

Requirement of the MEK5–ERK5 pathway for neural differentiation in *Xenopus* embryonic development

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Although previous studies have identified several key transcription factors in the generation process of the vertebrate nervous system, the intracellular signalling pathways that function in this process have remained unclear. Here we identify the evolutionarily conserved mitogen-activated protein kinase kinase 5 (MEK5)–extracellular signal-regulated kinase 5 (ERK5) pathway as an essential regulator in neural differentiation. Knockdown of *Xenopus* ERK5 or *Xenopus* MEK5 with antisense morpholino oligonucleotides results in the reduced head structure and inhibition of neural differentiation. Moreover, forced activation of the MEK5–ERK5 module on its own induces neural differentiation. In addition, we show that the MEK5–ERK5 pathway is necessary for the neuralizing activity of SoxD, a regulator of neural differentiation, and is sufficient for the expression of *Xngnr1*, a proneural gene. These results show that the MEK5–ERK5 pathway has an essential role in the regulation of neural differentiation downstream of SoxD and upstream of *Xngnr1*.

Keywords: MAP kinase; neural development; signal transduction; *Xenopus laevis*

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INTRODUCTION

During vertebrate embryogenesis, the neuroectoderm arises from uncommitted ectoderm. In *Xenopus laevis*, signals from the Spemann organizer induce neural tissues on the dorsal side of the ectoderm. The factors include Noggin, Chordin and Follistatin, which bind to bone morphogenetic protein-4 (BMP-4) in the extracellular space and inactivate BMP signalling (Lamb *et al*, 1993; Hemmati-Brivanlou *et al*, 1994; Sasai *et al*, 1995; Piccolo *et al*, 1996; Fainsod *et al*, 1997). As BMP signalling promotes epidermal differentiation and inhibits neural differentiation, the inhibition of BMP signalling by neural inducers promotes neural differentiation (Munoz-Sanjuan & Brivanlou, 2002; De Robertis & Kuroda, 2004). Recent studies identified the factors that were regulated by neural inducers, including several transcription factors, such as basic

helix–loop–helix (bHLH), Zic and Sox factors (Sasai, 1998). SoxD was identified as a gene induced by Chordin (Mizuseki *et al*, 1998). It is expressed widely in the ectoderm at the late blastula stages, and, after mid-gastrula stages, its expression is limited at the neuroectoderm. SoxD has a neuralizing activity, and its expression is enhanced by Zicr1, another mediator of neural differentiation. One of the genes functioning downstream of SoxD is *Xngnr1*, which is a member of the bHLH family. It is one of the earliest proneural genes and its transcript is detected in the neural plate at mid-gastrula stages (Ma *et al*, 1996). Several transcription factors have been identified, but the intracellular signalling pathways that regulate early neural development have not been fully defined.

The mitogen-activated protein (MAP) kinase pathways are among the conserved intracellular signalling pathways. So far, at least four independent MAP kinase pathways have been identified—the extracellular signal-regulated kinase (ERK) 1/2 pathway, the c-Jun amino-terminal kinase (JNK) pathway, the p38 pathway and the ERK5 pathway (Sturgill & Wu, 1991; Nishida & Gotoh, 1993; Robinson & Cobb, 1997; Davis, 2000; Kyriakis & Avruch, 2001). The ERK5 pathway is shown to be activated by oxidative stress, hyperosmolarity and growth factors, such as the nerve growth factor and epidermal growth factor (Abe *et al*, 1996; Kato *et al*, 1997, 1998; Kamakura *et al*, 1999). Mice that lack ERK5 or MAP kinase kinase 5 (MEK5), which is a direct activator of ERK5, are embryonic lethal and show cardiovascular defects (Regan *et al*, 2002; Sohn *et al*, 2002; Yan *et al*, 2003; Hayashi *et al*, 2004; Wang *et al*, 2005). However, the precise role of the MEK5–ERK5 module in embryonic developmental processes such as neural differentiation has not been fully understood.

In this study, we have identified the MEK5–ERK5 pathway as a novel signalling pathway that regulates neural differentiation. Our results show that activation of the MEK5–ERK5 pathway is necessary, and is sufficient, for neural differentiation in *Xenopus* early embryonic development. In addition, the MEK5–ERK5 pathway is required for SoxD-induced neural differentiation and is capable of inducing the expression of *Xngnr1*, suggesting that the MEK5–ERK5 pathway regulates neural differentiation downstream of SoxD and upstream of *Xngnr1*.

RESULTS AND DISCUSSION

ERK5 and MEK5 are essential for neural differentiation

We isolated complementary DNAs encoding *Xenopus* orthologues of ERK5 (xERK5) and MEK5 (xMEK5) from the *Xenopus*

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gastrula cDNA library (supplementary Fig 1 online). The DDBJ/EMBL/GenBank accession numbers for xERK5 and xMEK5 are AB213560 and AB213561, respectively. While this work was in progress, Klein *et al* isolated cDNAs (unpublished; GenBank accession numbers BC077412 and AAH68926) that were almost identical to the cDNA clones that we had isolated. Coexpression of ERK5 and MEK5 in animal caps showed that xERK5 is a downstream target of xMEK5 and that the MEK5–ERK5 pathway is conserved among vertebrates (supplementary Fig 2 online). xERK5 and xMEK5 were expressed maternally and throughout early embryogenesis (supplementary Fig 3 online). Whole-mount *in situ* hybridization showed that xERK5 was expressed widely in the presumptive mesoderm and ectoderm during the blastula and gastrula stages, and in the head and dorsal structures during the neurula and tailbud stages (supplementary Fig 4 online). Reverse transcription–PCR (RT–PCR) analyses with microsectioning showed essentially the same results (data not shown). xMEK5 showed an expression pattern similar to that of xERK5 (data not shown).

To determine the loss-of-function phenotypes, we designed antisense morpholino oligonucleotides against xERK5 (ERK5 MO) or xMEK5 (MEK5 MO). The co-injection of MOs with Myc-tagged xERK5 or xMEK5 messenger RNA showed that ERK5 MO and MEK5 MO specifically reduced the protein level of xERK5 and xMEK5, respectively (Fig 1A). Then, we injected ERK5 MO, MEK5 MO or control MO into the dorsal marginal zones of four-cell-stage embryos. Embryos injected with ERK5 MO or MEK5 MO were normal at the blastula and gastrula stages (data not shown). At the tailbud stages, embryos injected with ERK5 MO or MEK5 MO showed defects in the head region: a reduction or a complete loss of eyes and, in severe cases, loss of a cement gland. Embryos injected with control MO were normal (Fig 1B,C). We confirmed the specificity of ERK5 MO by rescue experiments with a mouse homologue of ERK5, mERK5, which was not downregulated by ERK5 MO (Fig 1A,D). In embryos injected with ERK5 MO, expression of a neural marker, N-tubulin, was reduced, especially in the head region (Fig 1E), whereas expression of a notochord gene, PA26 (Hikasa & Taira, 2001), was unaffected (Fig 1F). The result suggests that the phenotypes shown in Fig 1B,C are caused by defects in neural tissues. As previous studies have shown that ERK5 is important for cell survival in murine neural tissues (Liu *et al*, 2003; Yan *et al*, 2003), we tested the levels of cell death by TdT-mediated dUTP nick end labelling (TUNEL) assays. Embryos injected with ERK5 MO or MEK5 MO (supplementary Fig 5 online) did not show an increase of TUNEL-positive cells. This suggests that the defects in embryos that are injected with ERK5 MO or MEK5 MO are not caused by increased apoptosis.

The MEK5–ERK5 pathway regulates neural differentiation

We then examined the role of the MEK5–ERK5 pathway in more detail. Animal caps treated with activin, a member of the transforming growth factor- β family, express various molecular markers. These include neural markers (N-tubulin, NCAM and NeuroD), mesodermal markers (MyoDa, muscle actin and collagen type 2) and an endodermal marker (endodermis; Fig 2A). ERK5 MO suppressed induction of neural markers caused by activin, whereas it had no effect on the expression of mesodermal or endodermal markers (Fig 2A). This result suggests that the MEK5–ERK5 pathway is required specifically for neural differentiation. However, activin is not a direct inducer of neural markers: it first

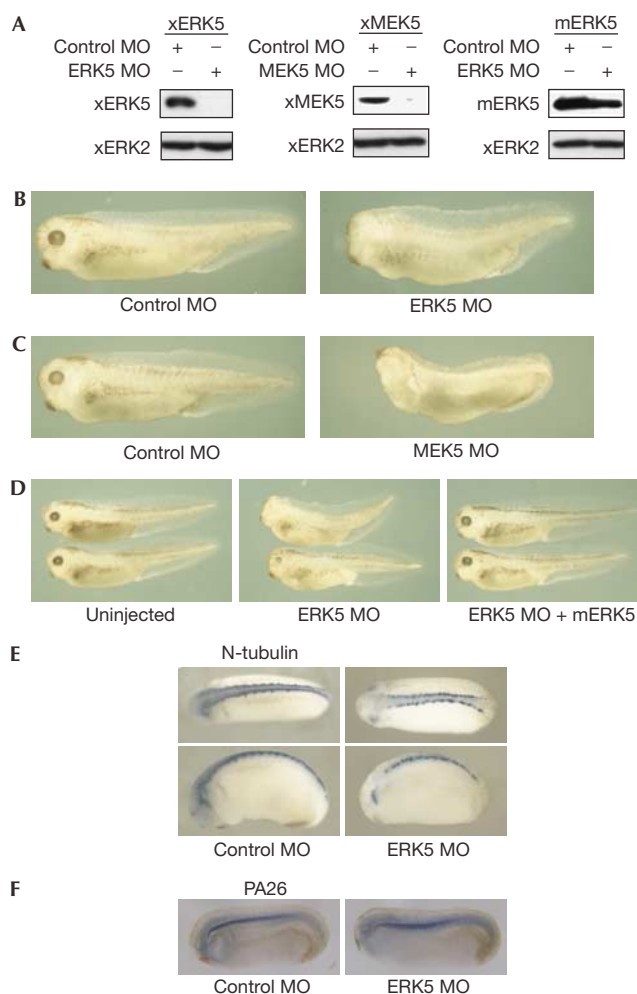


Fig 1 | ERK5 and MEK5 are essential for neural development.

(A) Indicated sets of morpholino oligonucleotides (MOs; 20 ng, left and right panels; 160 ng, middle panel) and messenger RNA (1.0 ng) were injected and the protein level was examined by immunoblotting with anti-Myc antibody (for *Xenopus* orthologues of ERK5 (xERK5; Myc–xERK5) and MEK5 (xMEK5; Myc–xMEK5)) or anti-ERK5 antibody (for mouse homologue of ERK5 (mERK5)). We used xERK2 as a loading control. (B) Control MO or ERK5 MO (20 ng) was injected into the dorsal marginal zone of four-cell-stage embryos. It was observed that 82% of embryos injected with ERK5 MO showed reduction or loss of eyes ($n=60$), whereas only 7% of embryos injected with control MO showed defects ($n=85$). (C) Control MO or MEK5 MO (200 ng) was injected as indicated in (B). It was observed that 72% of embryos injected with MEK5 MO showed reduction or loss of eyes ($n=99$), whereas only 12% of embryos injected with control MO showed defects ($n=110$). (D) mERK5 mRNA (0.7 ng) was co-injected with ERK5 MO (12 ng) for the rescue experiment. Only 11% of embryos that were injected with both ERK5 MO and mERK5 mRNA showed defects in the eyes ($n=141$), whereas 54% of embryos injected with ERK5 MO alone showed defects ($n=81$). (E,F) ERK5 MO or control MO (20 ng) was injected into the dorsal marginal zone and expression of N-tubulin or PA26 was examined by whole-mount *in situ* hybridization.

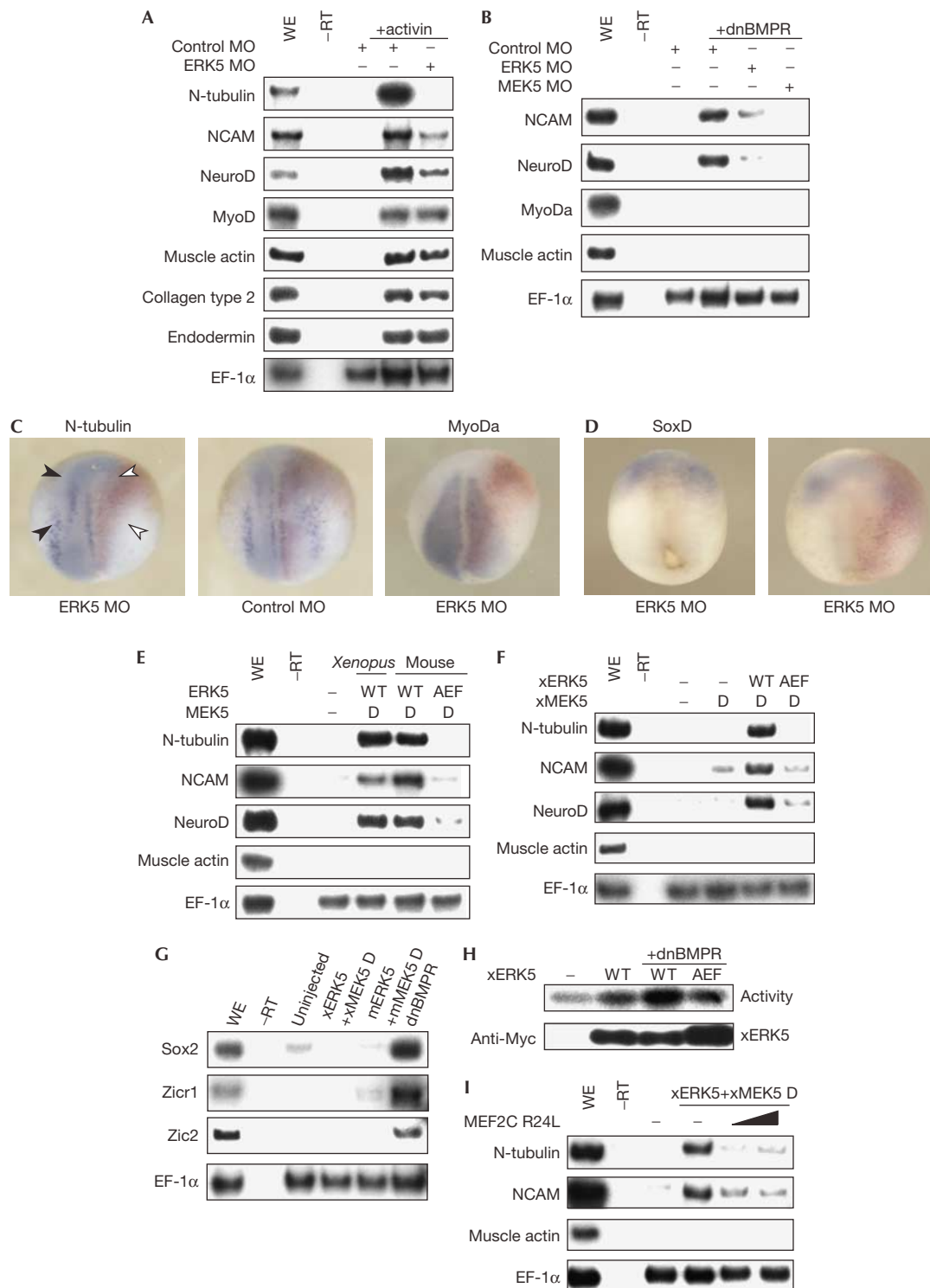
Fig 2 | Involvement of the MEK5–ERK5 pathway in neural differentiation. (A) Control morpholino oligonucleotide (MO) or ERK5 MO (20 ng in total) was injected into each blastomere of four-cell-stage embryos. Animal caps were treated with 10 ng/ml activin and cultured to stage 20. The expression of marker genes was examined. Elongation factor-1 α (EF-1 α) served as a loading control; WE, whole embryo. (B) Dominant-negative bone morphogenetic protein receptor (dnBMPR) messenger RNA (0.4 ng) with control MO (160 ng), ERK5 MO (20 ng) or ERK5 MO (160 ng) was injected and animal caps from the embryos were analysed by reverse transcription–PCR (RT–PCR). (C,D) ERK5 MO or control MO (20 ng) was injected into the right blastomeres of four-cell-stage embryos. The expression pattern of N-tubulin or MyoDa (neurula stages) (C) or SoxD (gastrula stage) (D) was analysed by whole-mount *in situ* hybridization. Nuclear localization signal– β -galactosidase mRNA was co-injected and used as a tracer (red staining). Dorsal views of embryos are shown with the anterior side at the top. (E,F) Indicated sets of mRNAs (1.0 ng) were injected and animal caps from the embryos were analysed by RT–PCR; WT, wild type. (G) Indicated sets of mRNAs were injected. Animal caps were cultured to stage 12 and analysed by RT–PCR. (H) Myc–xERK5 (*Xenopus* orthologue of ERK5) or Myc–xERK5 AEF mRNA (1.0 ng) was injected with dnBMPR mRNA (0.4 ng) and animal caps were cultured to stage 14. Activity of xERK5 was measured by kinase assays. The amounts of immunoprecipitated xERK5 were detected by anti-Myc antibody. (I) xERK5 and xMEK5 D (constitutively active xMEK5) mRNAs were injected with MEF2C R24L mRNA (1.0 or 2.0 ng) and animal caps were subjected to RT–PCR.

induces mesoderm, and then neural inducers, which are expressed in the mesoderm, induce neural tissues. To confirm that ERK5 MO suppresses induction of neural markers with no effect on mesoderm induction, we used a dominant-negative form of BMP receptor (dnBMPR) instead of activin. dnBMPR inhibits BMP signalling and mimics the neuralizing activity of neural inducers. ERK5 MO or MEK5 MO suppressed the expression of neural markers induced by dnBMPR in animal caps (Fig 2B). In embryos, ERK5 MO suppressed the expression of neural markers, without affecting the expression of mesodermal markers. Thus, embryos injected with ERK5 MO on the right blastomeres were analysed by whole-mount *in situ* hybridization. Although N-tubulin, a neural marker, was expressed normally on the left side (Fig 2C, left panel, black arrowheads), its expression on the right side was suppressed (Fig 2C, left panel, white arrowheads). Nuclear localization signal– β -galactosidase was used as a tracer. The observation that the reduction of expression of N-tubulin was observed even in the region with no LacZ staining suggests that ERK5 MO might function in a non-cell-autonomous manner in some regions. ERK5 MO had no effect on the expression of MyoDa, a mesodermal marker (Fig 2C, right panel). Expression of an early neural marker gene, SoxD, was unchanged in the presence of ERK5 MO (Fig 2D), which indicates that ERK5 is not required for neural induction. These results indicate that activation of the MEK5–ERK5 pathway is required for neural differentiation. Next, we examined whether activation of the MEK5–ERK5 pathway is sufficient for neural differentiation. Co-injection of xERK5 mRNA and constitutively active xMEK5 (xMEK5 D) mRNA caused the induction of neural markers such as N-tubulin, NCAM and NeuroD without mesoderm induction (Fig 2E,F). Forced activation of the MEK5–ERK5 pathway, however, was unable to induce expression of early neural markers, Sox2, Zic1 and Zic2 (Fig 2G). Overexpression of mouse homologues of ERK5 and MEK5 D, mERK5 and mMEK5 D, had essentially the same effect on gene expression (Fig 2E,G). These results also indicate that the MEK5–ERK5 pathway is essential for neural differentiation, but not for neural induction. To examine whether the kinase activity of ERK5 is required for its neuralizing activity, we used a non-activatable mutant of ERK5 (mERK5 AEF and xERK5 AEF), in which the activating phosphorylation sites were mutated. Injection of mRNAs of mERK5 AEF with mMEK5 D, or of xERK5 AEF with xMEK5 D, did not induce expression of neural marker genes (Fig 2E,F), indicating a requirement of the kinase activity of ERK5. To examine whether the MEK5–ERK5 pathway is activated in neural differentiation, the effect of expression of dnBMPR on the kinase activity of expressed xERK5 was studied. Expression of dnBMPR was able to activate xERK5 significantly (Fig 2H), suggesting that the

MEK5–ERK5 pathway lies downstream of neural inducers. As members of the myocyte enhancer factor-2 (MEF2) family are known to be direct targets of ERK5 (Kato *et al*, 1997), we examined the possible involvement of MEF2 in neural differentiation. We used dominant-negative MEF2C (MEF2C R24L), which has a mutation in the DNA-binding domain (Molkentin *et al*, 1996). Co-injection of MEF2C R24L inhibited the neural differentiation caused by activation of the MEK5–ERK5 pathway (Fig 2I), indicating that MEF2 may function downstream of ERK5 in this process.

Relationship with other regulators

We next examined the relationship of the MEK5–ERK5 pathway with some of the other known regulators of neural differentiation. A previous study had shown that inhibition of BMP signalling induces a transcription factor, SoxD, and the forced expression of SoxD causes ectopic neural tissues (Mizuseki *et al*, 1998). The injection of ERK5 MO or MEK5 MO suppressed the induction of neural tissues by SoxD (Fig 3A,B). These results indicate that the MEK5–ERK5 pathway is required for the neuralizing activity of SoxD. The crucial genes lying downstream of SoxD include Xngnr1, which is a proneural gene (Ma *et al*, 1996). In contrast to SoxD, induction of neural markers by Xngnr1 was not inhibited by ERK5 MO (Fig 3C). Furthermore, activation of the MEK5–ERK5 pathway, as well as the inhibition of BMP signalling, was able to induce the expression of Xngnr1 (Fig 3D). We then considered the possibility that expression of growth factors such as fibroblast growth factor (FGF) is induced downstream of SoxD, as previous studies have shown that FGF induces neural tissues in animal caps and dominant-negative FGF receptor inhibits the expression of neural genes in Noggin-treated animal caps (Kengaku & Okamoto, 1995; Launay *et al*, 1996) and that FGF can activate the MEK5–ERK5 pathway in mouse embryonic fibroblasts (Kesavan *et al*, 2004). It has also been reported that insulin-like growth factors (IGFs) are essential for anterior neural induction (Pera *et al*, 2001, 2003; Richard-Parpaillon *et al*, 2002). Then, we examined the expression levels of several growth factors after forced expression of SoxD. Interestingly, the expression level of FGF13, as well as that of FGF9, was found to be increased significantly by the expression of SoxD (Fig 3E). On the basis of these results, we propose a hypothetical model in which the MEK5–ERK5 pathway regulates neural differentiation downstream of SoxD and upstream of Xngnr1 (Fig 3F). It is possible that FGF13 is a candidate molecule lying between SoxD and the MEK5–ERK5 pathway, as it has been reported that FGF13 is expressed in the neural tube in the chick (Munoz-Sanjuan *et al*, 1999) and FGF13 induces the phosphorylation of ERK2 in rat hippocampal astrocytes (Greene *et al*, 1998).



Although it has been shown that SoxD is not essential for neural induction in the trunk CNS (Mizuseki *et al*, 1998), our results indicate that the MEK5–ERK5 pathway is essential for both anterior and posterior neural differentiation (Figs 1E,2C). We may speculate that unidentified molecules other than SoxD function upstream of the MEK5–ERK5 pathway in posterior neural differentiation.

CONCLUSION

In this study, we have shown a novel function of the MEK5–ERK5 pathway in neural differentiation. Genetic studies with ERK5- or MEK5-deficient mice showed that the MEK5–ERK5 pathway has an important role in cardiovascular development (Regan *et al*, 2002; Sohn *et al*, 2002; Yan *et al*, 2003; Hayashi *et al*, 2004;

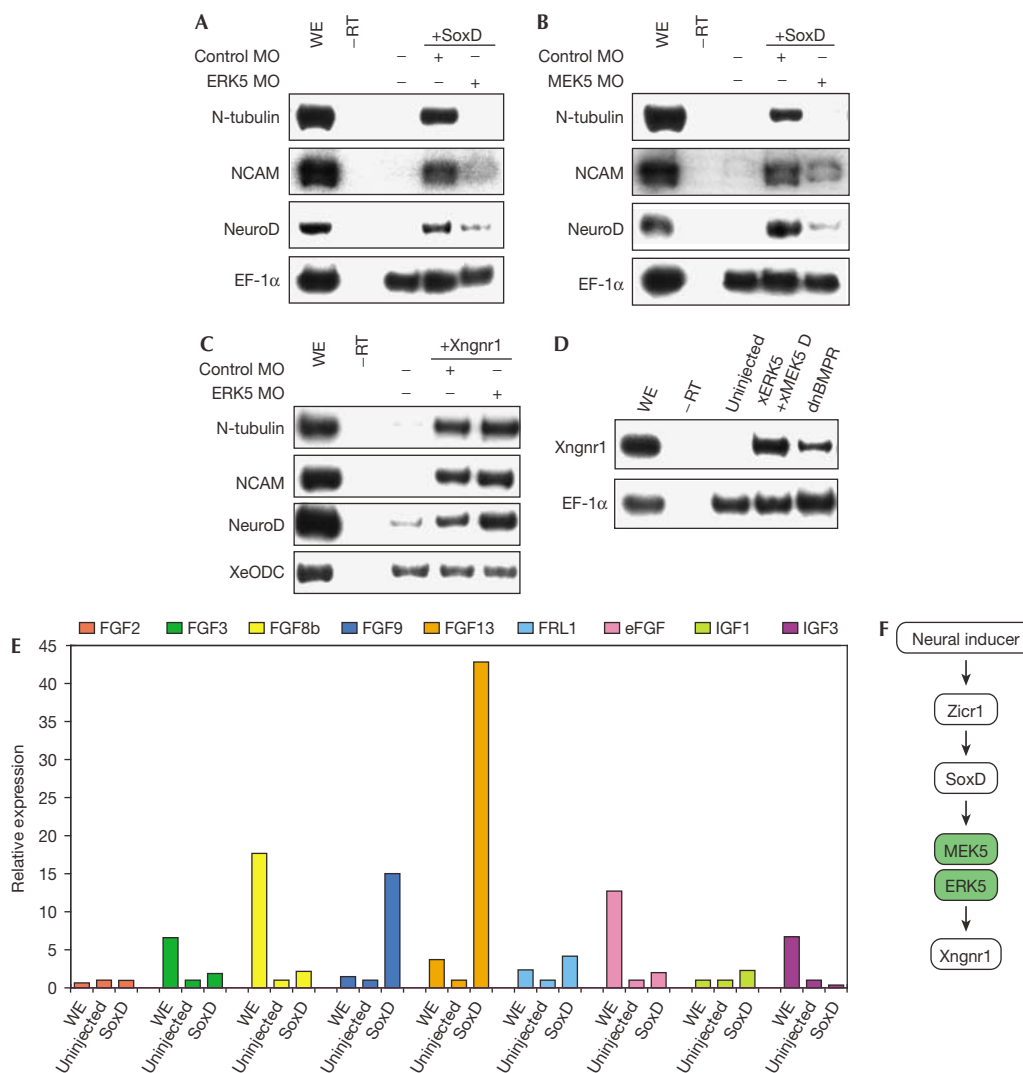


Fig 3 | Relationship between the MEK5-ERK5 pathway and other regulators of neural differentiation. (A,B) SoxD messenger RNA (0.4 ng) was injected with ERK5 morpholino oligonucleotide (ERK5 MO; 20 ng) or MEK5 MO (160 ng) and animal caps were analysed by reverse transcription-PCR (RT-PCR) Elongation factor-1 α (EF-1 α); WE, whole embryo. (C) Xngnr1 mRNA (40 pg) with control MO or ERK5 MO (20 ng) was injected at the animal hemisphere and animal caps were analysed. (D) xERK5 (*Xenopus* orthologue of ERK5) mRNA plus xMEK5 D (constitutively active MEK5) mRNA or dominant-negative bone morphogenetic protein receptor (dnBMPR) mRNA was injected. Animal cap explants from the embryos were cultured to stage 12 and analysed by RT-PCR. (E) SoxD mRNA (0.4 ng) was injected and animal caps were cultured to stage 14. Expression of fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs) was analysed by real-time RT-PCR. eFGF, embryonic FGF; FRL1, fibroblast growth factor receptor ligand 1. (F) A hypothetical model. The MEK5-ERK5 pathway regulates neural differentiation, probably downstream of SoxD and upstream of Xngnr1.

Wang *et al*, 2005). They also indicated that the mutant embryos show increased apoptosis and defects in endothelial cells, and thus growth retardation. Our study suggests that the inhibition of neural differentiation may be one of the reasons for growth retardation in the head region, which is consistent with the finding that growth retardation is severe in the head region. This implies that the essential role of the MEK5-ERK5 pathway in neural development is evolutionarily conserved in vertebrates. As the method to regulate specifically the activation of the MAP kinase modules, such as the MEK5-ERK5 module, has been well developed, our finding of the involvement of the MEK5-ERK5 module in neural differentiation could facilitate *in vitro* regulation of neural differentiation as well as further mechanistic studies on neural development.

METHODS

Embryonic manipulation, LacZ staining, whole-mount *in situ* hybridization and reverse transcription-PCR. *In vitro* fertilization, injection, LacZ staining, whole-mount *in situ* hybridization and RT-PCR were performed, as described (Kusakabe *et al*, 2001; Nitta *et al*, 2004). Recombinant human activin A was purchased from Genzyme-technie (MN, USA). For quantitative PCR analysis, we used 7300 Real Time PCR System (Applied Biosystems, CA, USA) with SYBR Green PCR Master Mix (Applied Biosystems). The sequences of primer pairs are described in the supplementary information online.

Morpholino oligos. Antisense MOs were obtained from Gene Tools Inc. (OR, USA). The morpholino oligo sequences were as

follows: ERK5 MO, 5'-CTTGCTCCTTCTGGCTCCGCCAT-3'; MEK5 MO, 5'-CAGGCCGAAGCCAAATAACAGCATC-3'; a standard control oligo (control MO), 5'-CCTCTTACCTCAGTTACAATTTATA-3'. Sequences complementary to the predicted start codon are underlined.

Antibody. We used anti-Myc antibody (9E10; Santa Cruz, CA, USA) or anti-ERK5 antibody (Sigma, MO, USA) for immunoblotting.

Kinase assays. Animal caps were crushed in a buffer containing 20 mM Hepes pH 7.5, 25 mM β -glycerophosphate, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1.5 mM $MgCl_2$, 2 mM EGTA, 50 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulphonyl fluoride, 1% aprotinin and 2 mM dithiothreitol. Myc-tagged xERK5 was immunoprecipitated and incubated for 15 min at 37 °C with 20 μ g of myelin basic protein in a buffer (15 μ l) containing 20 mM Tris-HCl (pH 7.5), 17 mM $MgCl_2$ and 50 μ M ATP (3 μ Ci of [γ - ^{32}P]ATP).

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

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