2.2. Functional domains

The N-terminal domain of ERK5a is important for cytoplasmic targeting (aa 1–77), association with the upstream kinase MEK5 (aa 78–139) and oligomerisation (aa 140–406) [12]. Unlike ERK1/2, ERK5 oligomerises in both stimulated and unstimulated conditions. The unique and very large C-terminal domain is required for the dynamic nuclear shuttling of ERK5 [14]. It contains both nuclear localisation and export signals (NLS and NES), and two proline rich (PR1 and PR2) regions that may serve as binding sites for proteins containing Src-homology 3 (SH3) domains [8,12,14]. Interestingly, the PR regions of the mouse and the human proteins differ significantly in their sequence. The identification of specific binding partners that interact with ERK5 may reveal significant differences in signalling mechanisms between human and mouse. The C-terminal tail also has an auto-inhibitory function that influences the activation of ERK5. This is demonstrated by enhanced ERK5 activity following deletions of the C-terminus [8,14]. These studies suggest a model where the C-terminal tail masks the CD domain preventing the interaction of ERK5 with its substrates. Upon stimulation, ERK5 phosphorylates its C-terminus thereby causing a conformational change that exposes the docking site and the NLS [14]. This explains the dynamic nuclear shuttling of ERK5. However, the cellular distribution of ERK5 varies largely between different cell types under non-stimulated conditions ranging from an overall diffuse pattern to predominantly nuclear [14].

2.3. Downstream targets

An important step in understanding the function of a MAPK cascade is to identify its downstream effectors. The transcription factors of the myocyte enhancer factor (MEF) family, MEF2A, C, and D are among the best-characterised substrates of ERK5 [15–17]. Phosphorylation of MEF2C by ERK5 enhances its transcriptional activity and subsequently leads to increased c-jun gene expression [15]. Consistent with this study, we have recently demonstrated that ERK5 is selectively required for the regulation of c-Jun expression following epidermal growth factor (EGF) stimulation of the cells, but not following UV radiation [18]. While MEF2D is a specific substrate of ERK5, MEF2A and MEF2C activities are controlled by both p38 MAPKs and ERK5 [15–17,19–21]. However, p38 MAPKs fail to compensate for the defect in MEKK2/3-induced transcription via MEF2A caused by *mek5* gene deletion, indicating that the ERK5 signalling pathway is essential for regulating MEF2A activity [22]. The C-terminal tail of ERK5 contains a MEF2-interacting region (aa 440–501) and a transcriptional activation domain (aa 664–789) essential for co-activation of MEF2 [23]. The functional importance of the tail in ERK5-mediated transcription via MEF2 is consistent with the inability of a C-terminal truncated ERK5 mutant that possesses an active protein kinase, to stimulate MEF2 activity [12].

Other direct substrates of ERK5 include Sap1, and c-Myc [10,24]. While the effect of phosphorylation of c-Myc by ERK5 remains unclear, ERK5-dependent phosphorylation of Sap1 enhances transcription via a serum response element that may be responsible for increasing expression of c-Fos [10,25]. In addition to acting on the c-fos promoter, the ERK5 signalling pathway stimulates the transcriptional activity of c-Fos and Fra-1 by a mechanism that implicates a kinase lying downstream of ERK5, which could be p90 ribosomal S6 protein kinase (RSK) [25]. Increased RSK activity may also be implicated in mediating the co-operative effect of ERK5 and ERK1/2 to regulate nuclear factor kappa B (NF-κB) function [26]. In contrast to MEF2, Fos does not interact with ERK5 [25]. However, similar to MEF2 regulation, a C-terminal truncated ERK5 mutant is unable to stimulate Fos activity [25]. This is consistent with the requirement of ERK5 to enter the nucleus for maximal Fos activation.

3. Regulation of ERK5 activity

3.1. Dual phosphorylation of ERK5 by MEK5

ERK5 activity is increased in response to growth factors, oxidative stress and hyperosmolarity via the dual phosphorylation of its TEY motif by MEK5 [27] (Fig. 1). The phosphorylation of ERK5 by MEK5 may contribute to stabilising ERK5 in an active conformation promoted by the auto-phosphorylation of its C-terminal tail [14,24,27]. The physiological significance of MEK5 was demonstrated by the analysis of *mek5* gene ablation in mice [22]. Similar to the *erk5*−/− embryos [28–30], the *mek5*−/− fetuses displayed abnormal cardiac development.

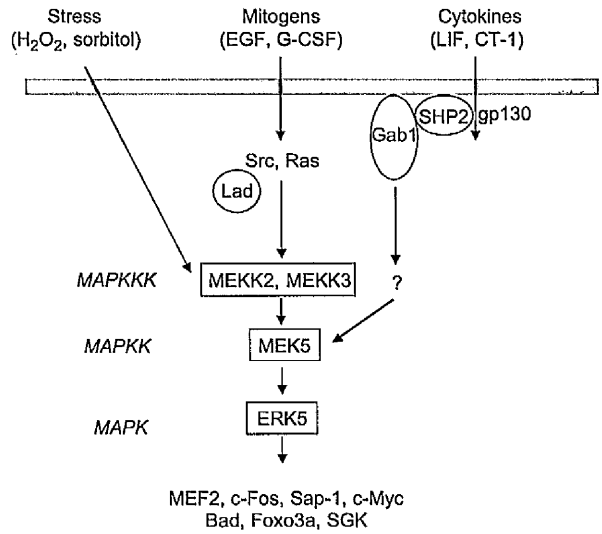

Fig. 1. Regulation of ERK5 by mitogens and extracellular stress stimuli. ERK5 is activated by dual phosphorylation at Thr and Tyr residues in the activation loop by MEK5. MEKK2 and MEKK3 activate MEK5 in response to mitogenic and stress stimuli. Mitogens stimulate ERK5 signalling via Ras dependent and independent mechanism. A functional Lad/MEKK2 complex is required to mediate Src-induced ERK5 activation. Cytokines including LIF and CT-1 induce the phosphorylation of Gab1 and SHP2 at Thr residues, thereby leading to the association of Gab1 to SHP2 and the activation of ERK5. Downstream targets of ERK5 are indicated.

The MEK5 cDNA encodes a 444-amino acid protein, which displays approximately 40% identity to known MEKs [8,31]. The alternative splicing of the mRNA gives rise to two isoforms with different N-termini, MEK5α (50 kDa) and MEK5β (40 kDa) [31]. The N-terminal extension of MEK5α is implicated in its restricted localisation to the particulate fraction while MEK5β is ubiquitously distributed and is primarily cytosolic [31]. MEK5β has been proposed to act as a kinase dead dominant negative variant that can suppress ERK5 signalling [32]. However, consistent with the ability of a MEK5β transgene to activate ERK5 in vivo [33], we found that MEK5β is an active enzyme [34]. Our results indicate that MEK5α is a stronger activator of ERK5 than MEK5β due to its higher affinity for ERK5. This led us to identify a novel MAPK docking site in the N-terminus of MEK5α that is distinct from the consensus motif identified in other MEKs [3]. It consists of a cluster of acidic residues at position 61 and 63–66 on the rat sequence. This domain is critical for MEK5α to promote maximal ERK5 activation and to increase transcription via MEF2 [34].

3.2. Upstream kinases

Both MEKK2 and MEKK3, but not MEKK1, are able to activate MEK5 in a stimulus and cell type specific manner [31,35,36] (Fig. 1). WNK1 (with no lysine (K)) was recently identified as a protein kinase upstream of MEKK2/3 in the ERK5 signalling pathway [37]. The N-terminal extension of MEK5α contains a phox and Bem1p (PB1) domain that mediates the binding interaction of MEK5α with MEKK2/3 [38]. Blocking the PB1-dependent formation of the MEKK/MEK5 complex prevents MEK5 activation [34,38]. This appears inconsistent with the ability of MEKK2 to increase MEK5β activity [34]. Indeed, MEK5β that lacks the PB1 domain is unlikely to bind MEKK2. To explain such discrepancy we propose that the N-terminus of MEK5α contains an auto-inhibitory domain that masks the phosphorylation sites of MEK5 by upstream kinases [34]. The interaction of MEK5 with MEKK2 affects the overall conformation of MEK5α so that Ser311 and Thr315 become accessible for phosphorylation.

As both MEKK2 and ERK5 interact with the N-terminal extension of MEK5α, it is possible that MEKK2 and ERK5 compete for binding to MEK5 rather than forming a ternary complex. Based on a similar model proposed to explain the organisation of the JNK signaling pathway [39], we hypothesise that MEKK2 and MEK5 form a complex that is dissociated upon activation, so that activated MEK5 becomes free to interact with its substrate ERK5 [34].

3.3. Adaptor/scaffold proteins

Adaptor/scaffold proteins facilitate and specify MAPK activation in response to physiological stimuli by forming multienzyme complexes [5]. For example, a functional interaction between MEKK2 and the Lck-associated adapter (Lad) is required for EGF-induced ERK5 activation via Src [40] (Fig. 1). Lad may be responsible for increasing the binding affinity between MEKK2 and MEK5, and for recruiting the MEKK2/MEK5 complex to the receptor. Grb2-associated binder 1 (Gab1) is another adaptor protein implicated in mediating ERK5 activation in cardiomyocytes in response to leukemia inhibitory factor (LIF) via the gp130 signal complex [41] (Fig. 1). LIF-induced tyrosine phosporylation of Gab1 and protein tyrosine phosphatase SHP2 leads to their association. Gab1/SHP2 interaction is crucial for LIF-induced elongation of cardiomyocytes via ERK5 [41]. The muscle specific protein kinase A (PKA) anchoring protein mAKAP also contributes to the transduction of the hypertrophic signal via ERK5 in cardiomyocytes [42]. MEK5 and ERK5 interact with the phosphodiesterase E4D3 (PDE4D3) and the cAMP-dependent exchange factor for the small GTPase Rap1 (Epac1) to form a functional complex with PKA and mAKAP. ERK5-mediated PDE4D3 phosphorylation decreases cAMP catabolism. The subsequent rise in cAMP concentration activates Epac1 that suppress LIF-induced ERK5 activation via Rap1 [42].

4. Role of ERK5 in cell survival

4.1. ERK5 regulation of neuronal survival

Suzaki et al. were the first to implicate ERK5 in the survival response of PC12 cells to oxidative stress [43]. The physiological significance of these results was provided by the demonstration that ERK5 contributes to the survival response of dorsal root ganglion (DRG) neurones to neuronal growth factor (NGF) [44]. TrkA receptors present at the surface of the extending axon auto-phosphorylate following the binding of NGF. Phosphorylated TrkA receptors are internalised into a "signalling endosome" that is retrogradely transported from the extending axon to the cell body where it activates ERK5. ERK5 then initiates a phosphorylation cascade resulting in the activation of the transcription factor Ca^{++}/cAMP response element binding protein (CREB) that regulates the transcription of survival and pro-apoptotic genes [44]. Evidence that ERK5 contributes to mediating the survival of neurones in the central nervous system via the activation of the transcription factor MEF2 has since been reported [45,46]. A more detailed account of the function of the ERK5 signalling pathway in neuronal survival can be found in a recent review by Cavanaugh [47].

These results raise the question of the in vivo role of ERK5 in the nervous system. Although *erk5* and *mek5* null mice displayed severe growth retardation in the head region [22,28–30], mice in which the *erk5* gene has been specifically deleted in the brain develop normally [48, our unpublished results]. This result contrasts with the reduced head structure in *Xenopus* associated with knock down of MEK5 or ERK5 expression using antisense morpholino oligonucleotides [49]. The defect is not caused by increased apoptosis but arises from the inhibition of neural differentiation associated with the specific suppression of neural markers. Differences in phenotypic abnormalities between mice and *Xenopus* may be explained by the presence of redundant signalling cascades in higher eukaryotes that compensate for the loss of the ERK5 pathway. Under physiological conditions, it remains highly likely that ERK5

participates in the normal development of the brain. The role of ERK5 during neurodegenerative diseases and in response to injury remains to be investigated.

4.2. Mechanisms of ERK5-mediated survival of mitotic cells

ERK5 is required to mediate the survival response of endothelial cells (ECs) to shear stress [50]. Over-expression of constitutively activated MEK5 restricts caspase-3 activity and inhibits apoptosis of ECs induced by serum deprivation or inflammatory stimuli, while inhibition of ERK5 activity with a dominant negative mutant or following gene deletion causes EC death. The mechanism by which ERK5 protects ECs from apoptosis implicates the phosphorylation of Bad at Ser136 and Ser112 independently of protein kinase B (PKB), since dominant negative ERK5 does not inhibit the phosphorylation of PKB in response to fluid shear stress [50].

In contrast to this result we found that PKB activation following sorbitol stimulation was hampered in the absence of ERK5 or MEK5 [our unpublished data]. Our study suggests that the ERK5 signalling pathway promotes the survival of fibroblasts by down-regulating Fas ligand (FasL) expression via PKB-dependent inhibition of Foxo3a activity (Fig. 2). This is consistent with a previous study where suppression of PKB signalling was reported to up-regulate FasL [51].

The serum- and glucocorticoid-induced kinase (SGK) is closely related to PKB by its sequence homology and its mechanism of activation. As PKB, SGK is able to inhibit Foxo3a activity by phosphorylation [52]. Since SGK is a key survival component of signal transduction in response to environmental stress stimuli [53] and a direct target of ERK5 [54], it will be interesting to examine whether ERK5-dependent regulation of SGK activity is implicated in mediating the survival function of the ERK5 signalling pathway.

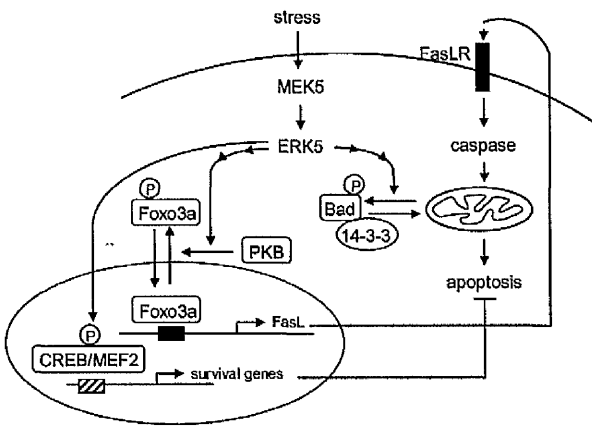

Fig. 2. Regulation of cell survival by the ERK5 cascade. Under stress conditions, the non-phosphorylated forms of Bad and Foxo3a translocate to the mitochondria and the nucleus where they increase cytochrome c release and *fasL* gene transcription, respectively. FasL-mediated caspase activation constitutes a positive feedback loop that enhances stress induced-apoptosis. ERK5 induces the phosphorylation of Bad and Foxo3a by PKB-independent and dependent mechanisms, respectively. In both cases, phosphorylation provides a mechanism by which ERK5 sequesters the proteins in the cytoplasm by promoting their interaction with 14-3-3, thereby blocking their apoptotic effect. The exact mechanisms by which ERK5 regulates Bad phosphorylation and PKB activity remain to be identified. In neurones, the ERK5 cascade contributes to mediating survival in response to growth factors via the activation of the transcription factors CREB and MEF2.

5. ERK5 and cell proliferation

The discovery that serum was a potent inducer of *c-jun* gene transcription via ERK5-induced MEF2C transcriptional activation provided the first evidence that the ERK5 signalling pathway was involved in regulating cell proliferation [15]. Consistent with this study, mitogens including EGF and granulocyte colony-stimulating factor (G-CSF), were subsequently found to transmit their growth promoting signals via ERK5 [55,56]. However, no marked difference was observed in the ability of *erk5−/−*, *mek5−/−*, and wild type fibroblasts to progress through S phase [22,57]. This suggests that the ERK5 signalling pathway may be important for promoting or regulating the proliferation of certain cell types under certain conditions.

The molecular mechanism by which ERK5 mediates its proliferative effect is not clear. It may implicate the activation of SGK, a protein kinase that is closely linked to the G1/S transition of the cell cycle [58]. The phosphorylation of SGK by ERK5 at serine 78 is required for mediating SGK activation and for promoting the entry of cells into S phase of the cell cycle in response to growth factor [54]. The transcriptional activation of the *cyclin D1* gene, a key cell proliferation checkpoint, the deregulation of which is frequently associated with neoplastic transformation, has also been shown to be regulated by the ERK5 cascade. The expression of a kinase-dead mutant of ERK5 blocks the synthesis of endogenous cyclin D1 protein induced by serum in a number of breast cancer cell lines [59]. Evidence from *jnk−/−* fibroblasts suggests that c-Jun is not involved in this process [60]. However, the requirement of ERK5 in regulating cyclin D1 expression is disputed by the ability of PD184352 to block increased levels of cyclin D1 and cell proliferation without affecting ERK5 activation [61].

6. ERK5 and cancer

6.1. Activation of ERK5 by oncogenes

Mutant *ras* has been identified in cancers of many different origins, including pancreas (90%), colon (50%), lung (30%), thyroid (50%), bladder (6%), ovarian (15%), breast, skin, liver, kidney, and some leukemias. Among the signalling pathways suspected to be involved in mediating the oncogenic effect of Ras is the ERK5 cascade. In certain cell types, including PC12, C2C12, and COS7 cells, ERK5 is activated by Ras [10,24,62]. Furthermore, foci induced by a dominant active mutant of Raf (Raf-BxB), a well characterised downstream effector of Ras, are increased in number by enhancing the activity of the ERK5 pathway and are decreased in number by disruption of MEK5

or ERK5 function [62]. This is consistent with the ability of Raf-1 to enhance the activation of ERK5 by Ras [62], although neither MEK5 nor ERK5 activity is increased following over-expression of Raf-BxB [24,62]. The requirement of ERK5 in cellular transformation and malignancy needs to be confirmed by testing the effect of *erk5* gene deletion on the ability of oncogenic Ras to induce typical morphological changes, loss of contact inhibition, and anchorage-independent growth of fibroblasts, and tumourigenicity in vivo.

In addition to Ras, many other oncogenes are able to activate ERK5. For example, Cot potently stimulates the activity of the *c-jun* promoter via JNK, p38 MAPK and ERK5 to induce neoplastic transformation in NIH3T3 cells [63]. Src is involved in the formation of a functional Lad/MEKK2/MEK5 complex that regulates MEF2 activity [40]. ERK5 synergises with ERK1/2 to promote Src-dependent cell proliferation [64,65]. In line with this study, concomitant activation of ERK5 and ERK1/2 causes the disruption of the actin cytoskeleton, a fundamental step of Src-dependent cell transformation [66]. ERK1/2 acts either through suppressing Rho activation or by down-regulating the levels of the Rho effectors, ROCKI and ROCK II, whereas the mechanism by which ERK5 regulates the process is unclear.

6.2. ERK5 in tumour cells

Receptor tyrosine kinases of the ErbB family are activated following interaction with members of the EGF family of ligands. Over-expression or mutations of these receptors in cancers is associated with poor prognosis, shorter disease-free intervals, increased risk of metastasis, and resistance to chemotherapy. The analysis of ERK5 in human-derived tumour cell lines has demonstrated a link between the presence of constitutively activated ERK5 and activated forms of ErbB2, ErbB3 and ErbB4 [67]. The ability of a dominant negative mutant form of ERK5 to decrease the growth rate of breast cancer cells suggests that the ERK5 pathway delivers accelerating-growth signals in human cancer via an ErbB-dependent mechanism [67]. The activity of the signal transducer and activator of transcription 3 (STAT3) is regulated by ErbB. Increased MEK5 expression in breast cancer cell lines expressing an activated form of STAT3 [68] provides a mechanism by which mutations in the *ErbB* genes may indirectly activate ERK5.

MEK5 expression has been assessed in 127 cases of prostate cancer and 20 cases of benign prostatic hypertrophy [69]. The results indicate that elevated MEK5 expression correlates with bone metastasis and unfavorable prognosis. One possible explanation implicates the deregulation of matrix metalloproteinase 9 activity associated with MEK5 over-expression which results in the degradation of the extracellular matrix surrounding the cancer cells, thus enabling them to invade [69].

More recently, IL-6 was shown to activate ERK5 in cells isolated from multiple myeloma patients [70]. Multiple myeloma is a carcinoma caused by the accumulation of terminally differentiated B cells in the bone marrow. A dominant negative form of ERK5 restricts the proliferation of myeloma cells and sensitises cells to the apoptosis-induced drugs [70].

6.3. ERK5 in tumour development

In addition to its contribution to the malignancy of tumour cells, genetic evidence that ERK5 is implicated in tumour-associated angiogenesis was provided by analysing the effect of the deletion of the *erk5* gene in vasculature development of melanoma and carcinoma xenografts [71]. The number of large blood vessels was greatly diminished in tumours growing in the flank region of the *erk5*−/− mice. The results indicate that ERK5 is required for both vascular endothelial growth factor (VEGF)- and fibroblast growth factor (FGF)-induced angiogenesis by a mechanism that implicates the phosphorylation of the ribosomal protein S6 via RSK.

7. ERK5 and heart function

7.1. Role of ERK5 during cardiovascular development

The targeted deletion of the *erk5* and *mek5* genes in mice has provided genetic evidence for an essential role of the ERK5 signalling pathway during heart development [22,28–30]. The phenotypes observed in these mice are almost identical. They die around embryonic day 10 (E10) due to cardiovascular defects that include disorganisation of the trabeculae and underdevelopment of the myocardium. Vasculogenesis and angiogenesis are impaired in both the embryo itself and the extraembryonic tissue including the yolk sac and placenta. However, the analysis of a mouse model featuring a conditional mutation in the *erk5* gene demonstrates that the cardiovascular defect is not a consequence of a placental defect [57]. Similar phenotypic abnormalities displayed by the *mef2*−/−, and *mekk3*−/− embryos identify the MEKK3/MEK5/ERK5/MEF2 cascade as an essential signalling cascade that controls early cardiovascular development [72,73].

The heart defect observed in conventional ERK5 knockout mutants was reproduced in embryos in which ERK5 was specifically ablated in ECs [57]. The irregular alignment and rounded morphology of the *erk5*−/− ECs surrounding the blood vessels in the developing heart is consistent with an essential role of ERK5 for the maintenance of vascular integrity. The normal development of mice lacking ERK5 in cardiomyocytes confirms that the abnormal development of the heart displayed by the *erk5*−/− embryo is a consequence of abnormal vasculogenesis and angiogenesis [57]. The requirement of ERK5 for the maintenance of vascular integrity is also indispensable in adult mice that display hemorrhages from multiple organs and lethality within 2–4 weeks after the induction of Cre recombinase [57]. The role of ERK5 for the survival of ECs in vitro [50] is consistent with the elevated apoptosis of ECs in vivo caused by the loss of ERK5 and decreased MEF2C activity [57]. Abnormal angiogenesis may also be caused by increased expression of VEGF due to deregulation of hypoxia inducible factor 1α (HIF1α) activity [29]. ERK5-induced HIF1α degradation occurs via an ubiquitin-dependent mechanism [74].

7.2. Role of ERK5 in cardiac hypertrophy

Cardiac hypertrophy involves the alteration of myocyte shape and extracellular matrix that results in wall thickening, followed by chamber dilation and myocardiac dysfunction. This process is initially compensatory for an increased workload, the prolongation of which leads to congestive heart failure, arrhythmia, and sudden death. Hypertrophy-stimulating factors including LIF, and oxidative and osmotic stress, activate ERK5 in cultured cardiomyocytes [33,41]. Over-expression of dominant active MEK5 elicits serial assembly of sarcomeres while dominant negative MEK5 blocks LIF-induced elongation of cardiomycoytes [41]. In vivo evidence that the ERK5 signalling pathway plays a role in the hypertrophic process is supported by the ability of over-expressed MEK5 to induce eccentric hypertrophy in the heart, which results in heart failure and sudden death [33]. This is consistent with increased activation of ERK5 during left ventricular hypertrophy [75]. It is worth noting that the deletion of seven amino acids in MEF2A, a well characterised downstream substrate of ERK5 [15,16], has been shown to be responsible for the onset of coronary artery disease/myocardial infarction (CAD/MI) in patients [76]. Whether other MEF2 family members are involved and whether ERK5 is the upstream kinase that regulates MEF2A function responsible for the pathogenetical manifestations of CAD/MI remains to be determined.

8. Conclusion

Efforts of many scientists in recent years has led to major progress in understanding the regulation of ERK5 and its function in vivo. In particular, genetically modified mice in which the *mek5* or *erk5* genes have been mutated have provided important information regarding the physiological relevance of the ERK5 signalling pathway during cardiovascular development. The potentially crucial role of ERK5 in cancers and heart diseases make this cascade highly attractive for the development of new therapeutic strategies to treat pathological conditions that are resistant to current therapies. Currently, very few downstream effectors of ERK5 that will constitute ideal targets for drug development have been identified. Proteomics-based screens need to be developed to discover novel regulatory molecules that transmit signals via ERK5. Such unbiased approaches may provide significant advances in understanding how cells proliferate and survive via the ERK5 signalling pathway.

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A Novel Mitogen-Activated Protein Kinase Docking Site in the N Terminus of MEK5α Organizes the Components of the Extracellular Signal-Regulated Kinase 5 Signaling Pathway

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The alternative splicing of the *mek5* gene gives rise to two isoforms. MEK5β lacks an extended N terminus present in MEK5α. Comparison of their activities led us to identify a novel mitogen-activated protein kinase (MAPK) docking site in the N terminus of MEK5α that is distinct from the consensus motif identified in the other MAPK kinases. It consists of a cluster of acidic residues at position 61 and positions 63 to 66. The formation of the MEK5/extracellular signal-regulated kinase 5 (ERK5) complex is critical for MEK5 to activate ERK5, to increase transcription via MEF2, and to enhance cellular survival in response to osmotic stress. Certain mutations in the ERK5 docking site that prevent MEK5/ERK5 interaction also abrogate the ability of MEKK2 to bind and activate MEK5. However, the identification of MEK5α mutants with selective binding defect demonstrates that the MEK5/ERK5 interaction does not rely on the binding of MEK5α to MEKK2 via their respective PB1 domains. Altogether these results establish that the N terminus of MEK5α is critical for the specific organization of the components of the ERK5 signaling pathway.

The mitogen-activated protein kinase (MAPK) signaling pathways regulate numerous physiological processes during development and pathogenesis (10). They consist of the sequential activation of protein kinases that include a MAPK, a MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK or MKK) and a MEK kinase (MEKK) (10). At least four MAPK subfamilies have been identified: ERK1/2, ERK5, c-Jun NH_2-terminal protein kinases (JNKs), and p38 MAPKs. MAPK activators include MEK1 and MEK2 for ERK1/2, MEK5 for ERK5, MKK4 and MKK7 for JNKs, and MKK3 and MKK6 for p38 MAPKs. MEKs/MKKs activate MAPK by dual phosphorylation on threonine (T) and tyrosine (Y) residues within a T-X-Y motif. A conserved cluster of two to five basic residues identified in the N terminus of MEK1 and -2 and MKK3, -4, -6, and -7 constitutes a consensus MAPK binding site that is necessary for the transmission of the signal (1, 28). A similar motif has been identified by sequence homology in MEK5, but its function as a docking site for ERK5 has not been proven (28).

The ERK5 signaling pathway regulates by phosphorylation the activity of a number of transcription factors including myocyte enhancer factor 2 (MEF2) (12, 13). Consistent with its role in stimulating gene expression through the regulation of MEF2 activity, ERK5 contributes in vitro to regulating muscle differentiation and neuronal survival (6, 16, 24). The analysis of mutant mice in which the *erk5* gene can be conditionally deleted has provided in vivo genetic evidence that ERK5 mediates the survival of endothelial cells (9). The loss of endothelial survival may be responsible for the cardiovascular defects observed in $erk5^{-/-}$ and $mek5^{-/-}$ embryos (21, 25, 29, 32). Being twice the size (815 amino acids) of the other MAPKs, ERK5 possesses a unique C-terminal tail that contains a MEF2-interacting domain and a potent transcriptional activation domain (11). The C-terminal tail has been shown to regulate the nuclear shuttling of ERK5, but its role in mediating ERK5 activation remains controversial (3, 31). In contrast to the other MAPK cascades, only one activator of ERK5, MEK5, has been cloned (7, 33).

We have recently provided genetic evidence that MEK5 is a nonredundant component of the ERK5/MEF2-dependent cell survival pathway (29). Alternative splicing of the mRNA gives rise to two isoforms with different N termini, MEK5α (50 kDa) and MEK5β (40 kDa) (7). The N-terminal extension of MEK5α has been implicated in its restricted localization to the particulate fraction, while MEK5β is ubiquitously distributed and is primarily cytosolic (7). In addition, it contains a phox and Bem1p (PB1) domain that mediates the binding interaction of MEK5α with the MEK kinases, MEKK2 and MEKK3 (17). Blocking the PB1-dependent formation of the MEKK2/MEK5 complex prevents MEK5 activation of ERK5 (17). Consistent with this study, MEK5β that lacks the PB1 domain has been identified as a kinase-dead dominant-negative variant that can suppress ERK5 signaling (4). However, this is disputed by the ability of a MEK5β transgene to activate ERK5 in vivo and to promote eccentric cardiac hypertrophy that progresses to dilated cardiomyopathy and sudden death (18).

To elucidate the functional consequence of differential splicing of the *mek5* gene, we have investigated the regulation of ERK5 by MEK5 isoforms. Our data demonstrate that both MEK5α and MEK5β are catalytically active enzymes, but the physical interaction of ERK5 with the N-terminal domain of MEK5α is critical for ERK5 activation. The docking site identified in the N terminus of MEK5α is unique since it does not match the consensus motif present in the other MAPK activa-

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tors (28). Further studies demonstrate that the docking of ERK5 to MEK5 is essential for the function of MEK5 as a cell survival factor.

MATERIALS AND METHODS

Cell culture, transfection, and preparation of lysates. COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 10 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere with 5% CO_2. Mouse embryonic fibroblasts (MEFs) established from *mek5*$^{-/-}$ embryos were described previously (29). Transfection was performed using the Lipofectamine (Invitrogen) or Metafectene (Biontex, Munich, Germany) reagents, following the manufacturer's instructions. A total of 3 μg of each plasmid DNA was used unless indicated otherwise. Proteins were extracted from cells in triton lysis buffer (TLB; 20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 10% glycerol, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Extracts were clarified by centrifugation (14,000 × *g* for 10 min at 4°C). The concentration of soluble proteins was quantified by the Bradford method (Bio-Rad).

Plasmid constructs. The epitope-tagged MEKK2 and MEKK3 (2), the dominant-active mutant MEK5α (MEK5D) (12), and the glutathione *S*-transferase (GST)-ERK5 (33) expression vectors were provided by C. Widmann, J.-D. Lee, and J. E. Dixon, respectively. Wild-type MEK5 was created by replacing D311 and D315 with S311 and T315 using a QuikChange XL Site-Directed Mutagenesis kit (Stratagene). N-terminal Flag-tagged MEK5α and MEK5β constructs were obtained by subcloning the cDNAs into p3*Flag-CMV-10 (where CMV is cytomegalovirus) vector (Sigma) using HindIII and NotI. Mammalian expression plasmids encoding full-length wild-type or mutant MEK5α, MEK5β, or N-terminal fragments of MEK5α fused to GST were created by in-frame ligation of the cDNAs into the pEBG vector (22) using SpeI and NotI. The ERK5 and MEK5 cDNAs were subcloned into pRSETA (BamHI/HindIII) and pGEX4T-1 (SalI/NotI) vectors to produce histidine (His) and GST fusion proteins, respectively. All constructs were confirmed by sequencing using the Big Dye kit (Applied Biosystems) on an ABI 377 sequencer.

Immunoblot analysis. Cell extracts (20 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% polyacrylamide gel) and electrophoretically transferred to Immobilon-P membrane (Millipore). Membranes were incubated with 3% nonfat dry milk for 30 min at room temperature and then probed overnight at 4°C with primary antibodies to Flag (Sigma), hemagglutinin (HA; Covance), ERK5 (Sigma), GST (Amersham Biosciences), or MEK5 (29). Immunocomplexes were detected by enhanced chemiluminescence (Pierce or Amersham Biosciences) with goat (Jackson Laboratories), rabbit, or mouse (Amersham Biosciences) immunoglobulin G coupled to horseradish peroxidase as the secondary antibody. Immunoblot signals were quantified with the ImageQuantifier software (BioImage, Jackson, MI) to normalize for protein expression prior to protein kinase assays.

Binding assays. Cell extracts (100 to 500 μg) were incubated at 4°C for 2 h with 1 to 2 μg of antibody and protein A agarose beads or with 1 to 5 μg of His- or GST-fusion protein and Ni-nitrilotriacetic acid (NTA) agarose (QIAGEN) or glutathione (GSH)-Sepharose (Amersham) beads, respectively. Complexes were washed three times with TLB and analyzed by immunoblotting. Affinity chromatography was performed as described previously (8).

Protein kinase assays. ERK5 and MEK5 protein kinase activity was measured in cell lysates following incubation with antibodies to ERK5, Flag, or GST and protein A agarose beads for 2 to 3 h at 4°C. Immunocomplexes were washed three times with TLB and twice with kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 2 mM dithiothreitol, 0.1% orthovanadate) prior to incubation for 30 min at 30°C in kinase buffer containing 50 μM [γ-^{32}P]ATP (10 Ci/mmol) and 1 μg of GST-MEF2 and GST-ERK5 or myelin basic protein (MBP) for ERK5 and MEK5 assays, respectively. The radioactivity incorporated into recombinant proteins was quantitated after SDS-PAGE by PhosphorImager analysis (Fuji FLA 3000).

Reporter gene expression assay. The reporter plasmid pG5E1bLuc (23) was transiently cotransfected together with a construct encoding the fusion protein GAL4-MEF2A (Hung-Ying Kao, Case Western University, Cleveland, Ohio) with or without expression vectors encoding wild-type or mutant MEK5α or MEK5β. A pRL-Tk plasmid encoding *Renilla* luciferase was cotransfected to monitor transfection efficiency. Aliquots of cell lysates were assayed for firefly and *Renilla* luciferase activities using a dual-luciferase reporter assay kit (Promega) on a Turner Designs 20/20 luminometer.

Cell survival assays. Cell viability was quantified by luciferase activity following transfection with the pCMV luciferase plasmid (15) or by flow cytometry using an anti-GST antibody coupled to Alexa Fluor 488 (Molecular Probes). Fluorescent cells were counted on a DakoCytomation Cyan flow cytometer equipped with a coherent Sapphire laser (488 nm; 20 mW) using a 530/40 nm band-pass filter.

RESULTS

Comparison of protein kinase activities of MEK5 isoforms. To further understand how MEK5 regulates ERK5, we compared the protein kinase activities of MEK5 isoforms. Lysates of COS-7 cells overexpressing ERK5 and dominant-active mutants of MEK5α or MEK5β were obtained. The activation of ERK5 by MEK5 was examined following immunoprecipitation of ERK5 using an anti-ERK5 antibody and recombinant MEF2 as a substrate (Fig. 1A and B). This assay demonstrated that MEK5α was a stronger activator of ERK5 than MEK5β regardless of the amount of MEK5 protein expressed. The analysis of the immune complexes by immunoblotting indicated that MEK5β interacted with ERK5 with a weaker affinity than MEK5α (Fig. 1A). Control experiments showed similar levels of expression of MEK5 isoforms. These observations suggested that the different activities of MEK5 isoforms were due to their distinct ability to interact with ERK5. Indeed, when MEK5 was coimmunoprecipitated with ERK5 using an anti-Flag antibody prior to the protein kinase assay, MEK5β activated ERK5 to a level similar to activation by MEK5α (Fig. 1A and B, IP:ERK5+MEK5). Similarly, ERK5 activation in cells transfected with 0.3 or 0.03 μg of MEK5α cDNA appeared higher than in experiments where only ERK5 was immunoprecipitated (Fig. 1A). In vitro activation of ERK5 by MEK5 contributes to increasing the levels of MEF2 phosphorylation, although we cannot totally exclude the presence of other protein kinases associated with the immunoprecipitated complexes.

The ability of MEK5 isoforms to regulate ERK5 in vivo was determined by testing their effect on the transcriptional regulation of MEF2 (Fig. 1C). *mek5*$^{-/-}$ fibroblasts were cotransfected with MEK5α or MEK5β together with the reporter plasmid pG5E1bLuc and a construct encoding GAL4-MEF2A. A pRL-Tk plasmid encoding *Renilla* luciferase was employed for monitoring transfection efficiency. The results demonstrated that only MEK5α increased the transcriptional activity of MEF2A as determined by the luciferase reporter assay (Fig. 1C).

To determine whether similar differences could be detected following activation of MEK5, we examined the effect of MEKK2 and MEKK3, two strong activators of the ERK5 signaling pathway (5, 26). COS-7 cells were cotransfected with wild-type MEK5α or MEK5β together with or without MEKK2 or MEKK3 and ERK5. MEK5 and ERK5 activity was measured following immunoprecipitation using recombinant ERK5 and MEF2 as substrates, respectively. At the basal level, MEK5α was more active than MEK5β, but no marked difference was detected in the ability of MEKK2 and MEKK3 to activate either isoform (Fig. 2A). However, the activation of ERK5 by MEKKs mediated via MEK5α was more potent than via MEK5β (Fig. 2B).

Together these data provide evidence that MEK5α and MEK5β are catalytically active enzymes and that the binding

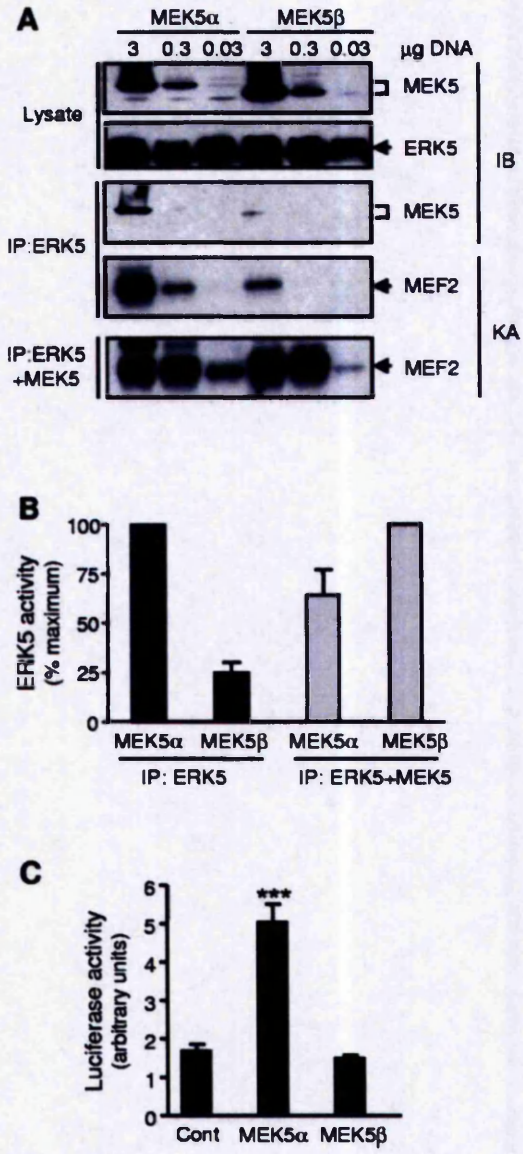

FIG. 1. Activation of ERK5 by MEK5 isoforms. (A and B) COS-7 cells were cotransfected with expression vectors encoding Flag epitope-tagged ERK5 and MEK5α or MEK5β. The expression of MEK5 and ERK5 in cell lysates and the presence of MEK5 in the complexes immunoprecipitated with an anti-ERK5 antibody were examined by immunoblot analysis (IB) using an anti-Flag antibody (A). ERK5 activity was measured by protein kinase assays (KA) following immunoprecipitation using an anti-ERK5 (IP:ERK5) or an anti-Flag (IP:ERK5+MEK5) antibody (A and B). The radioactivity incorporated into GST-MEF2C was quantitated after SDS-PAGE by phosphorimager analysis and expressed as a percentage of the maximum value. (B). Data of three independent experiments are shown (means ± standard error of the means). (C) The reporter plasmid pG5E1bLuc was transiently cotransfected with a construct encoding the fusion proteins GAL4-MEF2A without (Cont) or with MEK5α or MEK5β. MEF2 transcriptional activity was measured by a dual-luciferase reporter assay system. Values are expressed as ratios of firefly to *Renilla* luciferase activity. The data correspond to the means ± standard error of the means of three independent experiments (***, $P < 0.001$).

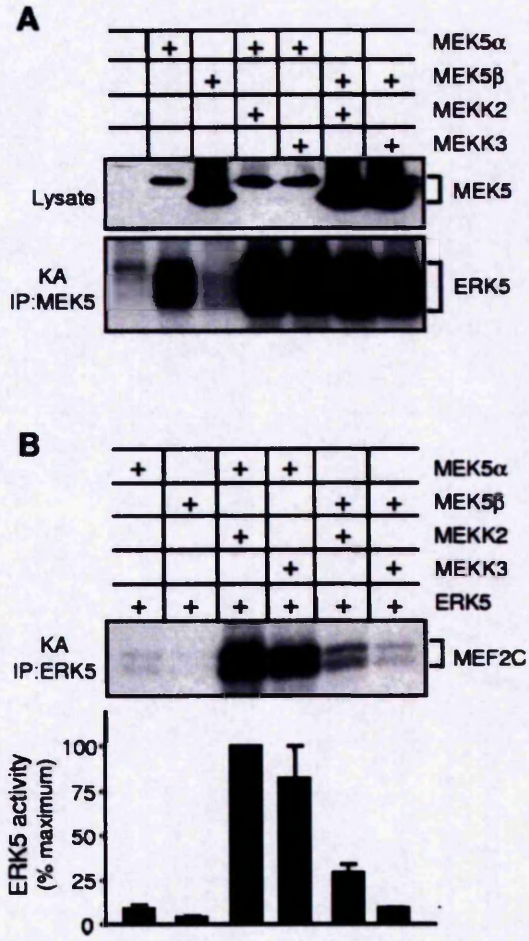

FIG. 2. Effect of MEKK2 and MEKK3 on ERK5 signaling. COS-7 cells were cotransfected with expression vectors encoding Flag-tagged MEK5α or MEK5β and HA-tagged MEKK2 or MEKK3 without (A) or with ERK5 (B). MEK5 and ERK5 activities were measured by protein kinase assays (KA) following immunoprecipitation using an anti-Flag (A) or an anti-ERK5 (B) antibody, respectively. The radioactivity incorporated into GST-MEF2C was quantitated after SDS-PAGE by phosphorimager analysis and expressed as a percentage of the maximum value (B). Data of three independent experiments are shown (means ± standard error of the means).

interaction between ERK5 and MEK5 contributes to mediating ERK5 activation.

MEK5α interacts with ERK5 via its N-terminal domain. The distinct affinity of MEK5 isoforms for ERK5 was confirmed by affinity chromatography. ERK5 protein produced in COS-7 cells was loaded onto GSH-agarose columns packed with equal amounts of bacterially expressed GST-MEK5α or GST-MEK5β. The elution was performed using increasing concentrations of KCl (0.01 to 1 M). The presence of ERK5 was detected in the eluates by immunoblot analysis. Whereas 10 mM KCl was sufficient to dissociate the ERK5/MEK5β complex, a significant proportion of ERK5 remained bound to MEK5α until the highest 1 M KCl concentration was used (Fig. 3A). Consistently, less ERK5 was detected in the first and the second washes and in the 10 mM KCl fraction of the

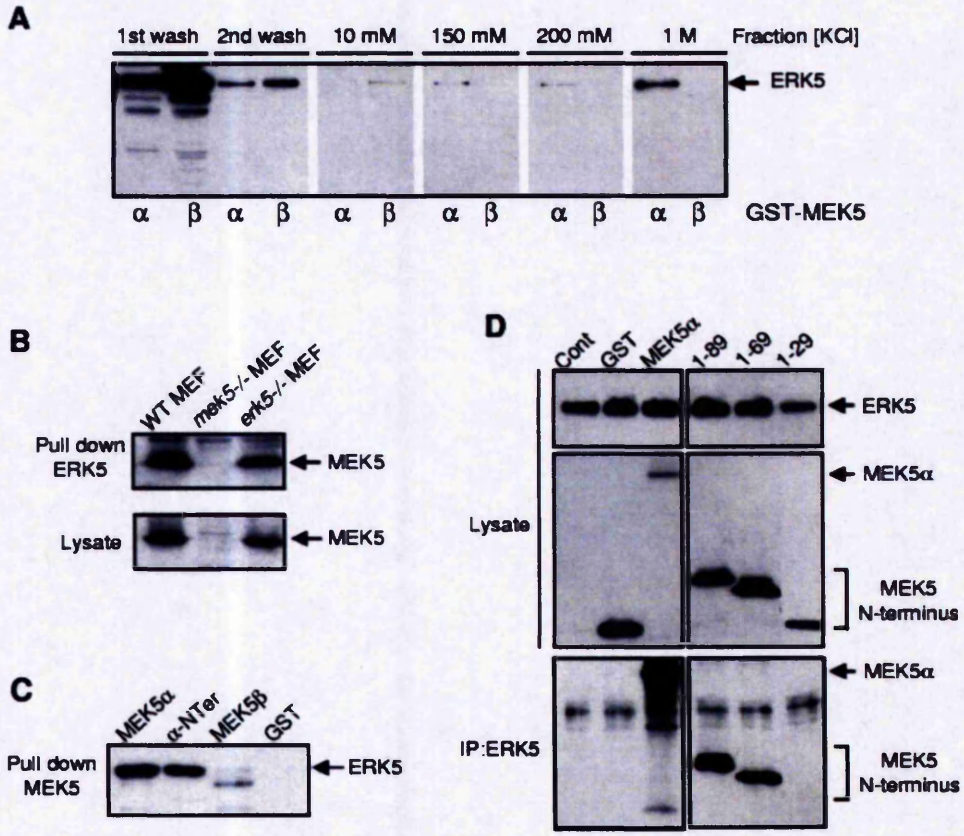

FIG. 3. Interaction of ERK5 with MEK5 isoforms. (A) Lysates (500 μg) of COS-7 cells expressing Flag-ERK5 were passed through a GST-MEK5α or a GST-MEK5β affinity chromatography column. Columns were washed twice prior to being eluted with increasing concentrations of KCl. The presence of ERK5 in the fractions was detected by immunoblot analysis using an anti-Flag antibody. (B) MEF extracts (500 μg) were incubated with bacterially expressed His-tagged ERK5 (1 μg). ERK5 was isolated by incubation with Ni-NTA agarose beads. The binding of MEK5 was examined by immunoblot analysis with an antibody to MEK5. (C) Lysate (250 μg) of COS-7 cells expressing HA-tagged ERK5 was incubated with 5 μg of GST, GST-tagged MEK5α, MEK5β, or α-Nter. MEK5 was isolated by incubation with GSH-agarose beads. The binding of ERK5 was examined by immunoblot analysis with an antibody to the HA epitope tag. (D) COS-7 cells were cotransfected with the expression vectors encoding Flag-ERK5 without (Cont) or with GST, MEK5α, or fragments of the N-terminal domain of MEK5α (amino acids 1 to 89, 1 to 69, and 1 to 29) fused to GST. Immunoblot analysis using an anti-Flag and an anti-GST antibody confirmed similar expression of ERK5 and MEK5 in cell lysates, respectively. ERK5 was isolated by incubation with anti-ERK5 antibody. The binding of MEK5 was examined by immunoblot analysis with an antibody to GST.

MEK5α compared to the MEK5β column. These studies clearly demonstrated that MEK5α displayed a higher affinity for ERK5 than MEK5β. To confirm the interaction between MEK5α and ERK5, bacterially expressed His-tagged ERK5 incubated with lysates of fibroblasts was precipitated, and the presence of MEK5 in the precipitates was detected by immunoblot analysis (Fig. 3B). Endogenous MEK5α expressed in wild-type and *erk5*$^{-/-}$ MEFs coprecipitated with ERK5. A control experiment shows that no protein was detected when lysate of *mek5*$^{-/-}$ MEFs was used (Fig. 3B).

To determine how the binding affinity of MEK5 isoforms is affected by alternative splicing, we investigated the interaction of ERK5 with the N terminus of MEK5α (amino acids 1 to 89 [α-Nter, where α indicates MEK5α]). ERK5-transfected COS-7 cells were incubated with an equal amount of bacterially expressed GST-MEK5α, GST-MEK5β, or GST-α-Nter. Immunoblot analysis of the complexes pulled down by GSH beads showed that the binding affinity of ERK5 for α-Nter was similar to that for MEK5α and significantly higher than for MEK5β (Fig. 3C). To identify more specifically the region of MEK5α responsible for the binding to ERK5, COS-7 cells were cotransfected with ERK5 together with different N-terminal fragments of MEK5α (Fig. 3D). ERK5 was immunoprecipitated, and the presence of MEK5 in the precipitates was detected by immunoblot analysis. Fragments 1 to 89 and 1 to 69 displayed affinity for ERK5 similar to that of the full-length MEK5α. In contrast, fragment 1 to 29 was unable to bind ERK5 (Fig. 3D). These studies indicate that amino acids 29 to 69 within the PB1 domain of MEK5 (17) are critical for mediating a strong binding interaction between MEK5 and ERK5.

N terminus of MEK5α contains a docking site for ERK5. The analysis of the N terminus of MEK5α revealed the presence of four acidic residues, EDED (amino acids 63 to 66), which are highly conserved in PB1 domains found in other proteins (14). To determine the role of these residues in me-

diating the binding of MEK5α to ERK5, we generated two mutants in which the EDED motif was either deleted (αΔ63–66, MEK5α with a deletion of amino acids 63 to 66) or replaced with aliphatic amino acids (α63LVLV66) (Fig. 4A). Mutants αK146L/K147L and αR153L/K154L were engineered to test whether the putative docking site present in both MEK5 isoforms and identified by sequence homology with other MAPKKs (28) was implicated in mediating the binding to ERK5.

An in vitro binding assay was performed using bacterially expressed His-tagged ERK5 and GST-fused wild-type and mutant MEK5α. MEK5 was precipitated, and the presence of ERK5 in the complexes was detected by immunoblot analysis (Fig. 4B). This assay revealed that the ability of MEK5α to bind ERK5 was dramatically affected following the deletion or the mutation of the EDED motif, while mutations in the proposed docking site had no effect. To confirm in vivo the role of the EDED motif, lysates of COS-7 cells overexpressing ERK5 and wild-type or mutant MEK5α were obtained. The presence of MEK5 was detected by immunoblotting following the immunoprecipitation of ERK5 (Fig. 4C). A control experiment shows equal amounts of ERK5 in the immune complexes. Consistent with the in vitro data, the αK146L/K147L and the αR153L/K154L mutants, but not the αΔ63–66 or the α63LVLV66 mutants, bind ERK5 as well as the full-length MEK5α.

The MEK5/ERK5 interaction was further characterized by engineering more specific mutations in the N terminus of MEK5α (Fig. 4A). Lysates of COS-7 cells overexpressing wild-type or mutant MEK5α were incubated with bacterially expressed His-tagged ERK5. ERK5 was precipitated, and the presence of MEK5 in the complexes was detected by immunoblot analysis (Fig. 5A). The result showed that the deletion of two acid residues in the EDED motif (Δ63–64) was sufficient to completely abolish the binding of ERK5 to MEK5 (Fig. 5A). The αΔ63 mutant in which one acidic residue was deleted displayed a lower affinity for ERK5 compared to the wild-type MEK5α. Outside of the EDED motif, a glutamic acid and an aspartic acid residue at positions 61 and 68 were replaced by a leucine and a valine residue, respectively (Fig. 4A). The E61L mutation significantly decreased the ability of MEK5 to bind ERK5, while the D68V mutation had no effect. This analysis demonstrates that acidic residues within the EDED motif and at position 61 are critical for mediating the MEK5α/ERK5 interaction.

Since the docking site is contained within the PB1 domain, we analyzed the ability of the mutants to bind MEKK2 (Fig. 5B). COS-7 cells were cotransfected with expression vectors encoding wild-type or mutant GST-MEK5α with HA-tagged MEKK2. MEK5 was precipitated, and the presence of MEKK2 in the complexes was detected by immunoblot analysis. Mutations in the EDED motif decreased the ability of MEK5 to interact with MEKK2. Similarly, the αD68V mutant that displayed normal affinity for ERK5 was unable to bind MEKK2. In contrast, the E61L substitution had no effect. These results indicate that the EDED motif and the D68 residue constitute an important sequence for mediating MEKK2/MEK5 interaction. The selective effect of the E61L and the D68V mutations on ERK5 and MEKK2 binding

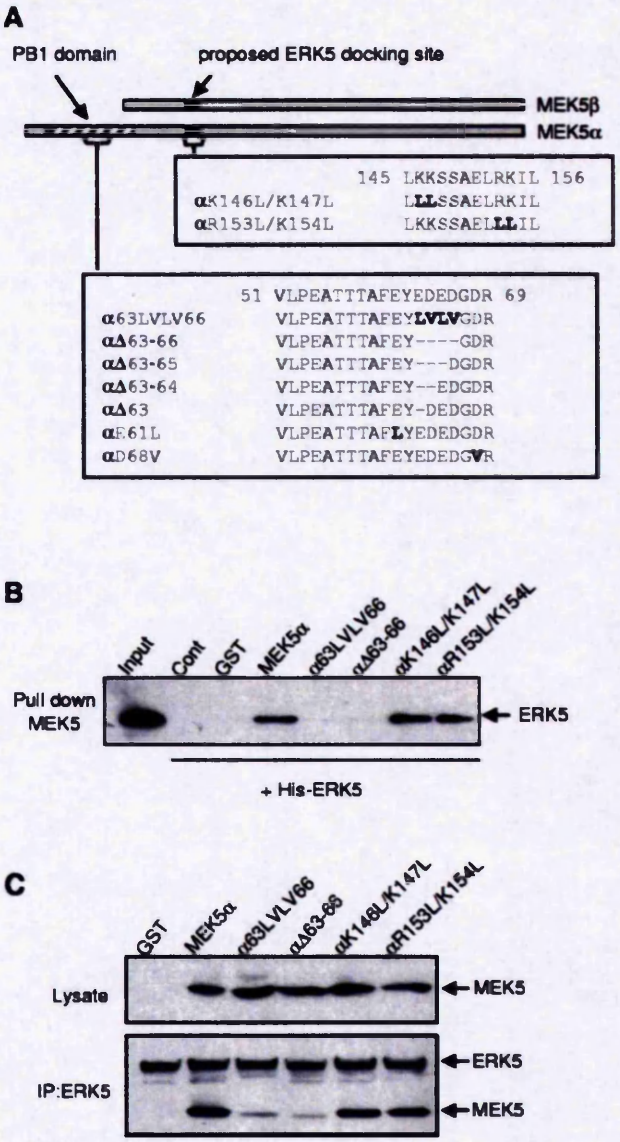

FIG. 4. Identification of a novel MAPK docking site in the N terminus of MEK5α. (A) Schematic representation of the MEK5α and the MEK5β sequences. The residues mutated in the PB1 domain and in the proposed ERK5 docking site are indicated in bold. The deletion is indicated by dashes. (B) Bacterially expressed His-ERK5 (1 μg) was incubated without (Cont) or with 1 μg of GST or GST-tagged wild-type and mutant MEK5α. MEK5 was isolated by incubation with GSH-agarose beads. The binding of ERK5 was examined by immunoblot analysis with an antibody to ERK5. One-tenth of the His-ERK5 used is shown (input). (C) COS-7 cells were cotransfected with the expression vectors encoding Flag-tagged ERK5 and GST or wild-type or mutant MEK5α fused to GST. ERK5 was isolated by incubation with an anti-Flag antibody. The binding of MEK5 was examined by immunoblot analysis with an antibody to the GST epitope tag. Immunoblot analysis of the lysates and of the immunoprecipitates with an anti-GST and an anti-ERK5 antibody shows similar levels of expression of wild-type and mutant MEK5α and ERK5, respectively.

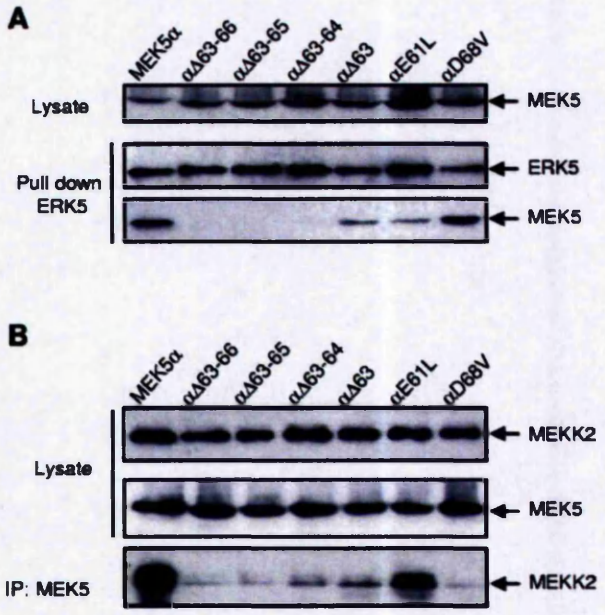

FIG. 5. Characterization of the ERK5 docking site in the N terminus of MEK5α. (A) Bacterially expressed His-ERK5 (1 μg) was incubated with extracts of COS-7 cells transfected with the expression vectors encoding wild-type or mutant MEK5α fused to GST. ERK5 was isolated by incubation with Ni^{2+}-NTA beads. The binding of MEK5 was examined by immunoblot analysis with an antibody to the GST epitope tag. (B) COS-7 cells were cotransfected with expression vectors encoding wild-type or mutant GST-MEK5α with HA-tagged MEKK2. MEK5 was isolated following precipitation with an anti-GST antibody. The binding of MEKK2 was examined by immunoblot analysis with an antibody to the HA epitope tag. The expression of MEK5 (A and B) and MEKK2 (B) in cell lysates and the presence of ERK5 (A) in the immune complexes were detected by immunoblot analysis using an anti-GST, an anti-HA, and an anti-ERK5 antibody, respectively.

clearly demonstrates that the binding of MEK5 to ERK5 does not rely on the PB1 domain.

The docking site of MEK5 is essential for mediating ERK5 activation. The role of the docking site in regulating MEK5 activity was investigated under basal conditions (Fig. 6). COS-7 cells were cotransfected with wild-type or mutant MEK5α. MEK5 activity was measured following immunoprecipitation using recombinant ERK5 or MBP as substrates (Fig. 6A and B). While the activity of the αK146L/K147L and the αR153L/K154L mutants remained comparable to the wild-type MEK5α, mutations in the EDED motif that affected the binding of MEK5 to ERK5 also prevented MEK5 from phosphorylating ERK5. Consistently, the αE61L mutant displayed a lower level of activity than the αD68V mutant or the wild-type MEK5α (Fig. 6B). A control experiment shows no marked difference in the ability of wild-type and mutant MEK5 to phosphorylate MBP, ruling out a deleterious effect of the mutations on protein kinase activity (Fig. 6A). The expression of MEK5α, αK146L/K147L, or αR153L/K154L in the *mek5*$^{-/-}$ fibroblasts caused a significant increase in luciferase activity. In contrast, αΔ63–66 and α63LVLV66 were unable to regulate MEF2-induced transcription (Fig. 6C). Together these data demonstrate that the binding of MEK5 to ERK5 via its docking site is required for activating the ERK5 cascade.

A similar defect was observed in the regulation of MEK5 by MEKK2 (Fig. 7). COS-7 cells were cotransfected with wild-type or mutant MEK5α together with or without MEKK2. Mutations in the docking site that affected the MEKK2/MEK5 interaction also abrogated the ability of MEKK2 to increase MEK5 activity. This is demonstrated using MBP as a substrate to exclude the effect of the mutations on MEK5 binding to ERK5. A similar result was obtained with MEKK3 (data not shown).

The survival function of MEK5 is dependent on its docking domain. Consistent with our previous data (29), we found that the *mek5*$^{-/-}$ MEFs were more sensitive than the wild-type cells to the toxic effect of sorbitol (data not shown). Cell viability assays that employ a luciferase plasmid showed that the ectopic expression of MEK5α but not of αΔ63–66 or α63LVLV66 mutants was able to partially restore this defect. *mek5*$^{-/-}$ MEF transfected with an empty vector or a vector encoding MEK5α mutants defective in ERK5 binding displayed between 40% to 60% decrease in luciferase activity following sorbitol treatment (Fig. 8A). In contrast, no marked decrease was observed in cells overexpressing MEK5α (Fig. 8A). To confirm the requirement of the docking site in mediating the protective effect of MEK5α, we tested the effect of wild-type and mutant MEK5α on the survival of transfected COS-7 cells in response to sorbitol (Fig. 8B). The number of viable COS-7 cells expressing MEK5 was quantified by flow cytometry using a fluorescence-coupled antibody against MEK5. The results showed that cell loss was significantly prevented by wild-type MEK5α but not by the αΔ63–66 or α63LVLV66 mutants. These results demonstrate that, unlike wild-type MEK5α, MEK5α mutants defective in their ability to bind and activate ERK5 are unable to protect cells against stress-induced death.

DISCUSSION

Consistent with the analysis of mutant mice in which the *erk5* gene has been deleted (9, 21, 25, 32), we have previously established the role of MEK5 in vivo as an activator of ERK5 and as an essential regulator of cell survival that is required for normal embryonic development (29). Here we demonstrate that the protective effect of MEK5 is dependent on its ability to bind ERK5. A novel MAPK docking site in the N terminus of MEK5 mediates the interaction between MEK5α and ERK5 that is critical for ERK5 activation. As purified recombinant MEK5 and ERK5 form a stable complex, the interaction between the two proteins is direct.

Based on sequence homology with the other MAPK activators, the docking site on MEK5 was predicted to correspond to a cluster of basic residues on positions 146 to 153 present in both MEK5 isoforms (28). Our data show that this was not the case. The substitution of lysine 146 and 147 or arginine 153 and lysine 154 to leucine did not affect the binding of MEK5α or MEK5β with ERK5 (Fig. 4B and C and 6A; data not shown). In addition, no marked differences were observed between the ability of these mutants and the wild-type proteins to activate ERK5 under basal conditions or following activation (Fig. 6A and 7 and data not shown). The region in the MEK5β isoform

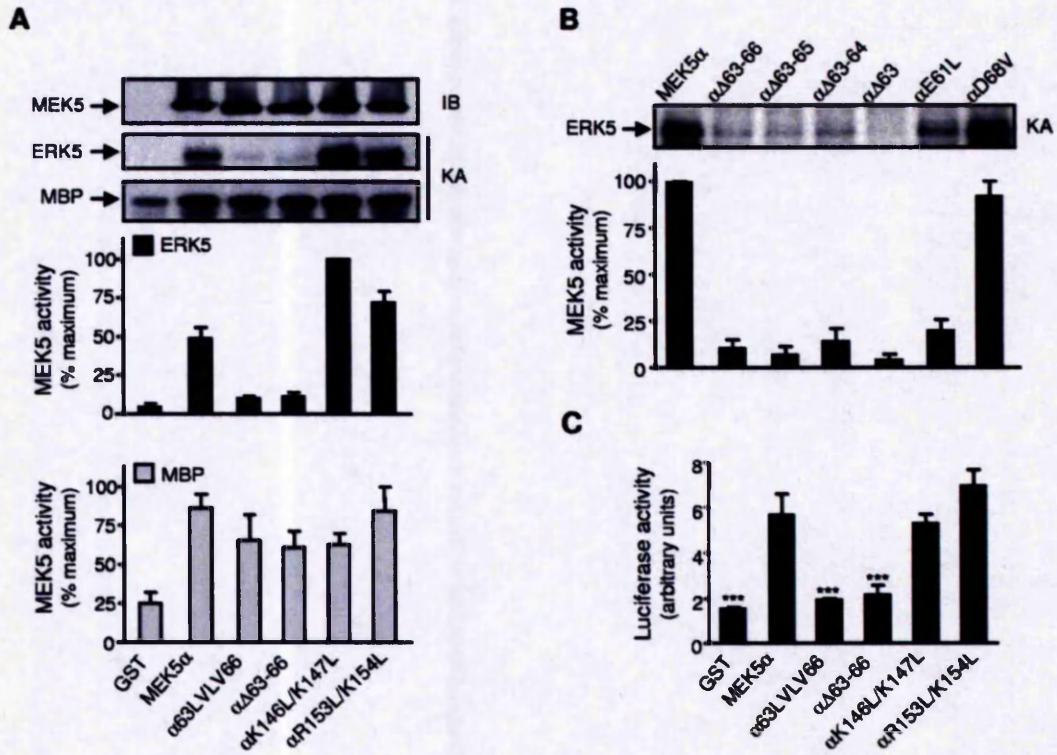

FIG. 6. Mutations in the MAPK docking site affect MEK5 activity. (A and B) COS-7 cells transfected with expression vectors encoding GST or wild-type or mutant MEK5α fused to GST. MEK5 activity was measured by protein kinase assay (KA) following immunoprecipitation with an anti-GST antibody. The radioactivity incorporated into GST-ERK5 or MBP was quantitated after SDS-PAGE by phosphorimager analysis and expressed as a percentage of the maximum value. Data of three independent experiments are shown (means ± standard error of the means). The presence of MEK5 in the immune complexes was detected by immunoblot (IB) analysis using an anti-GST antibody (A). (C) The reporter plasmid pG5E1bLuc was transiently cotransfected together with a construct encoding GAL4-MEF2A with expression vectors encoding GST, wild-type or mutant GST-MEK5α. MEF2 transcriptional activity was measured by a dual-luciferase reporter assay system. Values are expressed as ratios of firefly to *Renilla* luciferase activity. The data are the mean ± standard error of the means of three independent experiments (***, $P < 0.001$).

that contributes to the interaction with ERK5 (Fig. 1A and 3A and C) remains to be identified.

Consistent with its low binding affinity for ERK5, MEK5β that lacks the N terminus extension of MEK5α is a weak ERK5 activator (Fig. 1 and 2). However, when both MEK5 and ERK5 were coimmunoprecipitated, MEK5 isoforms displayed a similar ability to activate ERK5 (Fig. 1). These results lead us to conclude that MEK5β is an active enzyme, thereby emphasizing the functional importance of the interaction between MEK5 and ERK5 to mediating ERK5 signaling. The different activities of MEK5 isoforms are due to their distinct ability to interact with ERK5. Overall, our data do not support the idea that MEK5β is an enzyme-dead variant and a dominant-negative regulator of the ERK5 signaling pathway (4). Further studies are required to elucidate the function of MEK5β in vivo in order to understand the physiological significance of alternative splicing of the *mek5* gene.

The higher affinity of MEK5α compared to MEK5β for ERK5 (Fig. 3) suggested that the N-terminal extension of MEK5α was implicated in mediating the strong binding of MEK5 to ERK5. We found that the deletion of at least one acidic residue within the 63-EDED-66 motif or the mutation of a glutamic acid residue at position 61 in the N terminus of MEK5α dramatically decreased the binding interaction between MEK5 and ERK5 as well as the ability of MEK5 to activate ERK5 (Fig. 4, 5, and 6). These residues define a novel MAPK docking motif in the N-terminal domain of MEK5α within its PB1 domain (28). In contrast to the αE61L mutant, the αD68V mutant displayed normal affinity for ERK5 but was unable to interact with MEKK2 (Fig. 5). The identification of specific mutations in MEK5α that selectively affect the binding to ERK5 and MEKK2 clearly demonstrates that the interaction of MEK5α with its upstream and downstream kinases is mediated via distinct motifs. In particular, the formation of the MEK5/ERK5 complex does not rely on the PB1 domain of MEK5α. The region in the N terminus of ERK5 responsible for the binding to MEK5 corresponds to a stretch of 61 amino acids (amino acids 78 to 139) containing 11 basic residues (31). Altogether, these studies suggest that the interaction between ERK5 and MEK5 is triggered by opposite charges in the contact sites of the proteins.

The binding between MEKK2 or MEKK3 and MEK5α is mediated via their respective PB1 domains (17). The substitution of a conserved lysine residue at position 47 to an alanine residue in the N-terminal part of the PB1 domain of MEKK2 renders MEKK2 unable to bind MEK5 (17). In this study, we have identified residues 63 to 66 and 68 as critical amino acids of the PB1 domain of MEK5α (Fig. 5B and 7). Heterodimer-

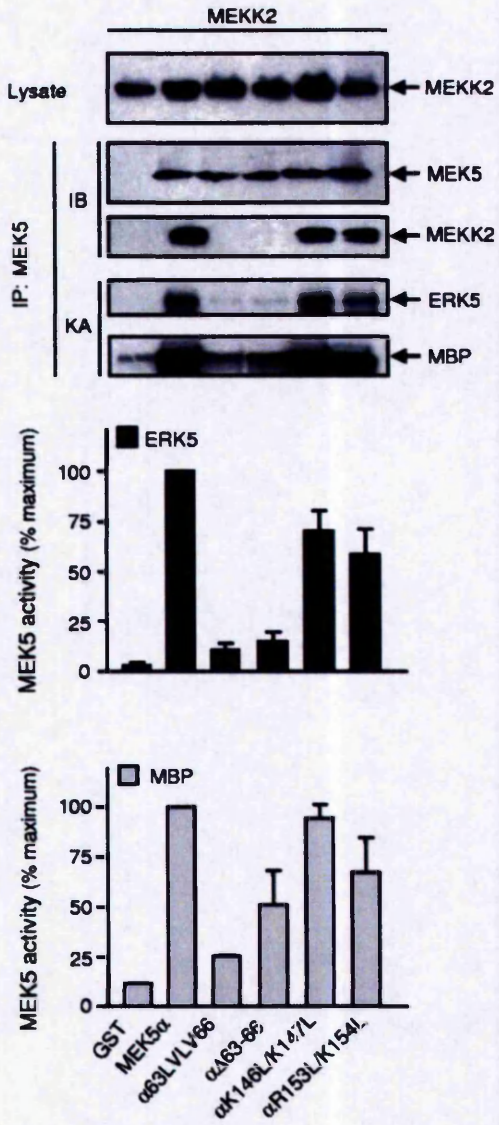

FIG. 7. Effect of mutations in the docking site on the regulation of MEK5 by MEKK2. COS-7 cells cotransfected with expression vectors encoding GST, wild-type or mutant GST-MEK5α, and HA-tagged MEKK2. MEK5 activity was measured by protein kinase assay (KA) following immunoprecipitation with an anti-GST antibody. The radioactivity incorporated into GST-ERK5 or MBP was quantitated after SDS-PAGE by phosphorimager analysis and expressed as a percentage of the maximum value. Data of three independent experiments are shown (means ± standard error of the means). The expression of MEKK2 in cell lysates and the presence of MEK5 and MEKK2 in the immune complexes were detected by immunoblot analysis (IB) using an anti-GST and an anti-HA antibody.

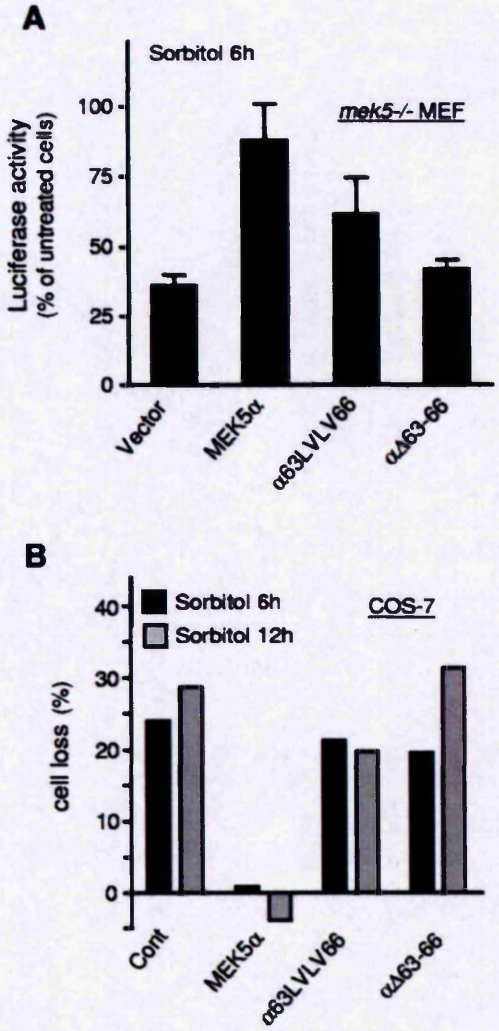

FIG. 8. The docking site is essential for the survival function of MEK5α. (A) *mek5*$^{-/-}$ fibroblasts were cotransfected with an empty vector (vector) or a vector encoding wild-type or mutant GST-MEK5α and luciferase (pCMV-luc) to monitor cell viability. At 36 h after transfection the cells were treated with 500 mM sorbitol for 6 h prior to being lysed, and the luciferase activity was measured. The values are normalized to the protein content. The data expressed as the percentage of treated versus untreated cells correspond to the means ± standard error of the means of three independent experiments. (B) COS-7 cells were transfected with an expression vector encoding wild-type or mutant GST-MEK5α and were treated with 500 mM sorbitol for the indicated times 24 h after transfection. The cells were fixed, stained with an anti-GST antibody coupled to Alexa Fluor 488, and analyzed by flow cytometry. The percentage of viable transfected cells identified by high fluorescence (>100-fold higher than baseline) was calculated. Cell loss represents the normalized values of treated versus untreated cells. The data are representative of two independent experiments.

ization between PB1 domains occurs via the interaction between conserved basic and acidic residues (19). Thus, residues 63 to 66 and 68 of MEK5 may constitute the binding site for lysine 47 of MEKK2. This interaction between MEK5 and MEKK2 is unique among the other MEK/MKK families (MEK1, MKK3, MKK4, MKK6, and MKK7) that have been found to bind their upstream kinases through a DVD site located in their C termini (27).

Mutations that prevent the formation of the MEK5α/MEKK2 complex abolish the activation of MEK5 by MEKK2 (Fig. 7). This observation appears to be inconsistent with the

ability of MEKK2 to increase MEK5β activity (Fig. 2A). Indeed, MEK5β that lacks the PB1 domain is unlikely to bind MEKK2. To explain this apparent discrepancy we propose that the N terminus of MEK5α contains an auto-inhibitory domain that masks the phosphorylation sites of MEK5 by upstream kinases. The interaction of MEK5 with MEKK2 affects the overall conformation of MEK5α so that S311 and T315 become accessible for phosphorylation. The recently solved structure of MEK1 (20) together with the identification of a conserved MEKK docking site in the C terminus of MEK1 (27) supports this general idea that the formation of the MEK/MEKK complex is not only required for mediating specificity but is also critical for making MEK receptive to phosphorylation by the bound MEKK.

In conclusion, as both MEKK2 and ERK5 interact with the N-terminal extension of MEK5α, we suggest that MEKK2 and ERK5 compete for binding to MEK5 rather than form a ternary complex. Based on a similar model proposed to explain the organization of the JNK signaling pathway (30), we hypothesize that MEKK2 and MEK5 form a complex that is dissociated upon activation. Activated MEK5 becomes free to interact with its substrate ERK5. Accordingly, the specific transmission of the signal within the ERK5 signaling pathway may not implicate a scaffold protein.

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The regulation of Bax by c-Jun N-terminal protein kinase (JNK) is a prerequisite to the mitochondrial-induced apoptotic pathway

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Abstract The signaling mechanism by which JNK affects mitochondria is critical to initiate apoptosis. Here we show that the absence of JNK provides a partial resistance to the toxic effect of the heavy metal cadmium. Both wild type and *jnk−/−* fibroblasts undergoing death exhibit cytosolic cytochrome *c* but, unlike wild type cells, the JNK-deficient fibroblasts do not display increased caspase activity and DNA fragmentation. The absence of apoptotic death correlates with a specific defect in activation of Bax. We conclude that JNK-dependent regulation of Bax is essential to mediate the apoptotic release of cytochrome *c* regardless of Bid and Bim activation.

Keywords: c-Jun N-terminal protein kinase; Apoptosis; Stress; Cytochrome *c*; Bcl-2 proteins

1. Introduction

Apoptosis is a physiological process by which cells undergo a programmed cell death (PCD) [1]. The characteristic morphological changes displayed by apoptotic cells reflect the activation of a tightly regulated intrinsic cell signaling machinery that leads to the activation of caspases [2]. The stress-induced caspase cascade is triggered by the mitochondrial release of cytochrome *c* that initiates the formation of an oligomeric multiprotein complex, the apoptosome, that includes the apoptotic protease-activating factor 1, and dATP or ATP [3,4]. How cytochrome *c* is released from the inter membrane space of the mitochondria remains a matter of intense debate [5]. The consensus model implicates the selective permeabilization of the outer mitochondrial membrane (OMM) by specific channels that involve Bcl-2 proteins [5,6]. This model offers the advantage of maintaining functional mitochondria and distinguishes apoptosis from necrosis where the release of cytochrome *c* occurs via the rupture of the OMM caused by the sustained opening of the permeability transition pore (PTP) [7].

The Bcl-2 family includes proteins that inhibit (e.g., Bcl-2, Bcl-x_L) and promote (e.g., Bax, Bak, Bid, Bim) apoptosis. Bcl-2, Bcl-x_L and Bak reside predominantly in the mitochondria, whereas Bax, Bid, and Bim reside mainly in the cytosol of healthy cells [8]. The accumulation of Bax at the mitochondria associated with a change in Bax conformation constitutes a critical checkpoint in the release of cytochrome *c* [9,10]. Activation of Bid correlates with its cleavage by caspase 8 and the generation of the death promoting fragment tBid [11,12]. Unlike Bid, Bim becomes hyperphosphorylated upon apoptotic stress and dissociates from the microtubule-associated dynein motor complex where it is normally sequestered [13].

Evidence suggests that the *c*-Jun NH_2-terminal protein kinase (JNK) cascade participates in the post-translational modifications of Bid and Bim [14–17]. Its requirement in mediating cytochrome *c* release was first demonstrated by the genetic analysis of homozygous *jnk1* and *jnk2* null (*jnk−/−*) mouse embryonic fibroblasts (MEFs) [17]. The essential role of Bax and Bak in mediating apoptosis via JNK is exemplified by the inability of a dominant active JNK to induce the death of cells deficient in Bax and Bak [18]. Whether JNK controls Bax/Bak function via the post-translational modification of Bid and Bim remains unresolved.

To further understand how cytochrome *c* is released from mitochondria we analyzed the effect of *jnk* gene deletion on the coordinated regulation of Bcl-2 proteins in response to cadmium (Cd). Our data demonstrate that the regulation of Bax by JNK is: (i) independent of Bim and Bid activation; and (ii) essential for mediating the apoptotic release of cytochrome *c*.

2. Materials and methods

2.1. Tissue culture and preparation of lysates

MEFs were cultured as previously described [17]. The cells were placed in 2% serum for 24 h prior to being treated with Cd chloride ($CdCl_2$). Cytosolic proteins were extracted in triton lysis buffer [17]. For detecting the cytosolic release of cytochrome *c* and apoptosis inducing factor (AIF), the cells were incubated at 4 °C for 15 min in mannitol buffer (10 mM HEPES, pH 7.2, 210 mM mannitol, 70 mM sucrose, 5 mM sodium succinate, 0.2 mM EGTA) containing 80 μg/ml digitonin. Supernatants were subsequently concentrated ~10-fold using Amicon Bioseparations centrifugal filters.

2.2. Biochemical studies

Lysates (50 μg) were analyzed by immunoblot [17]. Antibodies to cytochrome *c* (Pharmingen), AIF (Santa Cruz), caspase 3 (Cell Signaling Technology), poly (ADP-ribose) polymerase (PARP; Cell Signaling Technology), Bid (R&D Systems), Bim (Calbiochem), Bax

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Abbreviations: AIF, apoptosis inducing factor; JNK, c-Jun N-terminal protein kinase; MAPK, mitogen-activated protein kinase; MEFs, mouse embryonic fibroblasts; OMM, outer mitochondrial membrane; PCD, programmed cell death; PTP, permeability transition pore

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(Upstate), Bak (Upstate), actin (Calbiochem), or tubulin (Sigma) were used. Immunocomplexes were detected by enhanced chemiluminescence (Amersham-Pharmacia). JNK activity was measured in cell lysates (30 μg) by pull down protein kinase assay [17].

2.3. Cell viability and caspase assays

Cell viability was measured by crystal violet (CV) staining [17] and MTT assay. For the MTT assay, cells were incubated with 1 μg/μl of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide and lysed in DMSO. Caspase activity was measured by spectrofluorometer using the DEVD-AMC caspase 3 specific fluorogenic substrate.

2.4. Fluorescence microscopy

TUNEL was performed using the in situ Cell Death Detection kit (fluorescein) according to the manufacturer's instructions (Roche). For immunofluorescence the cells were fixed in methanol prior to being incubated with an anti-Bax antibody (clone 6A7, Sigma). Immune complexes were detected with a Texas red-conjugated anti-mouse immunoglobulin. Nuclei were stained with Hoechst 33342 (1 g/l). Fluorescence images were viewed with an Olympus Widefield microscope.

3. Results

The heavy metal cadmium (Cd) is a widespread industrial and environmental pollutant. Its toxicity is, at least in part, associated with its ability to cause necrotic and apoptotic cell death. Increased JNK activity was detected in wild type (wt) but not in *jnk1* and *jnk2* null (*jnk−/−*) MEFs incubated with 10 and 25 μM $CdCl_2$ with a maximum of 35- and 75-fold at 18 and 6 h, respectively (**Fig. 1A**). Control experiment showed no marked difference in the ability of Cd to increase p38 mitogen-activated protein kinase (MAPK) activity in both cell types (data not shown).

Consistent with the requirement of JNK in mediating apoptosis [17], *jnk−/−* MEFs were resistant to the toxic effect of 10 μM $CdCl_2$ that resulted in 50% death of the wt MEFs at 20 h (**Fig. 1B**, left panel). In contrast, wt and *jnk−/−* fibroblasts displayed similar sensitivity to 25 μM $CdCl_2$ resulting

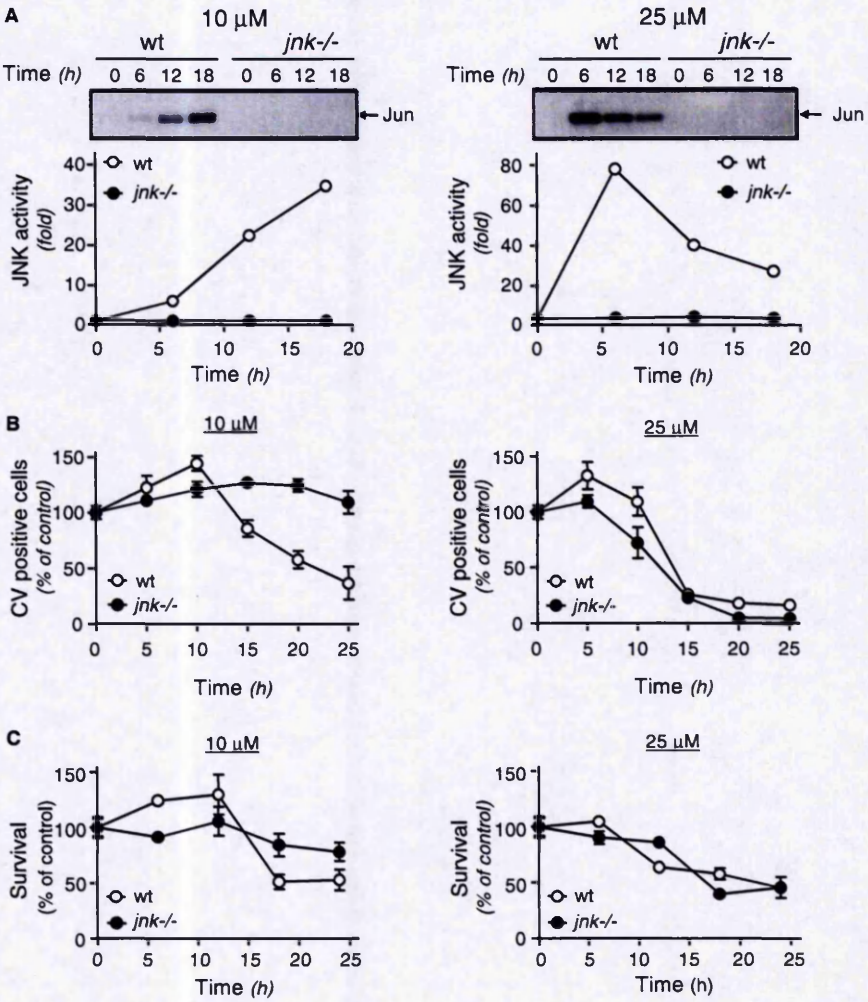

Fig. 1. JNK-deficient fibroblasts display distinct sensitivity to Cd toxicity. MEFs were treated with 10 or 25 μM $CdCl_2$ for the indicated times. (A) Endogenous JNK activity was measured by protein kinase assay. The radioactivity incorporated into recombinant c-Jun was quantitated by PhosphorImager analysis. Results are expressed as fold. Data are representative of two independent experiments. Cell survival was measured by (B), CV staining, and (C), MTT assay. The data represent the mean optical density expressed as % of control ± S.D. of two independent experiments performed in triplicate.

in 25% survival 15 h post-treatment (**Fig. 1B**, right panel). The finding that *jnk*−/− MEFs display distinct sensitivity to Cd toxicity was confirmed by MTT assay (**Fig. 1C**). Mitochondrial activity of wt but not *jnk*−/− cells was significantly impaired following incubation with 10 μM $CdCl_2$ for 18 h (**Fig. 1C**, left panel). In contrast, both cell types displayed a similar decrease in mitochondrial activity when incubated with 25 μM $CdCl_2$ (**Fig. 1C**, right panel). These results demonstrate that the protective effect of *jnk* gene deletion against Cd toxicity is dose dependent. High concentrations of Cd can trigger cell death via a JNK-independent mechanism.

To determine whether Cd triggers apoptotic death of fibroblasts, we examined its ability to induce DNA fragmentation, one hallmark of PCD [1]. Fragmented DNA was detected in wt fibroblasts treated with either 10 or 25 μM $CdCl_2$ (**Fig. 2A**). Concomitantly, the nuclei appeared shrunken with condensed chromatin (**Fig. 2A**). A similar change in the nuclear size was observed in the *jnk*−/− MEFs incubated with 25 μM but not 10 μM $CdCl_2$ (**Fig. 2A**). However, no TUNEL positive JNK-deficient cells were detected (**Fig. 2A**).

The requirement of JNK in mediating apoptosis was confirmed by the lack of caspase 3 activation in *jnk*−/− MEFs treated with 10 or 25 μM $CdCl_2$ (**Fig. 2B**). Immunoblot analysis demonstrated the proteolytic cleavage of caspase 3 in wt but not in *jnk*−/− MEFs following Cd treatment (**Fig. 2C**). The reduction of the inactive p32 proenzyme correlated with the appearance of the active p17 product and the cleavage of the poly (ADP-ribose) polymerase (PARP). Consistent with these results, the pancaspase inhibitor zVAD-fmk protected the wt but not the *jnk*−/− MEFs against Cd toxicity (data not shown). Altogether, these results provide strong genetic evidence that JNK is essential for mediating the apoptotic response of cells to Cd. In the absence of JNK, Cd triggers cell death via a caspase-independent mechanism.

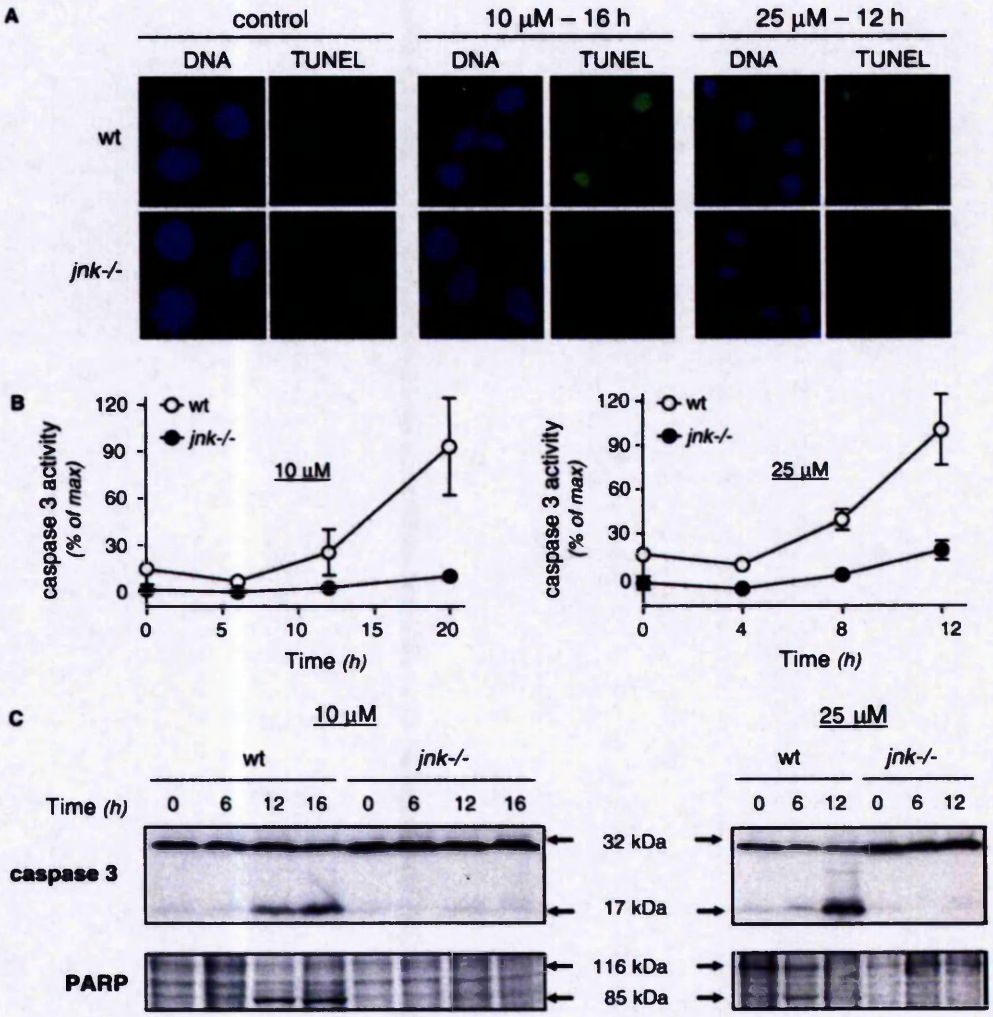

Fig. 2. Cd-induced apoptosis is JNK dependent. MEFs were treated with 10 or 25 μM $CdCl_2$ for the indicated times. (A) TUNEL (green) and Hoechst (blue) staining of the cells indicate increased DNA fragmentation in wt but not in *jnk*−/− fibroblasts treated with $CdCl_2$. Both cell types display similar changes in nuclear morphology when incubated with 25 μM $CdCl_2$. (B) Caspase 3 activity was measured by caspase assay. The data represent the mean caspase 3 activity expressed as % of maximum ± S.D. of two independent experiments performed in triplicate. (C) Cleavage of caspase 3 and PARP was followed by immunoblot analysis using specific antibodies. Immunoblots are representative of three independent experiments.

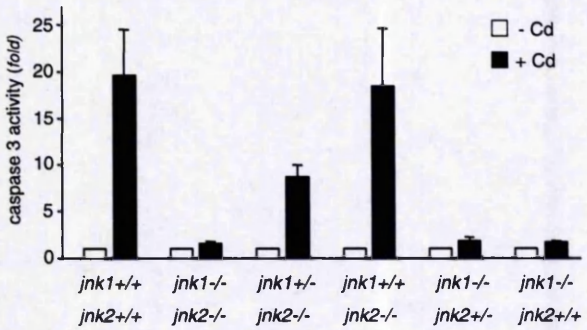

Fig. 3. Selective effect of *jnk1* and *jnk2* gene deletion on the apoptotic response of fibroblasts to Cd. MEFs carrying homozygous or heterozygous deletion of *jnk1* and *jnk2* genes were treated with 10 μM $CdCl_2$ for 16 h. Caspase 3 activity was measured by caspase assay. The data are expressed as fold ± S.E. of triplicate samples. Similar results were obtained in two independent experiments.

To determine the relative importance of JNK1 and JNK2 in mediating the apoptotic response of fibroblasts to 10 μM $CdCl_2$, we tested the effect of *jnk1* and *jnk2* deletion (**Fig. 3**). The tissue specific JNK3 isoform is not detected in MEFs [17]. No marked difference was observed between the ability of Cd to increase caspase 3 activity in wt and *jnk2* null (*jnk1+/+jnk2−/−*) fibroblasts. In contrast, cells carrying homozygous ablation of the *jnk1* gene (*jnk1−/−jnk2+/+* or *jnk1−/−jnk2+/−*) were resistant to Cd treatment. A greater role for JNK1 in mediating Cd-stimulated caspase 3 activation is consistent with the partial restoration of the wild type phenotype following heterozygous deletion of the *jnk1* gene in absence of JNK2 expression (*jnk1+/−jnk2−/−*).

To further establish the apoptotic defect caused by *jnk* gene deletion we examined the requirement of JNK in promoting the mitochondrial release of cytochrome *c*, one of the key events of apoptosis [3,4] (**Fig. 4**). 10 μM $CdCl_2$ promoted the release of cytochrome *c* and AIF in wt but not in *jnk−/−* MEFs (**Fig. 4A**, left panel). In contrast, a similar amount of cytochrome *c* and AIF was detected in the cytosolic fractions of wt and *jnk−/−* MEFs treated with 25 μM (**Fig. 4A**, right panel). These data indicate that the requirement of JNK for mediating Cd-induced cytochrome *c* release depends on the level of stress.

Next we demonstrated that neither zVAD-fmk (25 μM) nor cyclosporin A (CsA; 0.5 μM), an inhibitor of the PTP opening, blocked the ability of Cd to induce cytochome *c* release in fibroblasts (**Fig. 4B** and C). Similar results were obtained with 10 μM CsA (data not shown). Consistent with these data the decrease in cell viability following Cd treatment was not affected by the pretreatment of the cells with CsA (data not shown). We concluded that Cd-induced cytochrome *c* release

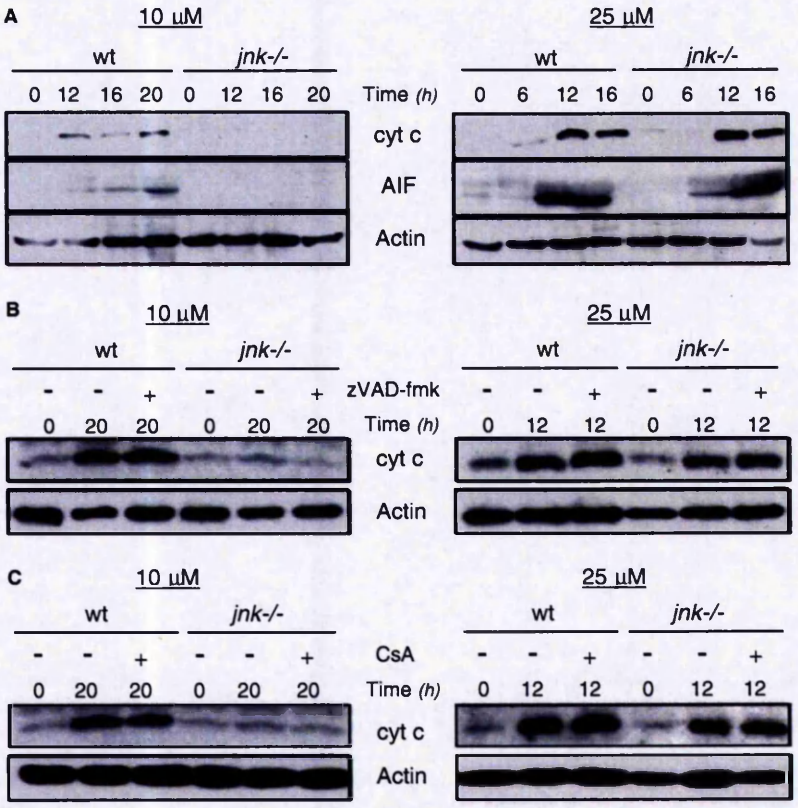

Fig. 4. The requirement of JNK for mediating cytochrome *c* release depends on the concentration of Cd. MEFs were treated with 10 or 25 μM $CdCl_2$ for the indicated times in the absence (A), or presence of zVAD-fmk (25 μM) (B), or CsA (0.5 μM) (C). Inhibitors were added 10 min prior to the agonist. Cytochrome *c* (cyt *c*) and AIF present in the cytosolic fraction were detected by immunoblot analysis. Detection of actin indicates equal protein loading. The results are representative of three independent experiments.

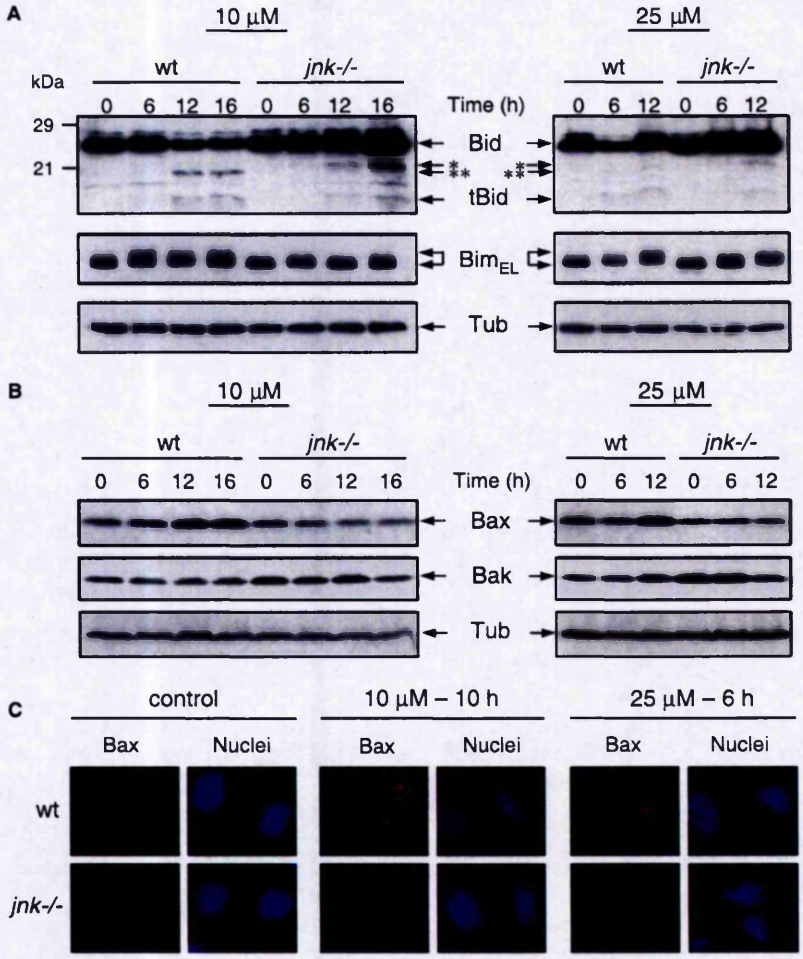

Fig. 5. JNK is essential for regulating Bax. MEFs were treated with 10 and 25 μM $CdCl_2$ for the indicated times. Expression of Bid and Bim (A), and Bax and Bak (B), was detected in cell lysates by immunoblot analysis using specific antibodies. (*) and (**) indicate the position of the 22 and 20 kDa Bid fragments, respectively. (C) Immunofluorescence was performed with a conformation specific anti-Bax antibody (clone 6A7, Sigma) and a goat anti-mouse antibody conjugated to Texas red. DNA was stained with Hoechst (blue).

does not implicate caspase activation or permeability transition, leading us to test the hypothesis that Cd initiates apoptosis via the regulation of Bcl-2 proteins.

The basal level of Bid, Bax, Bak and Bim expression was similar in wt and *jnk*−/− fibroblasts (**Fig. 5**). Cleavage of Bid in wt cells incubated with Cd correlated with loss of the full-length protein (25 kDa) and the concomitant appearance of Bid fragments at 20 and 15 kDa (tBid) (**Fig. 5A**). tBid was detected in the *jnk*−/− MEFs incubated with Cd. However, unlike in wt cells, this correlated with the accumulation of the full-length protein and the appearance of a 22 kDa fragment (**Fig. 5A**). We concluded that JNK is not essential for the appearance of tBid in response to stress. In addition to inducing the apoptotic cleavage of Bid, Cd increases Bid expression by a mechanism that is inhibited by JNK.

Bim is another BH3-only protein implicated in the intrinsic cell death signaling pathway. Incubation of the wt cells with 10 or 25 μM $CdCl_2$ caused distinct electrophoretic mobility shifts indicative of Bim being phosphorylated (**Fig. 5A**). Similar phosphorylation pattern of Bim was detected in the *jnk*−/− cells but only in response to 25 μM $CdCl_2$. These results indicate that JNK is the primary kinase to phosphorylate Bim, but its requirement can be overturned by increasing the level of stress.

No change in the level of Bak was detected in MEFs after Cd treatment (**Fig. 5B**). In contrast, a significant increase in Bax expression was detected in wt cells in response to 10 and 25 μM $CdCl_2$ after 12 and 6 h stimulation, respectively (**Fig. 5B**). This effect was prevented by *jnk* gene deletion. Immunofluorescence analysis using a conformation specific anti-Bax antibody **[19]** showed the presence of active Bax at the mitochondria in wt but not in *jnk*−/− MEFs stimulated with Cd (**Fig. 5C**). Previous studies have shown that this characteristic change in Bax conformation precedes the apoptotic release of cytochrome *c* in the cytosol **[10]**. Consistent with the requirement of Bax in Cd-induced apoptosis, we found that Bax was essential for mediating increased cytochrome *c* release and caspase 3 activation in response to 10 μM $CdCl_2$ treatment (**Fig. 6**).

These observations suggest that, in absence of JNK, cytochrome *c* is released from the mitochondria via a mechanism

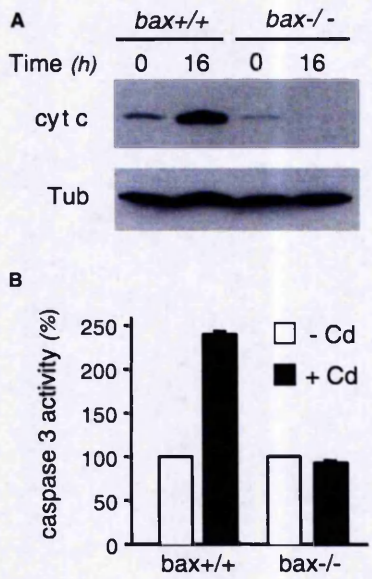

Fig. 6. The deletion of the *bax* gene prevents activation of the mitochondrial apoptotic pathway. MEFs were treated with 10 μM $CdCl_2$ for 16 h. (A) Bax is required for Cd-stimulated cytochrome *c* release. Cytochrome *c* (cyt *c*) present in the cytosolic fraction was detected by immunoblot analysis. Detection of tubulin (Tub) indicates equal protein loading. Similar results were obtained in two independent experiments. (B) Caspase 3 activity measured by caspase assay is increased after exposure of wild type (*bax*+/+) but not homozygous *bax* null (*bax*−/−) MEFs to Cd. The data are expressed as % ± S.E. of triplicate samples.

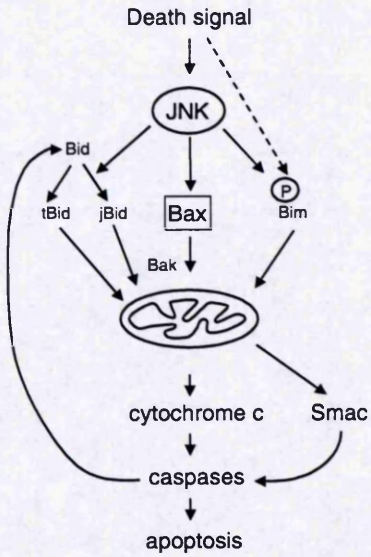

Fig. 7. Mechanism of JNK-mediated apoptotic release of cytochrome *c* via Bcl-2 proteins. JNK promotes the accumulation of active Bax at the mitochondria. In addition, JNK is essential for increasing Bax expression. jBid is generated from the cleavage of Bid by a JNK-dependent and caspase-independent mechanism. Its ability to alleviate caspase inhibition via the release of second mitochondria-derived activator of caspase (Smac) explains how JNK contributes to the efficient cleavage of Bid by caspase 8 and the generation of tBid. JNK-dependent and independent phosphorylation of Bim causes its dissociation from the dynein motor complex. The targeting of Bax, Bid, and Bim to the mitochondria promotes the release of cytochrome *c* that triggers the activation of the caspase cascade.

independent of Bax. Altogether these experiments clearly identify JNK as an essential regulator of Bax activity.

4. Discussion

Consistent with a previous study [18] our data provide direct genetic evidence that JNK is critical for mediating Bax activation in response to stress. Bax is not a JNK substrate (data not shown). The dissociation of the 14-3-3/Bax complex via JNK-dependent phosphorylation of 14-3-3 [20] constitutes a possible mechanism by which JNK promotes the accumulation of active Bax at the mitochondria. In addition, we demonstrate that JNK controls the expression of Bax. A previous study has shown that increased AP-1 activity by JNK induces the transcription of the *bax* gene in response to butyric acid [21]. However, real time PCR analysis indicates that Cd does not regulate the levels of *bax* mRNA (data not shown). Consistent with the ability of JNK to control the apoptotic response of MEFs to UV without requiring de novo gene expression [17], Cd induces Bax expression via a post-transcriptional mechanism that includes the stabilization of the protein by JNK.

In contrast to Bax, increased Bid expression following stress appears to be inhibited by JNK. This may be a consequence of a decrease in the efficiency of Bid cleavage in cells lacking JNK. A recent study showed that the activation of JNK by TNFα resulted in the generation of a distinct Bid cleavage product termed jBid (21 kDa) [14]. jBid promotes the specific release of Smac, thereby neutralizing inhibitors of apoptosis proteins and leading to increased caspase 8 activity. The indirect activation of caspase 8 by JNK via jBid provides an elegant mechanism to explain how JNK contributes to the efficient cleavage of Bid. Whether the 20 kDa Bid cleavage product identified in wild type MEFs corresponds to jBid remains to be confirmed.

Previous studies have shown that phosphorylation of Bim by JNK promotes its dissociation from the microtubule-associated dynein motor complex, thereby enhancing its pro-apoptotic activity [15,16]. Consistent with these findings we identified Bim as a downstream target of JNK. However the requirement of JNK can be bypassed by increasing the levels of stress. p38 MAPK which activity is upregulated by Cd (data not shown) may be the protein kinase responsible for Bim phosphorylation in the absence of JNK.

Altogether these studies provide a molecular link between JNK and the initiation of the mitochondrial apoptotic pathway (Fig. 7). The data support the hypothesis that Bax-dependent release of cytochrome *c* via JNK is a pre-determining step in the apoptotic process. The defect in Bax regulation associated with homozygous deletion of *jnk* genes prevents the formation of specific channels in the OMM [6]. Consequently, the release of cytochrome *c* in the *jnk*−/− MEFs results from the rupture of the OMM caused by the sustained opening of the PTP and subsequently mitochondrial matrix swelling, a mechanism implicated in necrotic cell death induced by high concentrations of Cd [7].

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