MOLECULAR AND CELLULAR BIOLOGY, May 2004, p. 4255–4266 Vol. 24, No. 10
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.10.4255–4266.2004
Copyright © 2004, American Society for Microbiology. All Rights Reserved.

Characterization of Mouse *Rsk4* as an Inhibitor of Fibroblast Growth Factor–RAS–Extracellular Signal-Regulated Kinase Signaling

Andrea Pomrehn Myers,[1] Laura B. Corson,[2] Janet Rossant,[2,3] and Julie C. Baker[1]*

Department of Genetics, Stanford Medical School, Stanford, California,[1] and Samuel Lunenfeld Research Institute, Mount Sinai Hospital,[2] and Department of Molecular and Medical Genetics, University of Toronto,[3] Toronto, Ontario, Canada

Received 28 October 2003/Returned for modification 5 December 2003/Accepted 2 February 2004

Receptor tyrosine kinase (RTK) signals regulate the specification of a varied array of tissue types by utilizing distinct modules of proteins to elicit diverse effects. The RSK proteins are part of the RTK signal transduction pathway and are thought to relay these signals by acting downstream of extracellular signal-regulated kinase (ERK). In this study we report the identification of ribosomal S6 kinase 4 (Rsk4) as an inhibitor of RTK signals. Among the RSK proteins, RTK inhibition is specific to RSK4 and, in accordance, is dependent upon a region of the RSK4 protein that is divergent from other RSK family members. We demonstrate that *Rsk4* inhibits the transcriptional activation of specific targets of RTK signaling as well as the activation of ERK. Developmentally, *Rsk4* is expressed in extraembryonic tissue, where RTK signals are known to have critical roles. Further examination of *Rsk4* expression in the extraembryonic tissues demonstrates that its expression is inversely correlated with the presence of activated ERK 1/2. These studies demonstrate a new and divergent function for RSK4 and support a role for RSK proteins in the specification of RTK signals during early mouse development.

Receptor tyrosine kinase (RTK) signals have established roles in a variety of physiological processes, including cellular proliferation and migration, tissue specification, and hormone regulation (reviewed in references 50, 53, and 65). RTK signals are relayed, in part, by the GTPase RAS and a cascade of kinases which include RAF, MEK, and extracellular signal-regulated kinase (ERK) (reviewed in reference 5). Though members of this pathway have been extensively studied, the mechanism by which the activation of these seemingly generic signals can illicit such a diversity of specific functions is still an area of intense investigation.

Studies of the p90 ribosomal S6 kinases (RSKs) indicate that these proteins may aid in the specification of RTK signals. The RSK proteins are intercellular serine/threonine kinases and were among the first identified targets of ERK (39, 64). Their protein structure comprises an ERK binding site as well as two distinct functional kinase domains; the C-terminal kinase domain is important for autophosphorylation, while the N-terminal kinase domain phosphorylates other target substrates (11, 26, 39, 76). In mammals, there are four $p90^{Rsk}$ genes, termed *Rsk1* to *-4* (RPS6K A1, A3, A2, and A6, respectively) (2, 47, 75, 77). Comparative analyses of RSK1-4 suggest that these proteins may have distinct roles for specifying ERK signals. Biochemical studies of RSK1-3 demonstrate that these proteins have different binding specificities for ERK and, after RTK stimulation, interact with ERK for different lengths of time (55, 76). In addition, RSK1 has limited interaction with identified targets of RSK2 (18), and *Rsk1*, *-2*, *-3*, and *-4* genes are expressed in different patterns during late embryonic stages and in adult tissues (2, 42, 75). The biochemical differences among the family members suggest that there may be functional differences among the RSK proteins on a cellular level. Moreover, the differential expression patterns of the *Rsk* genes suggest distinct roles for the specification of RTK signals during mammalian embryogenesis.

Genetic analysis of mice has shown that many components of the RTK pathway are critical for the formation and patterning of the extraembryonic tissues, the precursors of the placenta (27, 54, 58, 74). Analysis of activated ERK during early mouse development further demonstrates that RTK signals are stimulated in the extraembryonic tissue (21). We wanted to identify molecules that could modify the outcome of RTK signals in the developing mouse embryo. Given the well-established role of RTK signals in the extraembryonic tissue, we decided to use this tissue as a source for identifying molecules that can mediate RTK signals. Unfortunately, the mouse embryo is small and confined to the uterus during the time at which RTK signals in the extraembryonic tissue are most influential. To overcome these technical challenges, we devised a method to identify mediators of RTK signals in the extraembryonic tissue by exploiting the more tractable *Xenopus* embryo as a screening tool (6, 14). In *Xenopus* the RTK pathway is necessary for the formation of the mesoderm, a tissue that has been well studied (reviewed in reference 29). Based on the knowledge that the core proteins of RTK signaling are conserved across species and utilized for the formation of different types of tissues, we screened a mouse expression library containing extraembryonic tissue for molecules that, upon overexpression, could alter the fate of *Xenopus* mesoderm.

In this screen for RTK modulators, we identified ribosomal S6 kinase 4 (RSK4), the fourth and least-studied member of the RSK family (75). Though similar in structure to the other RSK family members, RSK4 has a function that is distinct from that of RSK1 to RSK3. Consistent with this functional divergence, the inhibitory activity of RSK4 for RTK signaling is dependent upon a region that is not conserved in the other RSK proteins. Analysis of RSK4 in the developing mouse

* Corresponding author. Mailing address: Stanford University, Genetics Department, Alway Room 337, 300 Pasteur Dr., Stanford, CA 94305. Phone: (650) 723-1082. Fax: (650) 725-1534. E-mail: jbaker@stanford.edu.

embryo demonstrates that *Rsk4* is at the right time and place for this functional activity. *Rsk4* expression is altered in mice without fibroblast growth factor (FGF) signals, suggesting that RTK signals regulate *Rsk4* in vivo. These data provide the first evidence that an RSK protein may play an important role in the regulation of RTK signaling and tissue patterning in the developing mammalian embryo. Moreover, this study provides further support for the notion that though similar, the RSK proteins have diverse and distinct functions in the regulation of RTK signals.

MATERIALS AND METHODS

Construction of the mouse expression library. Construction and subsequent gridding of the 6.5-day postcoitum (d.p.c.) mouse expression library has been previously described (6, 7, 14).

Construction of DNA plasmids. The expression library used for the screen was constructed with the pCS105 expression vector (35). The identified *Rsk4* gene was subcloned from this vector into pCS107 (6) at SalI/NotI sites. Using a PCR strategy, MYC-tagged clones were constructed by cloning products into the EcoRI site of MTpCS3+, a modified version of pCS2 (54, 56, 70) (for MTpCS3+ sequence, visit http://sitemaker.med.umich.edu/dlturner.vectors). The original *Rsk4*pCS105 plasmid was used as a template for the MYC-tagged *Rsk4* and Δ*1-96Rsk4* constructs. *Rsk2* pMT2, provided by C. Bjorbaek (24), and *Homo sapiens Rsk1* pKH3 and *H. sapiens Rsk3* pkH3 (55) were the templates for the Rsk2, -1, and -3 MYC constructs, respectively. Primers with EcoRI sequences were used to amplify the *Rsk4* open reading frame (ORF) (forward [F], 5′ CGTCAGAATTCTATGCTGAATTTTAGAAGGACACGCC; reverse [R], 5′ GCGTACGAATTCGGACTGAAGAGCACAAGACTCTTA), Δ*1-96Rsk4* (F, 5′ GCGTCAGAATTCTCCTGAAGCGGCGATGCTACCGTT; R, 5′ GCGTA CGAATTCGGACTGAAGAGCACAAGACTCTTA), *Mus musculus Rsk2* (F, 5′ AGCCTCTAGAAATGCC GCTGGCGCACGTGGCG; R, 5′ GAGCCTCT AGAACAGGGCTGTTGAGGTG). *H. sapiens Rsk1* (F, 5′ GCGTCAGGATT CTATGCCGCTCGCCC; R, 5′ GCGTCAGGATTCTCAGG GTGGTGG) and *H. sapiens Rsk3* (F, 5′ GCGTCAGGATTCTATGGACCTGAGCATG; R, 5′ GCGTCAGGATTCTCTACAGCCGCGTGG). Protein analysis using the TNT coupled reticulolysate system (Promega, Madison, Wis.) with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis for MYC demonstrated bands at the expected sizes. Δ*91-860Rsk4* was constructed from *Rsk4* in MTpCS3+. MTpCS3+*Rsk4* was cut with XhoI enzyme to excise all but the first 272 bp of the *Rsk4* gene. *Rsk4* in MTpCS3+ was also used as the template for the KΔQ221*Rsk4* construct. QuikChange site-directed mutagenesis (Stratagene, La Jolla, Calif.) was used with the following primer and its reverse complement: F, 5′ GGGCAGCTCTATGCAATGCAGGTGTTAAG AAAAGCTT. Mutations were confirmed by sequence analysis. The full length of the clone was sequenced to ensure that no other mutations were present. In vitro transcription and translation of this clone, followed by SDS-PAGE and Western blot analysis with MYC antibody, demonstrated that a protein at the expected size was formed.

Transcription of RNA. DNA constructs were linearized, and RNA was transcribed with an mMESSAGE Machine kit (Ambion, Austin, Tex.). The sources of the constructs are given in Table 1.

Microinjections. Female frogs were primed for ovulation with human chorionic gonadotropin (19). Embryos were collected into 0.1× MR solution (52), fertilized in vitro, and dejellied with 2.5% cysteine, pH 8.0. Embryos were transferred into 0.3× MR with 2.5% Ficoll for injection. Embryos were injected at the one-cell stage in either the presumptive ectoderm (for explant assays) or the presumptive mesoderm (for in situ analysis). Injection of *Xenopus laevis* oocytes was completed as previously described (63).

In situ hybridizations. Plasmids used to generate antisense probes are listed in Table 2.

X. laevis embryos were developed to stage 10.5 or 12.5 and fixed in MEMFA (52). Single in situ analysis was performed as described previously (30). Double in situ analysis was completed using a modified protocol (22) which has been previously described (14). Detection of β-galactosidase (β-Gal) protein was performed as described previously (62) with the replacement of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside with 6-chloro-3-indolyl-β-D-galactoside (Red-Gal; Research Organics, Cleveland Ohio).

Mouse in situ hybridizations were completed using a modified protocol (10, 72): embryos were dissected from pregnant Swiss Webster mice (Simmonson Lab, Gilroy, Calif.). Embryos were blocked with 2% BM Block (Roche, Indianapolis, Ind.) in 1× MAB buffer (52) and incubated at 4°C with anti-digoxigenin-AP Fab fragments (Roche) at a 1/2,000 dilution. Embryos were developed with BM Purple AP substrate (Roche). The antisense probe for *Rsk4* was synthesized by using the *Rsk4*Δ91-860 construct as a template.

TABLE 1. DNA constructs used in this study

RNA[a]	Vector	Linearization	Reference
Fgf8	pCS105	AscI	
Erk sem	pGEMHE	NheI	51
p21Ras	pSP64	EcoRI	71
M.m. Rsk2	MTpCS3+	ApaI	This work
H.s. Rsk3	MTpCS3+	ApaI	This work
X.l. FARsk1	pOTV-LIC	NotI	28
H.s. Rsk1	MTpCS3+	ApaI	This work
Noggin	pCS2+	NotI	61
Activin	pSP64T	AscI	68
Smad2	pCS105	AscI	7
Rsk4	MTpCS3+	ApaI	This work
Δ*1-96Rsk4*	MTpCS3+	ApaI	This work
Δ*91-860Rsk4*	MTpCS3+	ApaI	This work
*K*ΔQ221*Rsk4*	MTpCS3+	ApaI	This work

[a] *M.m.*, *M. musculus*; *H.s.*, *H. sapiens*; *X.l.*, *X. laevis*.

Ectodermal explants and RT-PCR assays. Embryos were injected into the presumptive ectoderm at the one-cell stage, developed to stage 8-9, and transferred to 0.75× NAM (52) solution for tissue excision. Explants were cultured in 0.75× NAM until stage 10.5. RNA was isolated, and reverse transcription (RT)-PCR was performed as previously described (73). Primers used are listed in Table 3.

Mouse whole-mount antibody analysis. Mouse whole-mount antibody staining was completed as previously described (21) with polyclonal phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204) antibody [Phospho-p44/42MAPK(Thr202/Tyr204)] (Cell Signaling Technology, Beverly, Mass.). Staining was detected using Tyramide signal amplification kit no. 15 (Molecular Probes, Eugene, Oreg.) or diaminobenzidine.

In vitro culture of mouse embryos. Embryos were dissected and cultured as described previously (21).

Protein assays and immunoblots. Embryos were injected with RNA at the one-cell stage. Ectoderm was excised at stage 8-9 and allowed to develop until stage 10.5 in 0.75× NAM. Tissue was lysed in NOP buffer (150 mM NaCl, 10 mM Tris [pH 7.5], 1 mM $MgCl_2$, 0.75 mM $CaCl_2$, 2% IGPal) with general protease inhibitors (Sigma, St. Louis, Mo.), 10 mM NaF (Sigma), 10 nM sodium orthovanadate (Sigma), and 5 mM β-glyceraldehyde (Sigma). Lysates were centrifuged at 4°C for 10 min, and the supernatant was removed. Samples were analyzed by SDS-PAGE and Western blotting with the following antibodies: Phospho-p44/42MAPK(Thr202/Tyr204) E10 monoclonal, p42 MAP kinase 3A7 monoclonal, and phospho-Mek1/2 (Ser217/221) (Cell Signaling Technology), anti-rabbit immunoglobulin, horseradish peroxidase (HRP)-linked whole antibody from donkey (Amersham, Piscataway, N.J.), and HRP-labeled polyclonal anti-mouse immunoglobulin (Pharmingen, San Diego, Calif.).

Kinase assays. One-cell-stage *Xenopus* embryos were injected with *Rsk4*, *Rsk2*, Δ1-96*Rsk4*, and *K*ΔQ221*Rsk4* RNA. Protein lysates were harvested at the gastrula stage and analyzed for kinase activity using the S6 kinase assay (Upstate, Lake Placid, N.Y.). Five experiments were analyzed for P^{32} transfer. The counts per minute (cpm) of *Rsk4*, *Rsk2*, and Δ1-96*Rsk4* were significantly different ($P < 0.05$) from the cpm of the control (uninjected embryo lysates) when analyzed individually with a matched *t* test. The cpm of KΔQ221*Rsk4* were not significantly different from that of the control lysates.

TABLE 2. Antisense probes used in this study

Probe	Vector	Reference
Xbra	pGEM7	60
*Sox17*β	pSPJC2L	36
Endodermin	pBS	57
Goosecoid	pG500	13
MyoD	pSP73	34
*Rsk4*Δ*91-860*	pCS107	This work

TABLE 3. Primers used in this study

Gene	Primer sequence[a]	Reference
Xbra	F, GGATCGTTATCACCTCTG R, GTGTAGTCTGTAGCAGCA	73
Ef1α	F, CAGATTGGTGCTGGATATGC R, ACTGCCTTGATGACTCCTAG	73
ODC	F, CAGCTAGCTGTGGTGTGG R, CAACATGGAAACTCACACC	1
Sox17β	F, AACTCCCACCAGCAGGCTACTTTG R, TGTCAATGTCACTCTCCAGATGTCC	
Gsc	F, ACAACTGGAAGCACTGGA R, TCTTATTCCAGAGGAACC	73
Endodermin	F, TATTCTGACTCCTGAAGGTG R, GAGAACTGCCCATGTGCCTC	57
NCAM	F, CACAGTTCCACCAAATGC R, GGAATCAAGCGGTACAGA	31
Sef	F, GTCGAATTCGCCTGCAACGACCAAGTGGC R, CTGCGAATTCGCCCTCGTGCTTCAGTTTG	
Sprouty2	F, CATTCCTTGTTTCAGGC R, GATGGGAGAGTCCTTGG	

[a] F, forward; R, reverse.

RESULTS

Identification of *Rsk4*. To identify molecules that regulate RTK signaling, we performed a functional screen in which pools of RNA from a 6.5-d.p.c. mouse expression cDNA library (14) were injected into the presumptive mesoderm of the one-cell *X. laevis* embryo. Embryos were cultured to stage 10.5 (midgastrulation) and analyzed for the expression of the T-box transcription factor, *Xbrachyury* (*Xbra*) (60), a downstream target of RTK signals and a molecular marker of mesoderm. Using this strategy, a pool of RNA was identified for its ability to disrupt the *Xbra* expression pattern in the absence of any noticeable cell death. From the pool, a single clone was identified which encoded a 4-kb transcript with a 2.6-kb ORF. The ORF exhibited similarity to the *Ribosomal S6 kinase* (*p90^{Rsk}*) family and 89% identity to the human *Rsk4* (RPS6KA6) gene (75), indicating that it is the mouse orthologue of human *Rsk4*.

Rsk4 is the fourth member of the mammalian *p90^{rsk}* gene family (39). Human *Rsk4* was originally identified in a positional cloning study as a candidate for an X-linked mental retardation syndrome, although no mutations within the *Rsk4* gene were identified in patients affected with the syndrome (75). To date, the biochemical and cellular characteristics of RSK4 have not been described. The cloning of *Rsk4* in a functional screen for *Xbra* inhibition suggested that *Rsk4* could modulate RTK signals.

As seen in Fig. 1, injection of *Rsk4* into the marginal zone of the developing embryos results in the disruption of normal *Xbra* expression (Fig. 1E). Upon further development, these embryos have severe defects, including patent blastopores and deformed neural tubes (Fig. 1F and G). The resulting tailbud-stage embryos are truncated along the anterior-posterior axis (Fig. 1H). These developmental abnormalities are consistent with the disruption of mesoderm and are similar to the phenotypes seen in embryos injected with other RTK inhibitors, such as dominant-negative forms of the FGF receptor and RAF proteins (3, 67).

***Xbra* inhibition by *Rsk4* is local.** To further characterize the effect of *Rsk4* on *Xbra*, one-cell-stage embryos were injected at the margin with both *Rsk4* and a lineage tracer, *lacz*. Embryos were allowed to develop until stage 10.5, fixed, and analyzed for both β-Gal activity and *Xbra* RNA. While embryos injected with *lacz* alone showed colocalization of β-Gal activity and *Xbra* RNA, embryos injected with *Rsk4* and *lacz* showed no cellular colocalization of β-Gal activity and *Xbra* RNA (Fig. 2B and D). Additionally, in double in situ analysis for *Rsk4* and *Xbra* performed on *Rsk4*-injected embryos, there was no colocalization of the two RNAs (Fig. 2L and N). These results indicate that *Rsk4* acts locally to inhibit *Xbra* expression.

***Rsk4* specifically disrupts the formation of mesoderm.** The inhibition of *Xbra* and the resulting developmental phenotype in *Rsk4*-injected embryos were consistent with a disruption of RTK signaling. To assess the specificity of this disruption, we studied the role *Rsk4* had in regulating markers of other tissues. *Rsk4*-injected embryos were analyzed at gastrula stages by in situ hybridization with markers for different types of mesoderm (Fig. 2). At stage 10.5, the mesoderm is patterned along the dorsal-ventral axis (reviewed in reference 48). In situ analysis of a dorsally restricted marker, *Goosecoid* (*Gsc*) (16), revealed that injection of *Rsk4* did not affect the specification of dorsal tissues (Fig. 2F). However, expression of *MyoD*, a helix-loop-helix transcription factor that marks the more ventral, somitic mesoderm (34), was disrupted in *Rsk4*-injected embryos at stage 12.5 (Fig. 2J). Analysis of *Sox17β* and *Endodermin*, markers of endoderm (36, 57) (both stained in Fig. 2H), demonstrated a slight reduction of expression in *Rsk4*-injected embryos compared to controls. *Endodermin* normally stains a subset of mesodermal cells (57), and we suspect that this slight reduction of expression of these markers is a loss of expression in these cells. *Rsk4*-injected embryos have severe developmental defects and often die prior to neurulation. To investigate the effect of *Rsk4* expression on the formation of neural tissue, the naive ectoderm of the one-cell *X. laevis* embryo was injected with the bone morphogenic protein antagonist and neural inducer *Noggin* (45, 61, 78) with and without *Rsk4* RNA. Injected ectodermal tissue was excised at the blastula stage (herein referred to as ectodermal explants)

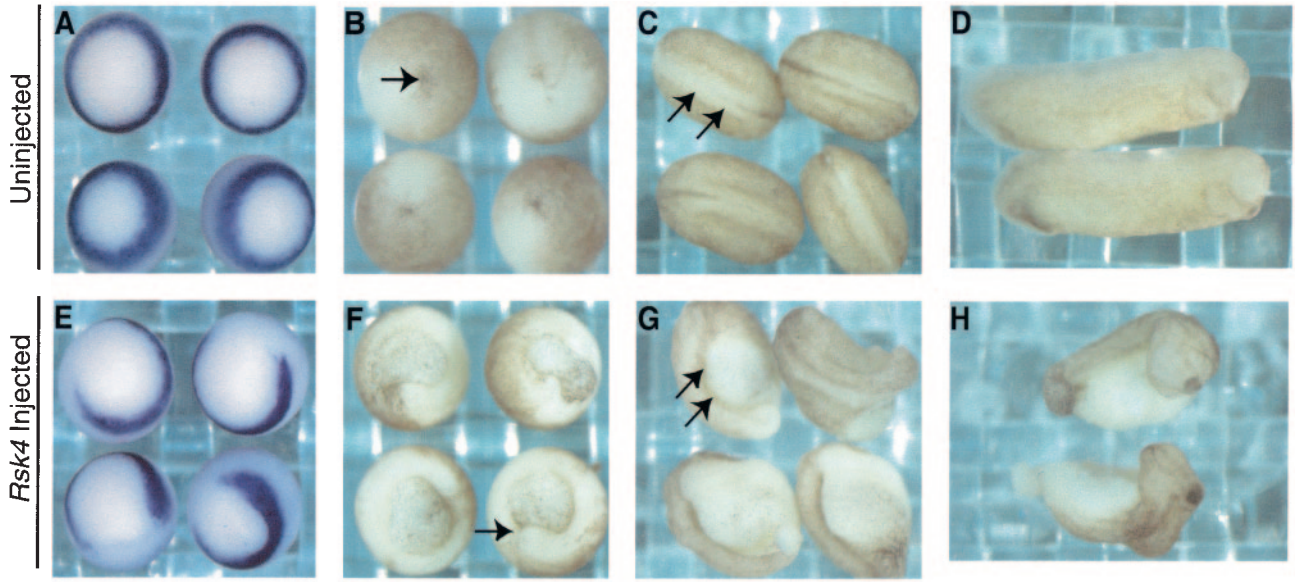

FIG. 1. *Rsk4* is identified in a functional expression screen. *Rsk4* was injected into the marginal zone of a one-cell embryo (E to H). Uninjected embryos were developed under the same conditions (A to D). In situ analysis shows that *Rsk4*-injected embryos at the gastrula stage (E) display disrupted *Xbra* expression compared to controls (A). Upon further development, *Rsk4*-injected embryos have a patent blastopore (F), fail to properly neurulate (G), and are truncated at the tailbud stage (H). The development of uninjected controls is also shown (B to D). The arrows demarcate the blastopore (B and F) and neural tube (C and G).

and cultured in vitro. At the neurula stage, RNA was isolated from the ectodermal explants and analyzed by RT-PCR to detect the neural marker *NCAM* (37). Results of this experiment showed that *Rsk4* does not inhibit the signals that induce neural tissue (Fig. 2O). This in situ analysis led us to conclude that *Rsk4* did not affect the expression of all tissue types and therefore was acting on a specific signaling pathway.

***Rsk4* disrupts FGF signaling.** The signaling pathways that regulate the specification, as well as the dorsal-ventral patterning, of the germ layers in *Xenopus* have been well studied. Cursory analysis of the tissue formation of *Rsk4*-injected embryos allowed for analysis of the effect of *Rsk4* overexpression on several pathways, including the FGF and transforming growth factor beta (TGF-β) pathways. Both the FGF and TGF-β signaling pathways are integral to the proper induction of *Xbra* (reviewed in reference 29). The TGF-β pathway can also induce endoderm and specify dorsal mesoderm (16, 33). These signaling pathways are not independent of each other, since TGF-β signals cannot induce general mesoderm in the absence of the FGF pathway. For example, in the presence of dominant-negative FGF receptor, ectopic TGF-β signals can no longer induce *Xbra* or *MyoD* but maintain the ability to induce the dorsal mesoderm marker *Gsc* (20, 44). The large effect of *Rsk4* overexpression on somitic mesoderm, but not dorsal or endodermal tissues, is consistent with a specific effect of *Rsk4* on the FGF pathway. To further investigate this possibility, the effect of *Rsk4* on the FGF and TGF-β pathways was analyzed in more detail.

To investigate the effect of *Rsk4* on the FGF and TGF-β pathways, an ectodermal explant assay was performed. *Activin*, encoding a TGF-β ligand, *Smad2*, encoding a mediator of TGF-β signals, and *Fgf8* were injected into the presumptive ectoderm of one-cell embryos, with or without *Rsk4*. Ectoderm explants were cultured to stage 10.5 and then harvested for RNA. RT-PCR was performed to detect downstream targets of the TGF-β and FGF pathways. As shown in Fig. 3, *Gsc*, a dorsal mesoderm marker, and *Sox17*β, an endoderm marker, are induced by *Activin*. (The level of *Smad2* RNA injected was not high enough to induce dorsal or endodermal genes in this experiment.) These inductions are maintained in the presence of *Rsk4*, indicating that *Rsk4* does not directly inhibit the TGF-β pathway (Fig. 3A). Analysis of *Xbra* demonstrated that the presence of *Rsk4* inhibited *Fgf8*, *Activin*, and *Smad2* from inducing *Xbra*. Previous studies have shown that if the FGF pathway is abrogated, TGF-β cannot induce *Xbra* (20, 44). Therefore, this result is consistent with *Rsk4* directly disrupting both the FGF pathway and *Xbra*-inducing capabilities of the TGF-β pathway or the FGF pathway only. We further investigated the effect of *Rsk4* on two FGF-specific targets, *Sprouty2* and *Sef* (49, 69). *Fgf8* enhanced the expression of both *Sprouty2* and *Sef*. This up-regulation was impeded by the presence of *Rsk4* (Fig. 3B), indicating that *Rsk4* inhibits FGF signals. Additionally, *Rsk4* inhibits the induction of *Xbra* by both FGF and TGF-β signals. Since the FGF pathway is required for TGFβ induction of *Xbra* and the injection of *Rsk4* does not impede the transcription of other downstream signals of TGF-β, we conclude that *Rsk4* is acting specifically on the FGF pathway.

Rsk4 inhibits the RAS-ERK pathway. In order to elucidate the molecular mechanism of *Rsk4* function, we investigated the effect of *Rsk4* on the RAS-ERK pathway, the mediator of FGF signals. The FGF ligands signal through an RTK receptor and the RAS-ERK pathway, a kinase cascade which includes the $p21^{RAS}$, RAF, MEK, and ERK proteins (reviewed in reference 5). Constitutively active molecules of the FGF receptor, $p21^{RAS}$, RAF, and ERK, can all induce *Xbra* (43, 46, 71). To investigate whether *Rsk4* affects this pathway, we injected em-

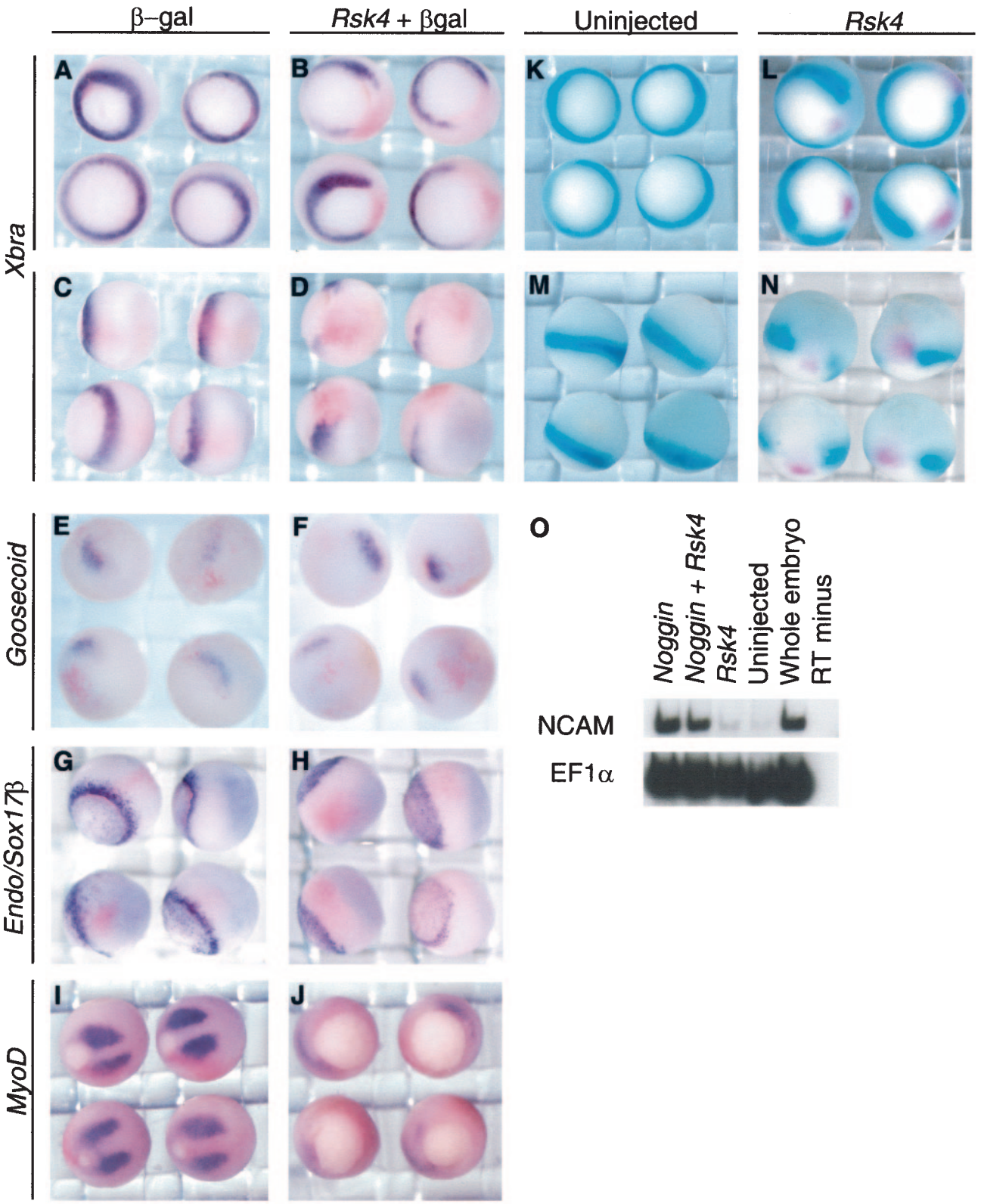

FIG. 2. Characterization of embryos injected with *Rsk4*. (A to J) *X. laevis* one-cell-stage embryos were injected at the margin with *lacZ* RNA (A, C, E, G, and I) or both *lacz* and *Rsk4* RNA (B, D, F, H, and J). Embryos were allowed to develop to stage 10.5 (A to H) or stage 12.5 (I and J), stained for β-Gal activity, and then analyzed by in situ hybridization. *Xbra* expression (A to D) is shown from the vegetal (A and B) and lateral (C and D) view. *Goosecoid* expression (E and F) is shown from a dorsal-vegetal view. *Sox17*β and *Endodermin* expression (G and H) are shown from a dorsal-vegetal view. *MyoD* expression is shown (I and J). *X. laevis* was injected into the margin of one-cell-stage embryos with *Rsk4* RNA. Double in situ analysis was performed for *Xbra* (turquoise) and *Rsk4* (magenta) (K to N). Uninjected controls are shown (K and M). The presumptive ectoderm tissue of one-cell embryos was injected with *Noggin* and *Noggin* with *Rsk4*. Ectodermal explants were excised and developed in vitro to neurula stage. RNA was isolated, and RT-PCR was performed for *NCAM,* a neural marker, and *EF1*α, a loading control (O).

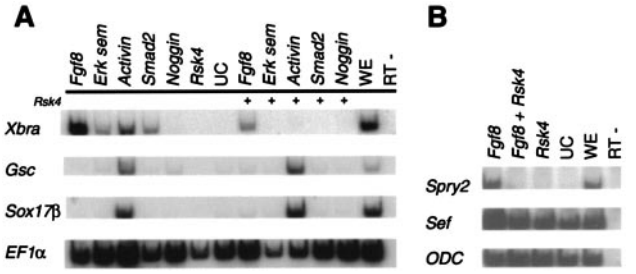

FIG. 3. *Rsk4* specifically blocks the Fgf pathway. (A) Embryos were injected into the presumptive ectoderm tissues with mesoderm inducing molecules with or without *Rsk4*. The ectodermal explants were excised and cultured to the gastrula stage. RNA was isolated, and RT-PCR was performed for *Xbra*, *Gsc*, *Sox17*β, and *EF1*α. (B) The presumptive ectodermal tissue of one-cell embryos was injected with *Fgf8* with or without *Rsk4*. Ectodermal explants were excised, and RNA was collected and analyzed by RT-PCR for *Sprouty2*, *Sef*, and *ODC,* a loading control. UC, uninjected control explants; WE, whole embryo; RT-, reverse transcriptase minus.

bryos with $p21^{Ras}$ and *Drosophila melanogaster* Erk^{sem} (51), encoding a constitutively active ERK. Both of these molecules are capable of inducing *Xbra* expression in *Xenopus* (43, 71). The presence of *Rsk4* disrupted the ability of both $p21^{Ras}$ and *D. melanogaster* Erk^{sem} to induce *Xbra* expression (Fig. 3A and 4A). These results indicate that *Rsk4* can block the RAS-ERK pathway and that *Rsk4* likely acts directly on or downstream of ERK.

To further investigate the effect of *Rsk4* on ERK, we analyzed the phosphorylation state of ERK in the presence of *Rsk4*. *Fgf8* was injected into the presumptive ectoderm of the one-cell-stage embryo, with or without *Rsk4* RNA. Ectodermal explants were excised and allowed to develop until gastrulation. As can be seen in Fig. 4B, embryos injected with *Fgf8* alone had active ERK as determined by the phosphorylation at Tyr202 and Thr204 (4). However, active ERK was either reduced or absent in embryos injected with *Fgf8* and *Rsk4.* That *Rsk4* is able to block the inducing capabilities of *D. melanogaster* Erk^{sem}, and active ERK was not present in *Rsk4*-injected embryos, suggests that *Rsk4* is acting at the level of ERK.

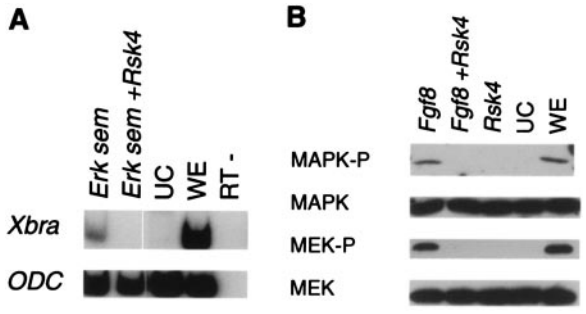

FIG. 4. *Rsk4* disrupts the RAS-MAPK pathway. (A) The presumptive ectodermal tissue of one-cell embryos was injected with the Erk^{sem} or $p21^{Ras}$, with or without *Rsk4*. RNA was isolated from injected tissue, and RT-PCR was performed for *Xbra* and *ODC*. (B) *Fgf8* was injected into the presumptive ectoderm of one-cell embryos with or without *Rsk4.* Ectodermal explants were excised and allowed to develop to gastrula stage. Proteins were isolated and analyzed by SDS-PAGE and Western blot techniques for $p42^{MAPK}$ and phospho-$p44/42^{MAPK(Thr202/Tyr204)}$. UC, uninjected control explants; WE, whole embryo; RT-, reverse transcriptase minus.

RTK inhibition is specific to *Rsk4*. The RSK proteins have high similarity (Fig. 5A), and RSK1, -2, and -3 have been used interchangeably in biochemical assays to illicit similar effects (55, 76). A few studies have suggested a role for RSK proteins in negative feedback inhibition (23, 24, 40); however, RSK1, -2, and -3 have not been shown to directly inhibit RTK signaling. To investigate whether the other *Rsk* genes were capable of *Xbra* disruption, *H. s*apiens *Rsk1*, *M.m. Rsk*2, and *H. sapiens Rsk3* (2, 77) were injected into the marginal zone of one-cell *X. laevis* embryos and analyzed by in situ analysis for *Xbra* expression at the gastrula stage. *Xbra* expression was not specifically disrupted by any these genes (Fig. 5C). To measure the protein level in the injected embryos, 20 embryos from each batch of injected embryos were removed prior to fixation. Protein was isolated from these embryos and analyzed by SDS and Western blotting for the MYC-tag protein. As can be seen in Fig. 5B, the embryos had similar amounts of protein.

Since the other *Rsk* homologs do not inhibit *Xbra*, we hypothesized that perhaps RSK4 was simply a more active isoform than the other RSK proteins. To investigate this, we analyzed the effects of the constitutively active *Rsk* clone, *FARsk1* (28). *FARsk1* was designed to mimic the hyperphosphorylated state of active RSK protein and has acidic residues at all of the known phosphorylation sites. Unlike its wild-type counterpart, *FARsk1* induces oocyte maturation in the absence of progesterone. *FARsk1* was injected into the marginal zone of the embryos and analyzed by in situ hybridization for *Xbra*. *Xbra* expression was not disrupted in embryos injected with this molecule (data not shown).

Conversely, we investigated whether *Rsk4* could mimic the activity of *FARsk1* in maturing *X. laevis* oocytes. Stage 6 oocytes were injected with *Rsk4* RNA, and germinal vesicle breakdown was measured both visually and by ERK activity. Unlike results with *FARsk1* (28), the *Rsk4*-injected oocytes did not mature in the absence of progesterone (data not shown). Since the *Rsk* genes are not interchangeable in our assays, we conclude that *Rsk4* has an intrinsic property that is sufficient for the disruption of the RAS-ERK pathway and is not found in the other *Rsk* genes.

Mutational analysis of *Rsk4*. To identify regions of *Rsk4* that confer *Xbra*-inhibiting activity, we deleted regions of the *Rsk4* gene that are divergent from the other *Rsk* genes (Fig. 6A). Members of the $p90^{RSK}$ family have a distinct protein structure, comprised of two functional kinase domains as well as an ERK (ERK1/2) docking site (26, 39, 76). Alignment of the *Rsk4* translated ORF with members of the $p90^{RSK}$ family shows that these domains are highly conserved (Fig. 5 and data not shown). However, mouse RSK4 has a 96-amino-acid extension at its N terminus that is not present in the published sequences of mouse RSK1-3 or human RSK4 (2, 41, 75). No known protein motifs are present in this extension. However, it contains a high number of positively charged residues. To test whether this N-terminal extension conferred the mesoderm-inhibiting activity of RSK4, a Δ*1-96Rsk4* construct was created. Further analysis of the clone demonstrated that it encodes a MYC-tagged protein of the expected size (Fig. 6B) and it is a functional kinase (see Materials and Methods). *Fgf8* RNA was injected into the presumptive ectoderm of one-cell embryos

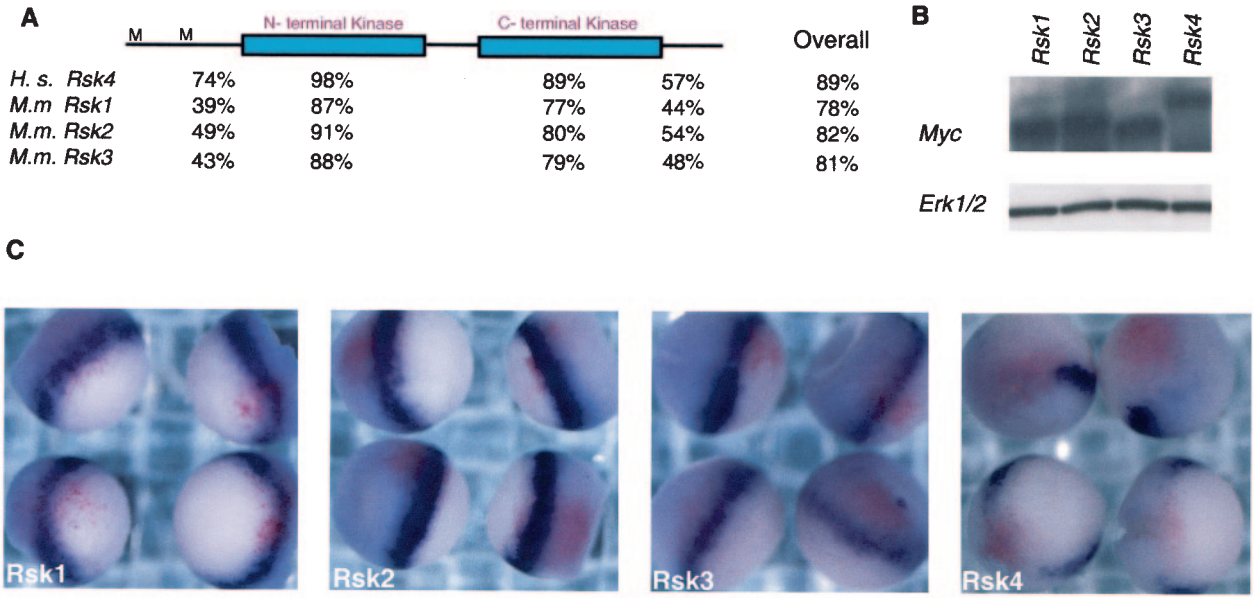

FIG. 5. The *Rsk* family. (A) Using DNA Star MegaAlign, the conversed region of RSK4 (97 to 860 amino acids) was aligned to mouse RSK1, RSK2, and RSK3 and human RSK4. The percent identities for the N terminus, N-terminal kinase, C-terminal kinase, and C-terminal domains are listed below the schematized protein structure. M marks the methionine at positions 1 and 97 in the full-length RSK4 protein. The second M is the start of the region of homology of RSK4 to the RSK family. (B) One-cell-stage embryos were coinjected at the marginal zone with *lacz* and *Rsk1, Rsk2, Rsk3*, or *Rsk4*. Embryos were allowed to develop to gastrula stages, at which point 20 embryos from each variable were removed for the isolation of protein and the remaining were fixed. Protein lysates were analyzed by SDS-PAGE and Western blotting with MYC antibody. (C) The fixed embryos were analyzed for β-Gal activity and *Xbra* expression.

with or without RNA generated from the Δ*1-96Rsk4* construct. Ectodermal explants were excised, RNA was generated, and RT-PCR analysis for *Xbra* was performed. While the full-length *Rsk4* was able to block the induction of *Xbra* by *Fgf8*, Δ*1-96Rsk4* could not (Fig. 6C). To test whether the N-terminal region alone could confer RSK4 activity, an N-terminal clone, *Rsk4*Δ91-860, was constructed and analyzed. This clone did not impede the induction of *Xbra* by *Fgf8* (Fig. 6C).

Since the N-terminus region was necessary but not sufficient for *Rsk4* activity, we wanted to investigate the relative importance of the kinase domain in mediating RSK4 activity. Members of the RSK protein family encode a protein with two distinct kinase domains (39). Previous mutational analysis of these kinase domains in other RSK family members has demonstrated differential activities for these domains. The carboxy-terminal kinase is generally required for autophosphorylation, while the N-terminal kinase domain is required for the phosphorylation of other target substrates (11, 26). A lysine-to-glutamine substitution at the predicted ATP binding site in the N-terminal kinase domain of RSK4 was created to remove the putative N-terminal kinase activity. The effect of this construct, K221Q*Rsk4*, on *Xbra* expression was analyzed by in situ hybridization. *Rsk4* or K221Q*Rsk4* was injected into the margin of one-cell embryos. The embryos were fixed at stage 10.5 and analyzed by double in situ hybridization. While *Rsk4* RNA did not colocalize with *Xbra* RNA (Fig. 6D, right panel), K221Q*Rsk4* and *Xbra* staining overlapped (Fig. 6D, left panel), indicating that the N-terminal kinase activity is likely required for *Rsk4* activity in our assay. Protein lysates isolated from embryos injected with similar amounts of K221Q*Rsk4* and *Rsk4* RNA had similar amounts of protein (Fig. 6B). In assays for kinase activity, lysates from *Rsk4*-injected embryos had significantly different cpm than those from uninjected embryos, while lysates isolated from embryos injected with K221Q*Rsk4* did not (see Materials and Methods). These results indicate that *Rsk4* is likely dependent on its N-terminal kinase activity for its *Xbra*-inhibiting capability.

***Rsk4* expression in the developing mouse gastrula.** After first identifying and characterizing mouse RSK4 as a negative regulator of the RAS-ERK pathway in *X. laevis*, we proceeded to investigate the role of RSK4 in the mouse embryo. Since the *Rsk4* gene was identified from an expression library derived from 6.5-d.p.c. mouse embryos, we examined the expression pattern of *Rsk4* during this developmental time. Whole-mount in situ analysis revealed that *Rsk4* localizes to the extraembryonic tissue of the mouse at 6.0 to 8.5 d.p.c. At 6.0 to 7.5 d.p.c., expression is restricted to central extraembryonic tissue: it is not expressed in the ectoplacental cone or the extraembryonic cells most proximal to the embryonic/extra-embryonic junction (Fig. 7A to C). *Rsk4* expression is strong in the chorion at 7.5 d.p.c. (Fig. 7D) and persists to 8.5 d.p.c. (Fig. 7E). Expression can also be seen in the allantois (Fig. 7E). The strong expression of *Rsk4* in the extraembryonic tissue suggests a possible role in the specification of these tissues.

FGF and RAS-ERK signaling are known to have important roles in the specification of the extraembryonic tissue. Mice with targeted mutations in *Fgf4*, *FgfR2*, *Grb2*, or *Mek1* exhibit peri-implantation lethality due in part to a lack of trophoblast proliferation (25, 27, 58, 74). Trophoblast stem cells have been derived from the extraembryonic ectoderm at these stages and require FGF-RAS-ERK signaling for maintenance of their stem cell properties (66). Analysis of the temporal and spatial expres-

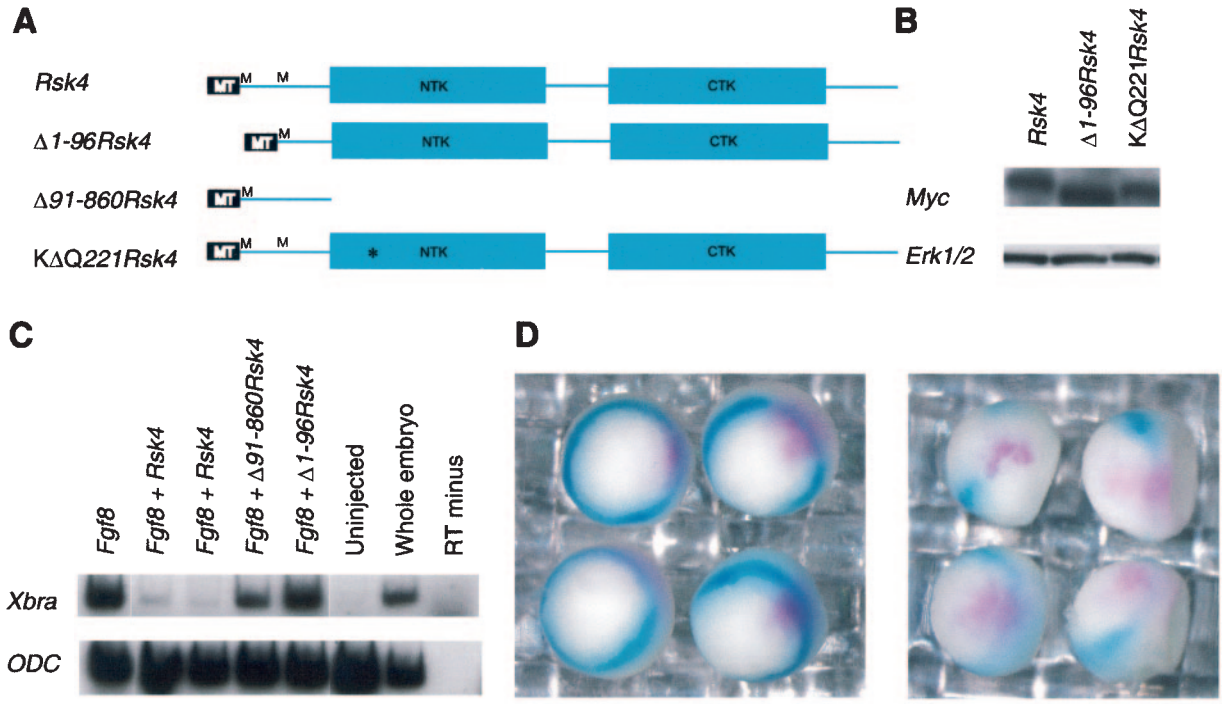

FIG. 6. *Rsk4* activity is dependent upon its N terminus and its N-terminal kinase. (A) Schematics of the RSK4 constructs, including Δ*1-96Rsk4,* Δ*91-860Rsk4*, and the KΔQ221*Rsk4* clones. (B) *Rsk4,* Δ1-96Rsk4*,* and the KΔQ221*Rsk4* RNAs were injected into one-cell-stage *Xenopus* embryos. At gastrula stage, protein was isolated and analyzed by SDS-PAGE and Western blotting with MYC antibody. (C) The presumptive ectoderm of a one-cell embryo was injected with *Fgf8* and the various *Rsk4* constructs. Ectodermal explants were excised, allowed to develop to gastrula stage, and analyzed by RT-PCR for the presence of *Xbra*. (D) Using site-directed mutagenesis, a K-to-Q transition at amino acid 221 was made in RSK4. RNA from this construct, KΔQ221*Rsk4* (left panel), and the original *Rsk4* construct (right panel) was injected into one-cell embryos at the marginal zone. Double in situ analysis was performed for detection of *Xbra* (turquoise) and *Rsk4* (magenta). Uninjected, uninjected control explants; RT minus, reverse transcriptase negative.

sion of dual phosphorylated ERK1 and -2 (active ERK) in the early developing mouse showed that there is strong ERK signaling in the extraembryonic ectoderm and ectoplacental cone (21). This pattern of ERK is restricted compared to the expression of *FgfR2*, encoding an RTK receptor, which is located throughout the extraembryonic ectoderm (17). To compare the expression of *Rsk4* and active ERK, RNA in situ analysis and whole-mount antibody staining was performed on similarly staged embryos (Fig. 8). The region of *Rsk4* expression is proximally and distally flanked by areas of tissue with active ERK. There is dark staining of active ERK in the ectoplacental cone and in a ring of tissue just proximal to the embryonic/extraembryonic junction. The juxtaposed nature of these two expression patterns suggests that *Rsk4* may be playing a role in restricting the domain of activated ERK in the extraembryonic tissue.

***Rsk4* expression is altered in embryos cultured in FGF inhibitor.** The localization of activated ERK just proximal to the embryonic/extraembryonic junction is dependent upon FGF signaling. Embryos cultured in the FGF inhibitor SU5402 lack prominent activated ERK in this ring of tissue (Fig. 9B, right embryo). In contrast, active ERK expression in the proximal ectoplacental cone persists. This indicates that FGF is required for active ERK expression at the embryonic/extraembryonic junction but not in the ectoplacental cone (21). We hypothesized that if *Rsk4* were regulated by FGF signaling, *Rsk4* expression would be altered in embryos cultured in SU5402. Therefore, we analyzed the expression pattern of *Rsk4* in embryos cultured in SU5402. A litter of 6.5-d.p.c. mice was dissected from a pregnant mouse and divided into two experimental treatments. One group was cultured in vitro in media supplemented with SU5402. The other was cultured in media with dimethyl sulfoxide (DMSO), the solvent of SU5402. These two groups were then further divided. Half of each group was examined for *Rsk4* expression, and half was examined for the presence of activated ERK. The expression patterns of both *Rsk4* and active ERK in the DMSO-cultured embryos were normal. In contrast, the mice cultured in SU5402 lacked active ERK expression in the ring just proximal to the extraembryonic/embryonic junction. Additionally, *Rsk4* expression in these embryos was expanded distally (Fig. 9D). The expansion of *Rsk4* into regions without active ERK but not into regions where active ERK was still present suggests that the formation of the *Rsk4* and active ERK boundary is dependent upon FGF signals.

DISCUSSION

RSK4 function is distinct from those of other RSK proteins. RTK signals are required for a variety of biological processes. The molecules comprising this pathway utilize distinct modules of proteins to render specific cellular responses. We demonstrate that the RSK proteins may be involved in generating subtle variations in different cellular environments. Studies

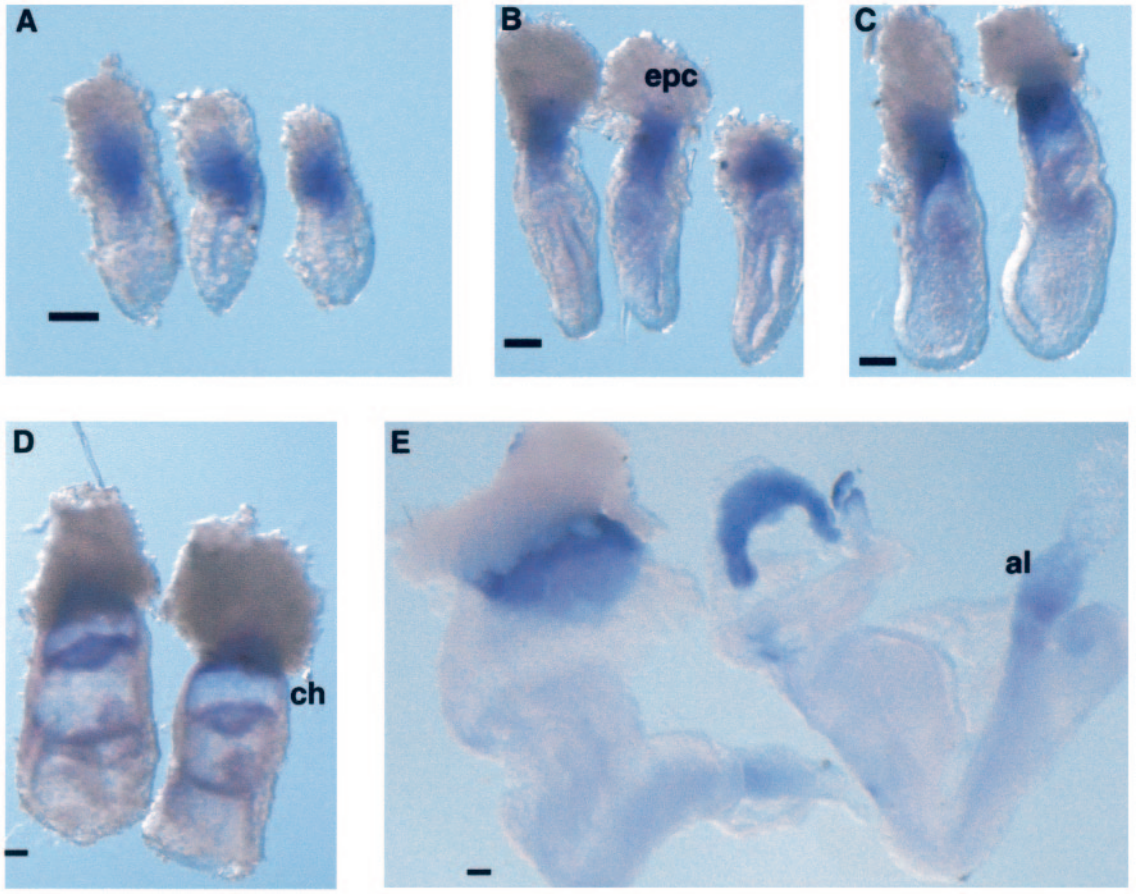

FIG. 7. *Rsk4* expression in the early developing mouse embryo. (A to E) In situ analysis was performed on 6.0- to 8.5-d.p.c. embryos. At 6.0 (A), 6.5 (B), and 7.0 (C) d.p.c., expression is restricted to the central region of the extraembryonic tissue; it is not expressed in the ectoplacental cone or the extraembryonic cells most proximal to the embryonic/extraembryonic junction. (D and E) *Rsk4* expression is strong in the extraembryonic ectoderm of the chorion at 7.5 d.p.c. (D) and persists to 8.5 d.p.c. (E). (E) Expression can also be seen in the allantois. Epc, ectoplacental cone; ch, chorion; al, allantois. Space bar = 100 μm.

have characterized RSK1 to -3 as downstream mediators of RTK signals (55, 76). In this study, we identified mouse RSK4. In contrast to the other RSK proteins, overexpression of RSK4 does not mediate, but rather inhibits, RTK signals. The ability of RSK4 to inhibit RTK signals is dependent upon the 96 amino acids at the amino-terminal end of the protein. This region is not conserved in the other RSK proteins. These data add to the growing evidence that though the RSK proteins are highly similar and can all be activated with stereotyped signals, subtle differences among them may result in distinct cellular roles (55, 76).

In vivo analysis of *Rsk4*. The extraembryonic patterning events that are regulated by RTK signals in the mouse are among the earliest in the developing mammal. Given the genetic and functional support for RTK signals in the extraembryonic tissue, we felt that this tissue was ideal for the identification of RTK modulators, despite some of the technical challenges. To facilitate the study of this small, less accessible tissue, we capitalized on the *Xenopus* embryo. Using an expression cDNA library from an early developing mouse embryo, we performed a functional screen with *Xenopus* to identify molecules that regulate and/or specify RTK signals and found *Rsk4*. Despite the obvious caveats of this nonconventional method, the subsequent demonstration of specific tissue expression of *Rsk4* validated our initial studies with the frog embryo. *Rsk4* is expressed in the extraembryonic tissue, a tissue in which the regulation of RTK signals is critical for proper development. Moreover, *Rsk4* is localized to the central region of the extraembryonic tissue, and its expression is flanked both distally and proximally by the expression of active ERK. The most distal expression of active ERK is a layer of cells that forms a discrete ring (21). Maintenance of such a specific pattern requires a tight regulation of local signals and therefore often involves intricate feedback loops. In the absence of FGF, an RTK ligand, active ERK is lost distally and *Rsk4* expression expands into the ring of cells that normally contains active ERK. This indicates that *Rsk4* and activated ERK signals have a dynamic interaction in vivo. This interaction may regulate the specification of the distal extraembryonic cells. Though the exact role of these distal cells remains unknown, they are located in an area of active signaling that likely plays a role in the patterning of both embryonic and extraembryonic tissues (reviewed in references 8 and 9). Further studies of *Rsk4* in this area may shed more light on the function of these

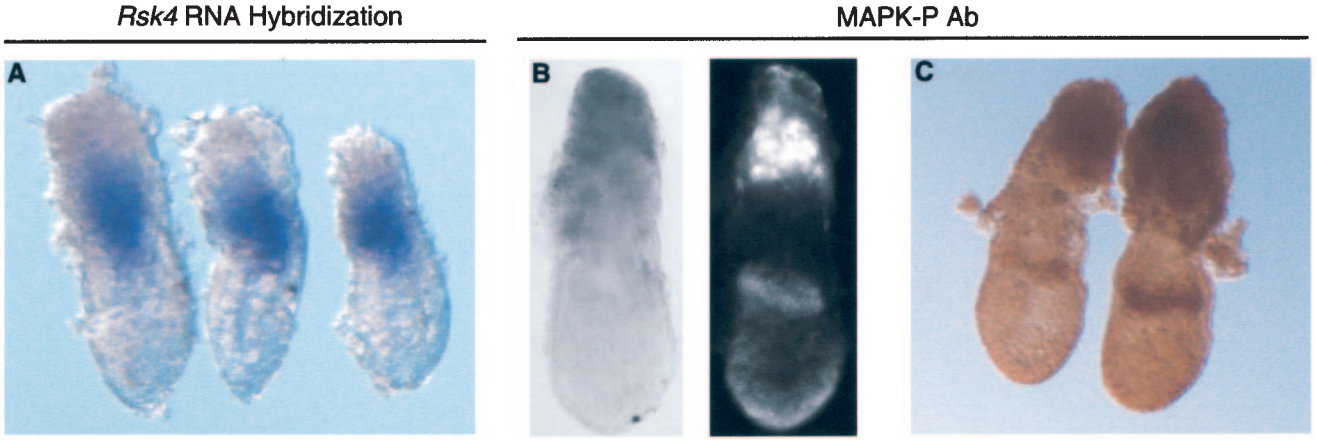

FIG. 8. Comparison of *Rsk4* and active MAPK expression localization. Embryos at stage 6.25 d.p.c. were analyzed for *Rsk4* RNA (A) and active MAPK protein expression (B and C). Whole-mount antibody stain for MAPK was detected by HRP visualization with both a tyramide amplification kit (B) and diaminobenzidine staining (C). The left panel in B is the light view of the same embryo under fluorescence on the right panel.

cells, but certainly data from this report provide the first evidence that an RSK may be involved in the modulation of RTK signals in the early developing embryo.

How and where does *Rsk4* act? Results from mutational analysis provide some information about how RSK4 might act. Site-directed mutagenesis of the lysine in the N-terminal kinase domain removed the ability of RSK4 to disrupt RTK signals. This suggests that the inhibition of RTK signals by RSK4 is a result of the phosphorylation of substrate. Candidates for the molecular target of RSK4 kinase activity are plentiful. RSK4 could act by directly phosphorylating and inhibiting a member of the RAS-ERK pathway. Alternatively, it could act indirectly by either activating an inhibitor or repressing expression of downstream transcriptional targets. These possibilities can all be supported by studies of the other RSK isoforms. In a study using rat PC12 epidermal growth factor (EGF)-cultured cells, RSK2 was shown to phosphorylate SOS, a membrane-tethered protein that mediates RTK activities (23). From this result, it was hypothesized that RSK2 could feedback negatively on the EGF pathway, though no further evidence in support of this idea has been put forth. Although hypothesized for RSK2, we do not support this mode of action for RSK4. RSK4 is able to inhibit constitutively active forms of RAS and ERK, which are both downstream of SOS. Therefore, we think it is more likely that RSK4 can inhibit RTK signaling at the level of or downstream of ERK. This model is supported in the literature, where it is shown that RSK isoforms localize to the nucleus upon RTK activation (12). Moreover, RSK2 has been shown to up-regulate *Estrogen Receptor* α and *c-Fos* transcription, suggesting a role for the RSK proteins in transcriptional regulation (15, 18, 38).

Deletion of the N terminus of RSK4, a region not conserved in the other RSK proteins, removes the ability of RSK4 to disrupt RTK signaling, indicating that these N-acids are required for RSK4 activity. Since BLOCK analysis (32) did not identify any known protein motifs, there is little indication of the exact role for this region in conferring RSK4 activity. Protein from the Δ*1-96Rsk4* construct maintains its ability to phosphorylate S6, suggesting that deletion of the N terminus does not disrupt the kinase ability of RSK4. Though no defined motifs were identified, the N terminus is comprised of many charged residues, suggesting that it could be targeting RSK4 to the nucleus or allowing RSK4 to interact with DNA. Analysis of *Sef* and *Sprouty2*, two negative feedback regulators of the FGF-RAS-ERK pathway, demonstrates that these genes are not transcriptionally regulated by RSK4. However, this does not exclude the possibility that RSK4 could be interacting with SEF and SPROUTY2 proteins or that RSK4 is interacting with other nuclear targets.

Our biochemical experiments suggest that RSK4 confers its *Xbra* inhibition by blocking signaling directly on or downstream of ERK, since RSK4 is able to block *D. melanogaster* Erk^{sem} from inducing its transcription. Consistent with RSK4 acting directly on ERK, tissues injected with *Fgf8* and *Rsk4* do not have phosphorylated ERK protein. Functional screens such as the one described in this report are inherently prone to the caveats of expressing genes at high, often nonphysiologic levels. Therefore, it is formally possible that RSK4, with its carboxy-terminal ERK binding site, is simply sequestering and inhibiting the activation of ERK to act as a dominant-negative molecule. We think this is unlikely for several reasons. First, the RSK4 putative N-terminal kinase activity is required for *Rsk4* activity. Additionally, the other RSK isoforms, in which the C-terminal ERK docking site is conserved (59), do not maintain this function. Finally, mutational analysis demonstrates that without the N terminus of the gene, *Rsk4* loses its ability to disrupt the FGF pathway. Since the conserved carboxy-terminal ERK binding site is still present on the Δ1-96*Rsk4* clone, its presence cannot be the sole reason for the described *Rsk4* function. Thus, while the data are consistent with RSK4 directly disrupting active ERK, the evidence that RSK4 requires its kinase activity and that the amino-terminally truncated RSK4 with an intact ERK binding site is not sufficient for its function suggests a mechanism of action that is more complex than simple sequestration of ERK by RSK4. The identification of the mechanism by which *Rsk4* inhibits RTK signals and the identification of other *Rsk4* targets will be exciting avenues for investigation. The use of trophoblast stem cells for biochemical assays in combination with RSK4 analysis

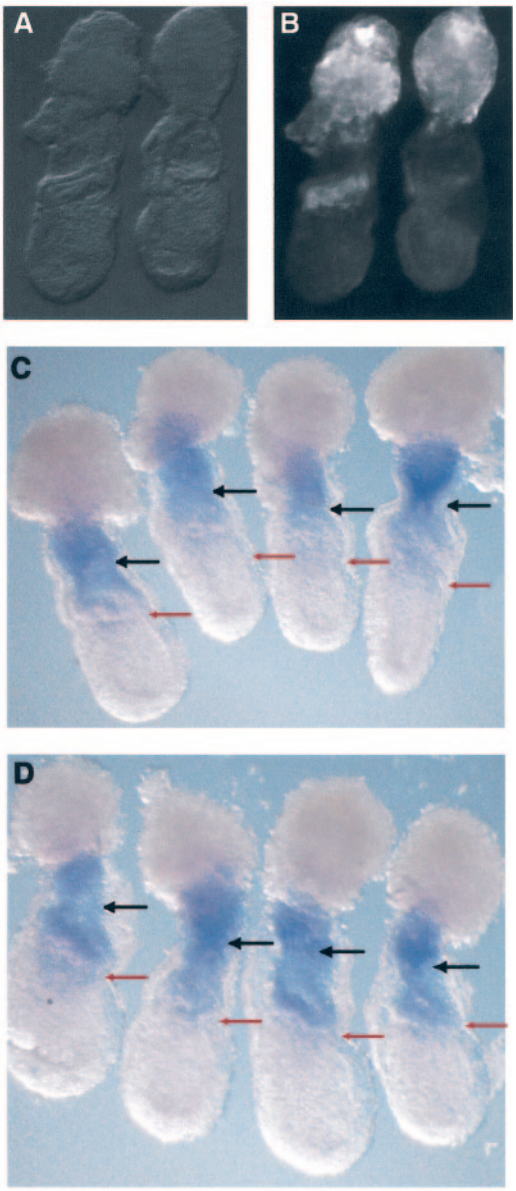

FIG. 9. *Rsk4* expression is altered in embryos cultured in FGF inhibitor. (A and B) 6.5-d.p.c. embryos were cultured in DMSO (embryos on the left of panels A and B) or SU5402, an FGF inhibitor (embryos on the right of panels A and B). Whole-mount antibody staining was performed for active MAPK. Embryos were visualized by light (A) and fluorescence (B) microscopy. (C and D) Embryos were cultured in DMSO (C) or SU5402 (D) and analyzed by in situ hybridization for *Rsk4.* Embryos cultured in SU5402 have distal expansion of *Rsk4* compared to DMSO controls. Red and black arrows demarcate the embryonic/extraembryonic junction and the distal boundary of normal *Rsk4* expression, respectively.

in genetically perturbed mouse models should aid in these pursuits.

Summary. As a result of the conservation of the RAS-ERK cascade and the requirement of this pathway in multiple organisms for the specification of different tissue types, we were able to identify *Rsk4* in a cross-species screen. Since its function is dependent upon a region of the gene that is divergent from the other *Rsk* family members, it would have been challenging to identify the full length of this gene by homology only. Among products of the *Rsk* genes, the ability to inhibit RTK signals is specific to RSK4, adding to the evidence that not all RSK functions are similar. *Rsk4* is expressed in the extraembryonic region of the developing gastrula in a pattern that is consistent with its activity. Further investigation of *Rsk4* in the contexts of general RAS-ERK signaling and the development of the extraembryonic tissues should add to these fields of investigation.

ACKNOWLEDGMENTS

We thank M. Whitman, J. Blenis, J. Farrell, J. Maller, and C. Bjorbaek for their generosity with DNA constructs. We thank J. Hebert, P. Roux, C. Smith, J. Myers, G. Barsh, S. Kim, P. Jackson, K. Knox, E. Ray, D. Ko, E. Chiao, A. Borchers, A. Hufton, and N. Mitiku for their sage scientific advice.

This work was supported by a grant from the National Institutes of Health (ROI HD 41557). Support for A.P.M. was provided by the Medical Scientist Training Program (GM07365).

REFERENCES

1. **Agius, E., M. Oelgeschlager, O. Wessely, C. Kemp, and E. M. De Robertis.** 2000. Endodermal nodal-related signals and mesoderm induction in Xenopus. Development **127:**1173–1183.
2. **Alcorta, D. A., C. M. Crews, L. J. Sweet, L. Bankston, S. W. Jones, and R. L. Erikson.** 1989. Sequence and expression of chicken and mouse rsk: homologs of *Xenopus laevis* ribosomal S6 kinase. Mol. Cell. Biol. **9:**3850–3859.
3. **Amaya, E., P. A. Stein, T. J. Musci, and M. W. Kirschner.** 1993. FGF signalling in the early specification of mesoderm in Xenopus. Development **118:**477–487.
4. **Anderson, N. G., J. L. Maller, N. K. Tonks, and T. W. Sturgill.** 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature **343:**651–653.
5. **Avruch, J., A. Khokhlatchev, J. M. Kyriakis, Z. Luo, G. Tzivion, D. Vavvas, and X. F. Zhang.** 2001. Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade. Recent Prog. Horm. Res. **56:**127–155.
6. **Baker, J. C., R. S. Beddington, and R. M. Harland.** 1999. Wnt signaling in Xenopus embryos inhibits bmp4 expression and activates neural development. Genes Dev. **13:**3149–3159.
7. **Baker, J. C., and R. M. Harland.** 1996. A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. Genes Dev. **10:**1880–1889.
8. **Beddington, R. S., and E. J. Robertson.** 1998. Anterior patterning in mouse. Trends Genet. **14:**277–284.
9. **Beddington, R. S., and E. J. Robertson.** 1999. Axis development and early asymmetry in mammals. Cell **96:**195–209.
10. **Belo, J. A., T. Bouwmeester, L. Leyns, N. Kertesz, M. Gallo, M. Follettie, and E. M. De Robertis.** 1997. Cerberus-like is a secreted factor with neutralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. Mech. Dev. **68:**45–57.
11. **Bjorbaek, C., Y. Zhao, and D. E. Moller.** 1995. Divergent functional roles for p90rsk kinase domains. J. Biol. Chem. **270:**18848–18852.
12. **Blenis, J., J. Chung, E. Erikson, D. A. Alcorta, and R. L. Erikson.** 1991. Distinct mechanisms for the activation of the RSK kinases/MAP2 kinase/pp90rsk and pp70-S6 kinase signaling systems are indicated by inhibition of protein synthesis. Cell Growth Differ. **2:**279–285.
13. **Blumberg, B., C. V. Wright, E. M. De Robertis, and K. W. Cho.** 1991. Organizer-specific homeobox genes in Xenopus laevis embryos. Science **253:**194–196.
14. **Borchers, A. G., A. L. Hufton, A. G. Eldridge, P. K. Jackson, R. M. Harland, and J. C. Baker.** 2002. The E3 ubiquitin ligase GREUL1 anteriorizes ectoderm during Xenopus development. Dev. Biol. **251:**395–408.
15. **Chen, R. H., P. C. Juo, T. Curran, and J. Blenis.** 1996. Phosphorylation of c-Fos at the C-terminus enhances its transforming activity. Oncogene **12:**1493–1502.
16. **Cho, K. W., B. Blumberg, H. Steinbeisser, and E. M. De Robertis.** 1991. Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene goosecoid. Cell **67:**1111–1120.
17. **Ciruna, B. G., and J. Rossant.** 1999. Expression of the T-box gene Eomesodermin during early mouse development. Mech. Dev. **81:**199–203.
18. **Clark, D. E., C. E. Poteet-Smith, J. A. Smith, and D. A. Lannigan.** 2001. Rsk2 allosterically activates estrogen receptor alpha by docking to the hormone-binding domain. EMBO J. **20:**3484–3494.
19. **Condie, B. G., and R. M. Harland.** 1987. Posterior expression of a homeobox gene in early Xenopus embryos. Development **101:**93–105.
20. **Cornell, R. A., and D. Kimelman.** 1994. Activin-mediated mesoderm induction requires FGF. Development **120:**453–462.
21. **Corson, L. B., Y. Yamanaka, K. M. Lai, and J. Rossant.** 2003. Spatial and

temporal patterns of ERK signaling during mouse embryogensis. Development **130:**4527–4537.
22. **Doniach, T., and T. J. Musci.** 1995. Induction of anteroposterior neural pattern in Xenopus: evidence for a quantitative mechanism. Mech. Dev. **53:**403–413.
23. **Douville, E., and J. Downward.** 1997. EGF induced SOS phosphorylation in PC12 cells involves P90 RSK-2. Oncogene **15:**373–383.
24. **Dufresne, S. D., C. Bjorbaek, K. El-Haschimi, Y. Zhao, W. G. Aschenbach, D. E. Moller, and L. J. Goodyear.** 2001. Altered extracellular signal-regulated kinase signaling and glycogen metabolism in skeletal muscle from p90 ribosomal S6 kinase 2 knockout mice. Mol. Cell. Biol. **21:**81–87.
25. **Feldman, B., W. Poueymirou, V. E. Papaioannou, T. M. DeChiara, and M. Goldfarb.** 1995. Requirement of FGF-4 for postimplantation mouse development. Science **267:**246–249.
26. **Fisher, T. L., and J. Blenis.** 1996. Evidence for two catalytically active kinase domains in pp90^{rsk}. Mol. Cell. Biol. **16:**1212–1219.
27. **Giroux, S., M. Tremblay, D. Bernard, J. F. Cardin-Girard, S. Aubry, L. Larouche, S. Rousseau, J. Huot, J. Landry, L. Jeannotte, and J. Charron.** 1999. Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. Curr. Biol. **9:**369–372.
28. **Gross, S. D., A. L. Lewellyn, and J. L. Maller.** 2001. A constitutively active form of the protein kinase p90Rsk1 is sufficient to trigger the G_2/M transition in Xenopus oocytes. J. Biol. Chem. **276:**46099–46103.
29. **Harland, R., and J. Gerhart.** 1997. Formation and function of Spemann's organizer. Annu. Rev. Cell Dev. Biol. **13:**611–667.
30. **Harland, R. M.** 1991. In situ hybridization: an improved whole-mount method for Xenopus embryos. Methods Cell Biol. **36:**685–695.
31. **Hemmati-Brivanlou, A., and D. A. Melton.** 1994. Inhibition of activin receptor signaling promotes neuralization in Xenopus. Cell **77:**273–281.
32. **Henikoff, S., and J. G. Henikoff.** 1994. Protein family classification based on searching a database of blocks. Genomics **19:**97–107.
33. **Henry, G. L., I. H. Brivanlou, D. S. Kessler, A. Hemmati-Brivanlou, and D. A. Melton.** 1996. TGF-beta signals and a pattern in Xenopus laevis endodermal development. Development **122:**1007–1015.
34. **Hopwood, N. D., A. Pluck, and J. B. Gurdon.** 1989. MyoD expression in the forming somites is an early response to mesoderm induction in Xenopus embryos. EMBO J. **8:**3409–3417.
35. **Hsu, D. R., A. N. Economides, X. Wang, P. M. Eimon, and R. M. Harland.** 1998. The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. Mol. Cell **1:**673–683.
36. **Hudson, C., D. Clements, R. V. Friday, D. Stott, and H. R. Woodland.** 1997. Xsox17α and -β mediate endoderm formation in Xenopus. Cell **91:**397–405.
37. **Jacobson, M., and U. Rutishauser.** 1986. Induction of neural cell adhesion molecule (NCAM) in Xenopus embryos. Dev. Biol. **116:**524–531.
38. **Joel, P. B., J. Smith, T. W. Sturgill, T. L. Fisher, J. Blenis, and D. A. Lannigan.** 1998. pp90^{rsk-1} regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. Mol. Cell. Biol. **18:**1978–1984.
39. **Jones, S. W., E. Erikson, J. Blenis, J. L. Maller, and R. L. Erikson.** 1988. A Xenopus ribosomal protein S6 kinase has two apparent kinase domains that are each similar to distinct protein kinases. Proc. Natl. Acad. Sci. USA **85:**3377–3381.
40. **Kinuya, M., K. Takishima, and G. Mamiya.** 2000. Detection of kinases that phosphorylate 14–3–3 binding sites of Raf-1 using in situ gel kinase assay. Biol. Pharm. Bull. **23:**1158–1162.
41. **Kispert, A., R. J. Stoger, M. Caparros, and B. G. Herrmann.** 1999. The mouse Rsk3 gene maps to the Leh66 elements carrying the t-complex responder Tcr. Mamm. Genome **10:**794–802.
42. **Kohn, M., H. Hameister, M. Vogel, and H. Kehrer-Sawatzki.** 2003. Expression pattern of the Rsk2, Rsk4 and Pdk1 genes during murine embryogenesis. Gene Expr. Patterns **3:**173–177.
43. **LaBonne, C., B. Burke, and M. Whitman.** 1995. Role of MAP kinase in mesoderm induction and axial patterning during Xenopus development. Development **121:**1475–1486.
44. **LaBonne, C., and M. Whitman.** 1994. Mesoderm induction by activin requires FGF-mediated intracellular signals. Development **120:**463–472.
45. **Lamb, T. M., A. K. Knecht, W. C. Smith, S. E. Stachel, A. N. Economides, N. Stahl, G. D. Yancopolous, and R. M. Harland.** 1993. Neural induction by the secreted polypeptide noggin. Science **262:**713–718.
46. **MacNicol, A. M., A. J. Muslin, and L. T. Williams.** 1993. Raf-1 kinase is essential for early Xenopus development and mediates the induction of mesoderm by FGF. Cell **73:**571–583.
47. **Moller, D. E., C. H. Xia, W. Tang, A. X. Zhu, and M. Jakubowski.** 1994. Human rsk isoforms: cloning and characterization of tissue-specific expression. Am. J. Physiol. **266:**C351–C359.
48. **Munoz-Sanjuan, I., and A. H.-Brivanlou.** 2001. Early posterior/ventral fate specification in the vertebrate embryo. Dev. Biol. **237**:1–17.
49. **Nutt, S. L., K. S. Dingwell, C. E. Holt, and E. Amaya.** 2001. Xenopus Sprouty2 inhibits FGF-mediated gastrulation movements but does not affect mesoderm induction and patterning. Genes Dev. **15:**1152–1166.
50. **Nystrom, F. H., and M. J. Quon.** 1999. Insulin signalling: metabolic pathways and mechanisms for specificity. Cell Signal **11:**563–574.
51. **Oellers, N., and E. Hafen.** 1996. Biochemical characterization of rolledSem, an activated form of Drosophila mitogen-activated protein kinase. J. Biol. Chem. **271:**24939–24944.
52. **Peng, H. B.** 1991. Xenopus laevis: practical uses in cell and molecular biology. Solutions and protocols. Methods Cell Biol.**36:**657–662.
53. **Porter, A. C., and R. R. Vaillancourt.** 1998. Tyrosine kinase receptor-activated signal transduction pathways which lead to oncogenesis. Oncogene **17:**1343–1352.
54. **Rossant, J., and J. C. Cross.** 2001. Placental development: lessons from mouse mutants. Nat. Rev. Genet. **2:**538–548.
55. **Roux, P. P., S. A. Richards, and J. Blenis.** 2003. Phosphorylation of p90 ribosomal S6 kinase (RSK) regulates extracellular signal-regulated kinase docking and RSK activity. Mol. Cell. Biol. **23:**4796–4804.
56. **Rupp, R. A., L. Snider, and H. Weintraub.** 1994. Xenopus embryos regulate the nuclear localization of XMyoD. Genes Dev. **8:**1311–1323.
57. **Sasai, Y., B. Lu, S. Piccolo, and E. M. De Robertis.** 1996. Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps. EMBO J. **15:**4547–4555.
58. **Saxton, T. M., A. M. Cheng, S. H. Ong, Y. Lu, R. Sakai, J. C. Cross, and T. Pawson.** 2001. Gene dosage-dependent functions for phosphotyrosine-Grb2 signaling during mammalian tissue morphogenesis. Curr. Biol. **11:**662–670.
59. **Smith, J. A., C. E. Poteet-Smith, K. Malarkey, and T. W. Sturgill.** 1999. Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK in vivo. J. Biol. Chem. **274:**2893–2898.
60. **Smith, J. C., B. M. Price, J. B. Green, D. Weigel, and B. G. Herrmann.** 1991. Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. Cell **67:**79–87.
61. **Smith, W. C., and R. M. Harland.** 1992. Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. Cell **70:**829–840.
62. **Smith, W. C., and R. M. Harland.** 1991. Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. Cell **67:**753–765.
63. **Sohaskey, M. L., and J. E. Ferrell, Jr.** 2002. Activation of p42 mitogen-activated protein kinase (MAPK), but not c-Jun NH(2)-terminal kinase, induces phosphorylation and stabilization of MAPK phosphatase XCL100 in Xenopus oocytes. Mol. Biol. Cell **13**:454–468.
64. **Sturgill, T. W., L. B. Ray, E. Erikson, and J. L. Maller.** 1988. Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. Nature **334:**715–718.
65. **Tan, P. B., and S. K. Kim.** 1999. Signaling specificity: the RTK/RAS/MAP kinase pathway in metazoans. Trends Genet. **15:**145–149.
66. **Tanaka, S., T. Kunath, A. K. Hadjantonakis, A. Nagy, and J. Rossant.** 1998. Promotion of trophoblast stem cell proliferation by FGF4. Science **282:**2072–2075.
67. **Tang, T. L., R. M. Freeman, Jr., A. M. O'Reilly, B. G. Neel, and S. Y. Sokol.** 1995. The SH2-containing protein-tyrosine phosphatase SH-PTP2 is required upstream of MAP kinase for early Xenopus development. Cell **80:**473–483.
68. **Thomsen, G., T. Woolf, M. Whitman, S. Sokol, J. Vaughan, W. Vale, and D. A. Melton.** 1990. Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. Cell **63:**485–493.
69. **Tsang, M., R. Friesel, T. Kudoh, and I. B. Dawid.** 2002. Identification of Sef, a novel modulator of FGF signalling. Nat. Cell Biol. **4:**165–169.
70. **Turner, D. L., and H. Weintraub.** 1994. Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. Genes Dev. **8:**1434–1447.
71. **Whitman, M., and D. A. Melton.** 1992. Involvement of p21ras in Xenopus mesoderm induction. Nature **357:**252–254.
72. **Wilkinson, D.** 1992. In situ hybridisation: a practical approach. IRL Press, Oxford University, Oxford, United Kingdom.
73. **Wilson, P. A., and D. A. Melton.** 1994. Mesodermal patterning by an inducer gradient depends on secondary cell-cell communication. Curr. Biol. **4:**676–686.
74. **Xu, X., M. Weinstein, C. Li, M. Naski, R. I. Cohen, D. M. Ornitz, P. Leder, and C. Deng.** 1998. Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. Development **125:**753–765.
75. **Yntema, H. G., B. van den Helm, J. Kissing, G. van Duijnhoven, F. Poppelaars, J. Chelly, C. Moraine, J. P. Fryns, B. C. Hamel, H. Heilbronner, H. J. Pander, H. G. Brunner, H. H. Ropers, F. P. Cremers, and H. van Bokhoven.** 1999. A novel ribosomal S6-kinase (RSK4; RPS6KA6) is commonly deleted in patients with complex X-linked mental retardation. Genomics **62:**332–343.
76. **Zhao, Y., C. Bjorbaek, and D. E. Moller.** 1996. Regulation and interaction of pp90(rsk) isoforms with mitogen-activated protein kinases. J. Biol. Chem. **271:**29773–29779.
77. **Zhao, Y., C. Bjorbaek, S. Weremowicz, C. C. Morton, and D. E. Moller.** 1995. RSK3 encodes a novel pp90^{rsk} isoform with a unique N-terminal sequence: growth factor-stimulated kinase function and nuclear translocation. Mol. Cell. Biol. **15:**4353–4363.
78. **Zimmerman, L. B., J. M. De Jesus-Escobar, and R. M. Harland.** 1996. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. Cell **86:**599–606.