

RGS proteins inhibit Xwnt-8 signaling in *Xenopus* embryonic development

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SUMMARY

RGS family members are GTPase activating proteins (GAPs) that antagonize signaling by heterotrimeric G proteins. Injection of *Xenopus* embryos with RNA encoding rat RGS4 (rRGS4), a GAP for G_i and G_q, resulted in shortened trunks and decreased skeletal muscle. This phenotype is nearly identical to the effect of injection of either *frzb* or dominant negative Xwnt-8. Injection of human RGS2, which selectively deactivates G_q, had similar effects. rRGS4 inhibited the ability of early Xwnt-8 but not Xdsh misexpression to cause axis duplication. This effect is distinct from axin family members that contain RGS-like

domains but act downstream of Xdsh. We identified two *Xenopus* RGS4 homologs, one of which, *Xrgs4a*, was expressed as a Spemann organizer component. Injection of *Xenopus* embryos with *Xrgs4a* also resulted in shortened trunks and decreased skeletal muscle. These results suggest that RGS proteins modulate Xwnt-8 signaling by attenuating the function of a G protein.

Key words: RGS, Xwnt-8, GAP, G protein, *Xenopus*, Signal transduction

INTRODUCTION

Heterotrimeric GTP-binding proteins (G proteins) are intracellular signaling molecules that are activated by seven transmembrane receptors (Gilman, 1987; Hamm and Gilchrist, 1996; Neer, 1995). Heterotrimeric G proteins are composed of α , β and γ subunits and there are multiple types of each subunit. Seven transmembrane receptors for hormones, cytokines and other agonists have guanine nucleotide exchange factor activity that substitutes GTP for GDP on the α subunit (G α), resulting in dissociation of the trimer into active G α -GTP monomer and G $\beta\gamma$ dimer. Both G α -GTP and G $\beta\gamma$ can regulate a variety of intracellular enzymes and ion channels. RGS family members are proteins that promote the intrinsic GTPase activity of the α subunit of heterotrimeric G proteins, resulting in the rapid deactivation of the G α subunit and reformation of the inactive G $\alpha\beta\gamma$ heterotrimer (Berman et al., 1996; Druey et al., 1996; Hepler et al., 1997; Hunt et al., 1996; Watson et al., 1996). RGS proteins are able to deactivate G proteins of the G_i, G_q and G_{12/13} but not the G_s families when examined in vitro.

The role of G proteins in early embryonic development is best understood in the nematode *C. elegans*. In worms there is a single G β subunit, GPB-1, which can bind to a variety of G α subunits (Zwaal et al., 1996). In larvae that contain maternal but no zygotic GPB-1, development proceeds until the first larval stage; however, the larvae die soon after hatching and exhibit little muscle activity. If maternal GPB-1 is also reduced, the orientation of mitotic spindles are randomized, resulting in abnormal cell positions. These embryos die with randomly distributed tissues, suggesting that maternal G proteins are important for the orientation of early cell divisions.

Previous work in *Xenopus* has established that G_i, G_o, G_q and G_s proteins are present in oocytes and early embryos (Otte et al., 1992; Gallo et al., 1996; Wilson et al., 1997). G α_i and G α_s protein levels are stable throughout development; however, G α_q family member protein levels increase markedly after the mid-blastula transition (Gallo et al., 1996). Microinjection of RNA encoding the α subunits of G_i, G_o, and G_s in early *Xenopus* embryos results in minimal phenotypic abnormalities (Otte et al., 1992). Overexpression of G α_i inhibits the disappearance of the blastocoel. Microinjection of pertussis toxin, a specific inhibitor of G_i and G_o proteins (G_i family members), has little effect on *Xenopus* pattern formation or neural development. Many of the extracellular factors that play a role in early *Xenopus* pattern formation, such as transforming growth factor- β and fibroblast growth factor family members, do not signal via heterotrimeric G proteins. Members of the transforming growth factor- β family, such as activin and bone morphogenetic proteins, signal via receptor serine kinases that phosphorylate intracellular proteins with transcriptional activity (Smad proteins; Massague, 1996). Fibroblast growth factors signal via receptor tyrosine kinases that activate the small G protein ras but not heterotrimeric G proteins (MacNicol et al., 1993; Kouhara et al., 1997). The receptors for wnt proteins have been identified as seven transmembrane receptors, but their ability to bind to and directly activate heterotrimeric G proteins has not been documented (Bhanot et al., 1996; Yang-Snyder et al., 1996). In one report, injection of pertussis toxin, a specific inhibitor of G_i proteins, into Zebrafish embryos blocked Xwnt5a-stimulated calcium efflux (Slusarski et al., 1997). This result suggests that a G_i family member mediates embryonic Xwnt-5a signaling.

There is little information available about the role of RGS proteins in early embryonic development. Recently, the protein axin was shown to play a role in modulating axis formation in murine development (Zeng et al., 1997). Mutations in the gene encoding axin are responsible for the fused mouse phenotype. Exogenous expression of axin in *Xenopus* embryos perturbs wnt signaling. Axin contains domains that are homologous to Disheveled (dsh) and RGS proteins; however, axin has not been demonstrated to have GAP activity. Furthermore, an important function of axin family members is to assemble a signaling complex that includes GSK-3 β , APC and β -catenin (Behrens et al., 1998; Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Sakanaka et al., 1998; Yamamoto et al., 1998).

To characterize the role of RGS proteins in early development, we performed microinjection experiments leading to overexpression of RGS4 or RGS2 in early embryonic development. We recently demonstrated that overexpression of RGS4 in rat cardiomyocytes inhibits heterotrimeric G protein-mediated signaling (Tamirisa et al., 1999). In the current report, we present data demonstrating that RGS overexpression in *Xenopus* embryos inhibits trunk and muscle development and blocks the effect of misexpressed Xwnt-8. We also describe the identification and functional characterization of Xrgs4a, a *Xenopus* RGS homolog.

MATERIALS AND METHODS

Plasmid constructions

The cDNAs encoding human wild-type RGS2, rat wild-type RGS4, rat frame-shift mutant form of RGS4 (fs-RGS4), rat point mutant form of RGS4 (N128A-RGS4) and *Xenopus* wild-type Xrgs4a were subcloned into a frog expression vector (pSP64T) (Druey et al., 1996; Heximer et al., 1997). The plasmids encoding full-length Xwnt-8 were gifts of Dr Randall Moon (University of Washington, USA). The plasmid encoding Xdsh was a gift of Dr Sergei Sokol (Harvard Medical School, USA). Synthetic capped RNA was transcribed from linearized plasmids as described (MacNicol et al., 1993; Muslin et al., 1994).

Embryo injections and animal cap assays

In vitro fertilization of *Xenopus laevis* eggs was performed as described previously (MacNicol et al., 1993). Embryos were staged as described by Nieuwkoop and Faber (1967). RNA was injected into both blastomeres of a 2-cell embryo for global expression experiments. RNA was injected into a single ventral-vegetal blastomere of a 16-cell embryo for axial twinning experiments. Blastomeres were injected with 2–10 nl of water containing RNA at a concentration of 5–500 ng/ μ l. Embryos were maintained at 18°C.

For animal cap assays, RNA or plasmid DNA was injected into both blastomeres of a 2-cell embryo. Animal caps were isolated manually from stage-8 embryos and were stimulated with recombinant activin (gift of Dr Greg Longmore, Washington University, USA) for 18–24 hours as previously described (MacNicol et al., 1993).

RNA purification and RT-PCR

Total RNA was obtained from *Xenopus* animal caps, oocytes, whole embryos and adult heart tissue by use of RNAzol (Tel-test), according to the manufacturers instructions. RT-PCR analyses were performed as previously described (Cui et al., 1995). Primers corresponding to gooseoid (Hemmati-Brivanlou et al., 1994) and histone H4 (Niehrs et al., 1994) were used as previously described (Hoppler et al., 1996). Primers for Xrgs4a were designed from its 3' UTR region: forward, 5'-TGTACAAGACACTACTGT-3'; reverse, 5'-CCAAGGCTAAA-GTTGCAG-3'. Primers for ornithine decarboxylase (ODC) were:

forward, 5'-AATGGATTTTCAGAGACCA-3'; reverse, 5'-AGAGTG-GTGTGTGGAATC-3' (Bassez et al., 1990).

Histological and immunocytochemical analysis of embryos

Embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4. Dehydrated embryos were embedded in Paraplast, sectioned at 7 μ m by use of a rotary microtome, and stained with Hematoxylin and Eosin. For immunocytochemical analysis, the sections were deparaffined and incubated with the muscle-specific antibody 12/101 (Kintner and Brockes, 1984) (Developmental Studies Hybridoma Bank). Bound antibody was visualized by alkaline phosphatase-conjugated secondary antibody and Vector red color substrate (Vector Laboratories). Sections were counterstained with Hematoxylin.

Whole-mount in situ hybridization

Albino or wild-type *Xenopus* embryos were dejellied and vitelline membranes were manually dissected. Embryos were fixed in 4% paraformaldehyde in PBS at pH 7.4. Whole-mount in situ hybridization was performed by the method of Harland (1991). All probes were generated by use of the Message Machine Kit (Ambion) with DIG RNA Labeling Mix (Boehringer Mannheim). The Xrgs4a probe consisted of the 3'-UTR of the full-length cDNA.

In vitro binding assays

The binding of histidine-tagged rat RGS4 protein to G α_q subunits present in *Xenopus* blastula-stage (stage 8) protein lysate was performed according to the method of Watson et al. (1996) with minor modifications. In brief, *Xenopus* stage-8 embryo lysate (0.5 mg protein) in buffer A (0.5% Nonidet P-40, 137 mM NaCl, 10 mM Tris, pH 7.5, 3 mM DTT, 6 mM MgCl₂, 0.2 U/ml aprotinin, 25 μ M leupeptin) containing 100 μ M GDP, 30 μ M AlCl₃ and 10 mM NaF, was incubated for 30 minutes at 5°C with histidine-tagged rat RGS4 (10 μ g). Lysate was added to Ni²⁺-nitrilotriacetic acid (NTA) agarose beads equilibrated with buffer A containing 20 mM imidazole, 100 μ M GDP, 30 μ M AlCl₃ and 10 mM NaF. The flow-through was collected for later analysis. Bound proteins were eluted with 500 mM imidazole, resolved by SDS/PAGE, transferred to nitrocellulose membranes and detected by western blot analysis with antiserum WO82 (G α_q).

Cloning of RGS homologs

RT-PCR was performed with a Stratagene kit and the primers of Chen et al. (1996) to obtain RGS protein homologs. PCR products were subcloned into pGEM-T according to the manufacturer's instructions. DNA sequencing of subcloned PCR products was performed using the ABI/prism kit with a Pharmacia 377 automated sequencer. BLAST searches (National Center for Biotechnology Information) were performed by use of the DNA and putative amino acid sequences of the RT-PCR products.

RACE-PCR was performed to obtain the entire coding region of Xrgs4a. The Marathon cDNA Amplification Kit and the SMART RACE cDNA Amplification Kit (Clontech) were used according to the manufacturer's instructions. PCR products containing the entire coding region and 5'- and 3'-untranslated regions of Xrgs4a were obtained independently from the two kits. Separate PCR products were sequenced to exclude the possibility of PCR-induced mutagenesis. The GenBank accession number of Xrgs4a is AF263451.

RESULTS

Overexpression of RGS4 in early *Xenopus* embryos

To assess the role of RGS proteins in early development, we first performed microinjection experiments that produced

global overexpression of RGS4, a GAP for G_i and G_q family members. RNA encoding full-length wild-type rat RGS4 (rRGS4) was microinjected into the vegetal pole of both blastomeres of 2-cell *Xenopus* embryos. A frame-shift mutant form of rRGS4 (fs-RGS4) that does not yield a polypeptide product was used for control injections.

Compared with control RNA-injected embryos, wild-type rRGS4-injected embryos displayed marked phenotypic abnormalities, which first became apparent during neurulation. By the tailbud stage, 40-50% of rRGS4 embryos were severely foreshortened with relatively normal head development, consistent with a defect in axial structure formation (Fig. 1). 8-10% of embryos were not foreshortened, but exhibited a bent anteroposterior axis (Table 1). The appearance of rRGS4-injected embryos was similar to the previously described phenotypes of frzb-injected embryos and dominant negative Xwnt-8-injected embryos (Hoppler et al., 1996; Leyns et al., 1997; Wang et al., 1997).

Histological analysis of rRGS4-overexpressing embryos revealed a shortened trunk with a marked decrease in skeletal muscle compared with that of control embryos (Fig. 2A,B). This decrease in skeletal muscle was similar to the defects noted in frzb-injected embryos (Leyns et al., 1997; Wang et al., 1997). In many truncated embryos, there was a substantial increase in the size of the cement gland. Immunohistochemical analysis of rRGS4 overexpressing embryos with the 12/101 monoclonal antibody confirmed the decrease and relative disorganization of skeletal muscle in the rRGS4 overexpressing embryos (Fig. 2A,B) (Kintner and Brockes, 1984). In addition, in several of these embryos, the retina was disordered and exhibited marked infolding in a pattern similar to that seen in frzb-injected embryos (Fig. 2C,D) (Leyns et al., 1997).

To evaluate alterations in gene expression in rRGS4 overexpressing embryos, whole-mount in situ hybridization experiments were performed on gastrula-stage embryos. XmyoD expression was markedly reduced when these embryos were injected with RNA encoding rRGS4 (Fig. 2E,F), consistent with the reduced skeletal muscle that was observed on immunohistochemical analysis. In contrast, Xbra (Fig. 2G,H) expression was unaffected in rRGS4-injected embryos.

Specific inhibition of G_i protein function in *Xenopus* embryos

To determine whether the phenotypic effect of rRGS4

overexpression was due to inactivation of G_i proteins, 2-cell embryos were injected at the vegetal pole with pertussis toxin, a selective inhibitor of G_i family members. PTX-injected embryos did not exhibit the same phenotype as those injected with rRGS4 and were morphologically normal (Table 1). Previous work has demonstrated that RGS proteins can promote the deactivation of several G protein families, including G_q , which are insensitive to pertussis toxin (Heximer et al., 1997).

To ascertain that the effect of wild-type rRGS4 microinjection resulted from the GAP activity of rRGS4 and not from some unrelated activity, 2-cell embryos were injected with RNA encoding a point mutant form of rRGS4 (N128A-RGS4), which yields a polypeptide product that differs from that produced by wild-type rRGS4 at only one residue: asparagine-128 is mutated to alanine. The N128A-RGS4 mutant has previously been demonstrated to be defective in GAP activity and G_α binding (Srinivasa et al., 1998). Injection of 2-cell embryos with RNA encoding N128A-RGS4 did not result in a shortened or bent anteroposterior axis and those embryos were morphologically normal (data not shown).

Overexpression of RGS2 in *Xenopus* embryos

Although RGS2 and RGS4 are homologous in their respective RGS domains, there are several important differences in the amino acid composition of the two proteins. It was recently shown that RGS2, in contrast to RGS4, is a selective inhibitor of G_q proteins with minimal GAP activity in vitro and in vivo toward G_i proteins (Heximer et al., 1997, 1999). The ability of rRGS4, but not PTX, to interfere with axial structure formation implied that the effect of rRGS4 was due to inhibition of a G_q -related activity. To test this hypothesis, we overexpressed RGS2 in early *Xenopus* embryos. When 2-cell embryos were injected with human RGS2 (hRGS2) RNA, marked inhibition of trunk development was noted in nearly 40% of embryos (Table 2).

RGS4 blocks Xwnt-8-induced axis duplication and mesoderm ventralization

The phenotype of rRGS4-injected embryos was similar to that of those overexpressing frzb (Leyns et al., 1997; Wang et al., 1997). Frzb antagonizes the early developmental activity of injected Xwnt-8, causing axis duplication and blocking the later ability of injected Xwnt-8 to cause ventralization. To

Table 1. Morphological abnormalities following injection of mRNA encoding RGS4 into 2-cell *Xenopus* embryos

Injected	Total survivors	Phenotype at stage 28			
		Normal	Bent axis	Short	Bifurcated tail
Control (fs-RGS4)	86	78 (90%)	4 (5%)	0	4 (5%)
Pertussis toxin	86	79 (92%)	3 (3%)	1 (1%)	3 (3%)
Wild-type RGS4	50	10 (20%)	4 (8%)	33 (66%)	3 (6%)

Embryos were injected at the 2-cell stage into the vegetal pole of both blastomeres with control RNA (fs-RGS4), pertussis toxin or wild-type RGS4 RNA.

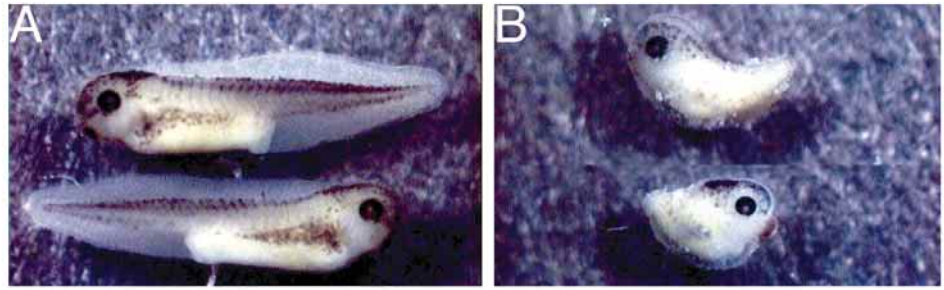
The phenotypes of injected embryos were scored at stage 28 and depicted as the number of embryos that were observed showing each phenotype. The results represent the cumulative data from four independent experiments. Values in parentheses are the percentage of total survivors after each injection.

Table 2. Inhibition of axial structure formation by injection of RNA encoding RGS2 into 2-cell *Xenopus* embryos

Injected	Total survivors	Phenotype at stage 28			
		Normal	Bent axis	Short	Bifurcated tail
Control (fs-RGS4)	29	28 (97%)	0	0	1 (3%)
Pertussis toxin	32	30 (94%)	0	0	1 (6%)
Wild-type RGS2	30	12 (40%)	7 (23%)	11 (37%)	0

Morphological abnormalities in embryos injected at the 2-cell stage into the vegetal pole of both blastomeres with 1-2 ng of control RNA (fs-RGS4), pertussis toxin or wild-type human RGS2 RNA. The phenotypes of injected embryos were scored at stage 28 and depicted as the number of embryos that were observed with each phenotype. The results represent the cumulative data from two independent experiments. Values in parentheses are the percentage of total survivors after each injection.

Fig. 1. RGS4 RNA injection at the 2-cell stage inhibits axial structure formation of *Xenopus* embryos. (A) Control embryos were injected at the 2-cell stage into the vegetal pole of both blastomeres with 1-2 ng of frame-shifted rat RGS4 RNA (fs-RGS4). These embryos were indistinguishable from uninjected embryos. (B) Experimental embryos were injected at the 2-cell stage into the vegetal pole of both blastomeres with 1-2 ng of synthetic wild-type rat RGS4 RNA. Note the shortening of the body axis in these embryos.



determine whether rRGS4 could also block the early effect of misexpressed Xwnt-8, rRGS4 RNA was coinjected into a single ventral-vegetal blastomere of 16-cell embryos with Xwnt-8 RNA. In embryos injected with 10 pg Xwnt-8 and 10 ng control RNA, axis duplication was noted in 47% of embryos ($n=86$). In embryos injected with 10 pg Xwnt-8 plus 1 ng rRGS4 RNA, axis duplication was markedly reduced to only 5% of embryos ($n=80$) (Fig. 3A-C). Axis duplication was not reduced in embryos coinjected with Xwnt-8 RNA and pertussis toxin (40% axis duplication, $n=55$). Injection of a single ventral-vegetal blastomere of a 16-cell embryo with 1 ng rRGS4 RNA alone had no effect on morphological development.

Injection of the intracellular signaling protein Xdsh into a ventral-vegetal blastomere of a 16-cell embryo also causes axis duplication. To determine whether rRGS4 could block the effect of misexpressed Xdsh, rRGS4 RNA was coinjected with Xdsh RNA. In embryos injected with Xdsh RNA, axis duplication was noted in 29% of embryos ($n=38$). Axis duplication was not reduced in embryos coinjected with Xdsh and rRGS4 RNA (32% axis duplication, $n=34$) (Fig. 3D). These results suggest that rRGS4 inhibits Xwnt-8 signaling upstream of, or parallel to, the activity of Xdsh.

In postblastula embryos, Xwnt-8 misexpression leads to ventralization of mesodermal tissues. Delayed misexpression of Xwnt-8 can be achieved by injection with CSKA-Xwnt8 plasmid DNA that is not transcribed until the mid-blastula transition (Christian and Moon, 1993). To determine whether rRGS4 could

block this delayed effect of Xwnt-8, activin-stimulated animal caps isolated from injected embryos were analyzed for the expression of *goosecoid*, a dorsal marker gene, and *histone H4*, a control gene (Hoppler et al., 1996). As expected, activin

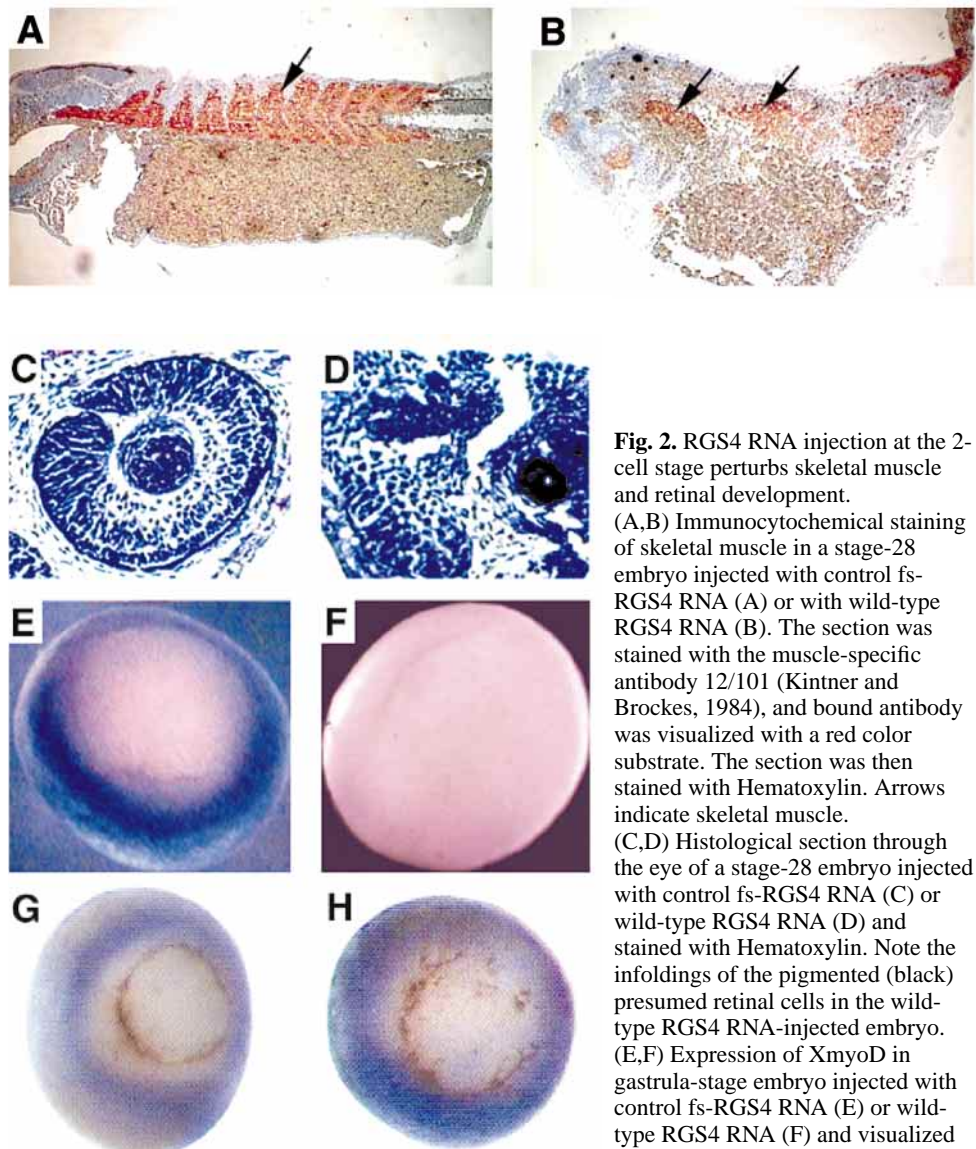


Fig. 2. RGS4 RNA injection at the 2-cell stage perturbs skeletal muscle and retinal development.

(A,B) Immunocytochemical staining of skeletal muscle in a stage-28 embryo injected with control fs-RGS4 RNA (A) or with wild-type RGS4 RNA (B). The section was stained with the muscle-specific antibody 12/101 (Kintner and Brockes, 1984), and bound antibody was visualized with a red color substrate. The section was then stained with Hematoxylin. Arrows indicate skeletal muscle.

(C,D) Histological section through the eye of a stage-28 embryo injected with control fs-RGS4 RNA (C) or wild-type RGS4 RNA (D) and stained with Hematoxylin. Note the infoldings of the pigmented (black) presumed retinal cells in the wild-type RGS4 RNA-injected embryo.

(E,F) Expression of XmyoD in gastrula-stage embryo injected with control fs-RGS4 RNA (E) or wild-type RGS4 RNA (F) and visualized by whole-mount in situ

hybridization. (G,H) Expression of Xbra in gastrula-stage embryo injected with control fs-RGS4 RNA (G) or wild-type RGS4 RNA (H) and visualized by whole-mount in situ hybridization.

stimulation resulted in increased *gooseoid* expression that was almost completely blocked by CSKA-Xwnt-8 injection, compared with baseline values. rRGS4 blocked the ability of CSKA-Xwnt-8 to inhibit activin-stimulated *gooseoid* gene expression (Fig. 4A,B).

RGS4 binds to *Xenopus* G α_q subunits

Xenopus embryo microinjection experiments suggested that rRGS4 blocks axial structure formation and the effect of misexpressed Xwnt-8 by deactivating a G $_q$ -related protein. In vitro experiments were performed to assess the validity of this hypothesis. *Xenopus* blastula-stage protein lysates were treated with GDP and AIF $_4^-$ to place G proteins in a conformation that mimics the transition state (Watson et al., 1996). Treated embryonic lysates were mixed with histidine-tagged rRGS4 protein and then added to Ni $^{2+}$ nitrilotriacetic acid (NTA)-agarose beads. Bound proteins were washed extensively and then eluted with 500 mM imidazole. Bound proteins were evaluated by immunoblotting using an anti-G α_q specific antibody. Under these conditions, G α_q subunits derived from *Xenopus* embryonic lysates bound to immobilized rRGS4 but not to control beads (Fig. 5). These experiments demonstrate that rRGS4 can bind to G α_q subunits derived from blastula-stage *Xenopus* embryos.

Identification of *Xenopus* homologs of RGS proteins

The marked effect of RGS protein overexpression on early development led us to investigate the possibility that *Xenopus* homologs of RGS proteins are expressed in embryos and that they play an important physiological role in development. We first investigated the possibility that RGS proteins are maternally expressed in *Xenopus* oocytes. We performed reverse transcription-polymerase chain reaction (RT-PCR) using primers that had previously been employed in the identification of RGS-r (Chen et al., 1996). We analyzed 22 RT-PCR products and found that they all encoded the identical sequence containing an RGS core domain. This sequence was homologous at the DNA level to human RGS4 (67% identity) and has been named Xrgs4a. At the amino acid level, this *Xenopus* clone was most homologous to murine RGS4 (Fig. 6A). Xrgs4a was much less homologous to the RGS-like domain of human axin than it was to human or murine RGS4 (Fig. 6B).

To identify RGS proteins expressed in later development and in adult life, we performed RT-PCR using adult *Xenopus* heart RNA and sequenced 12 products. Six of these products encoded sequences that contained an RGS core domain: four were identical to the products obtained with oocyte RNA (Xrgs4a), one product (Xrgs4b) was different from the oocyte clone but was also highly homologous to human RGS4 (65% identity at the DNA level) (Fig. 6A); and one was weakly homologous to RGS3. Xrgs4b was much less homologous to the RGS-like domain of human axin than it was to human or murine RGS4 (Fig. 6B).

A full-length cDNA encoding Xrgs4a was obtained by RT-PCR using the RACE protocol with *Xenopus* oocyte RNA. The full-length cDNA obtained independently from two RACE cDNA amplification kits was found by DNA sequencing to be identical, excluding the possibility of PCR-induced mutagenesis. The putative amino acid sequence of full-length Xrgs4a was highly homologous to human, mouse and rat RGS4 with about 70% amino acid identity (Fig. 6C).

The RT-PCR results demonstrated that Xrgs4a mRNA was present in *Xenopus* oocytes. To determine whether Xrgs4a protein was also present, we performed immunoblot

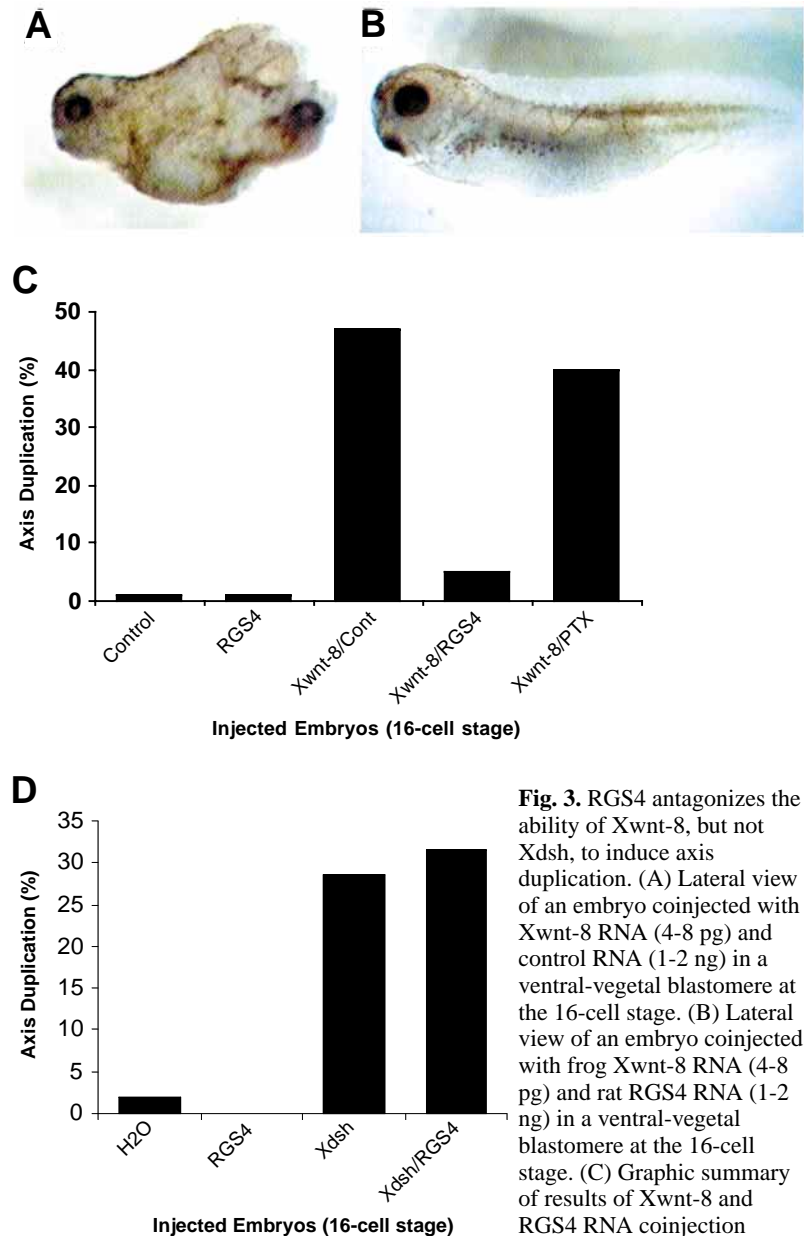


Fig. 3. RGS4 antagonizes the ability of Xwnt-8, but not Xdsh, to induce axis duplication. (A) Lateral view of an embryo coinjected with Xwnt-8 RNA (4-8 pg) and control RNA (1-2 ng) in a ventral-vegetal blastomere at the 16-cell stage. (B) Lateral view of an embryo coinjected with frog Xwnt-8 RNA (4-8 pg) and rat RGS4 RNA (1-2 ng) in a ventral-vegetal blastomere at the 16-cell stage. (C) Graphic summary of results of Xwnt-8 and RGS4 RNA coinjection experiments as described in

A and B. Cumulative data from four independent experiments was used to calculate the percentage of injected embryos with axis duplications. (D) Graphic summary of results of Xdsh and RGS4 coinjection experiments. Embryos were coinjected with Xdsh RNA and control (1-2 ng) or RGS4 RNA (1-2 ng) in a ventral-vegetal blastomere at the 16-cell stage. The results represent the cumulative data from three independent experiments.

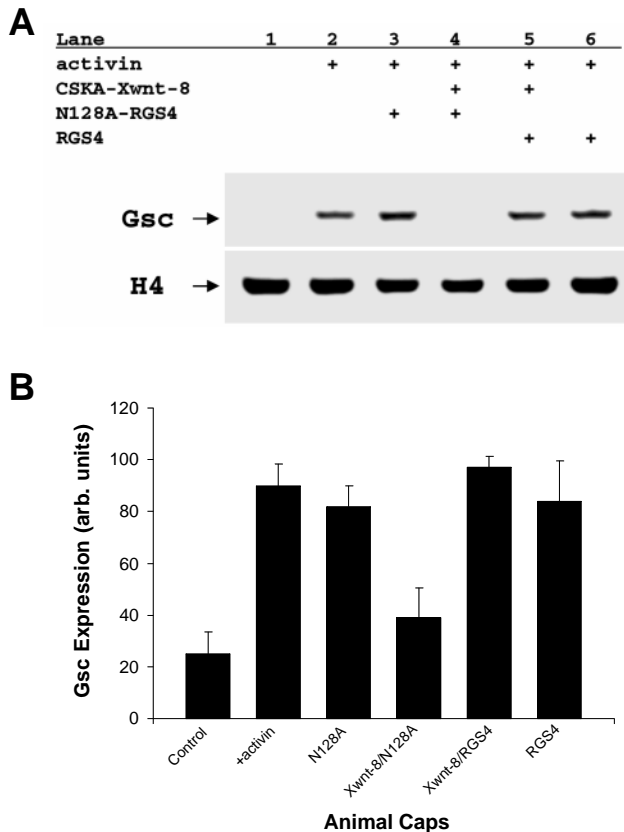


Fig. 4. RGS4 antagonizes the ability of Xwnt-8 to ventralize activin-induced mesoderm in animal caps. (A) *Xenopus* embryos were coinjected at the 2-cell stage with CSKA-Xwnt8 plasmid DNA (Hoppler et al., 1996) and N128A-RGS4 or wild-type RGS4 RNA. Animal caps were isolated from uninjected and injected embryos and treated with activin (Hoppler et al., 1996). RNA was isolated from animal caps and used for RT-PCR. The various treatments are summarised at the top. Below: ethidium bromide-stained agarose gel demonstrating Goosecoid (Gsc) and H4 expression in *Xenopus* animal caps. (B) Graphic summary of results obtained in RT-PCR experiments as described in A. Animal caps were isolated from uninjected or injected embryos and were treated with buffer (column 1) or 8 units/ml activin (columns 2-6). Ethidium bromide-stained agarose gels were analyzed by computerized densitometry using NIH Image. Goosecoid (Gsc) expression was normalized by H4 expression in each case. Values are mean + s.e.m. of three separate determinations.

experiments using a monoclonal anti-human RGS4 antibody and these experiments demonstrated that *Xenopus* oocytes contain Xrgs4a (or a highly related) protein (data not shown). The *Xenopus* RGS4 homolog, similar to human RGS4, migrated in SDS-polyacrylamide gels like a 32-33 kDa protein (Zhang et al., 1998).

Biological activity of Xrgs4a

To determine whether Xrgs4a regulates embryonic development, 2-cell embryos were injected with RNA encoding full-length Xrgs4a. Injection of Xrgs4a RNA resulted in phenotypic abnormalities that were similar to those observed with rRGS4. A prominent suppression of trunk development was observed in most embryos (Table 3). Increasing the dose of Xrgs4a RNA resulted in more severe phenotypic abnormalities (data not shown).

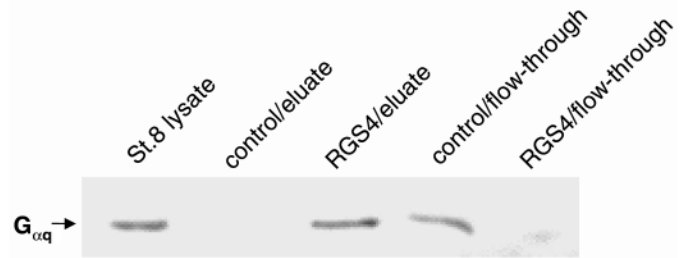


Fig. 5. Binding of recombinant RGS4 to *Xenopus* G α_q subunits. *Xenopus* blastula-stage (stage 8) embryonic protein lysates were treated with GDP and AlF $_4^-$ and incubated with histidine-tagged rat RGS4 protein in buffer A (or with buffer A alone). Complexes containing RGS4 protein were immobilized by the use of Ni $^{2+}$ -NTA chromatography. Bound proteins were eluted with 500 mM imidazole. Proteins in the flow-through and eluate fractions from Ni $^{2+}$ -NTA chromatography were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with an anti-G α_q antibody.

Table 3. Inhibition of axial structure formation by injection of RNA encoding Xrgs4a into 2-cell *Xenopus* embryos

Injected	Total survivors	Phenotype at stage 40		
		Normal	Bent axis	Short
Control (no injection)	105	96 (91%)	5 (5%)	4 (4%)
Control (H $_2$ O)	15	15 (100%)	0	0
Xrgs4a mRNA	38	6 (16%)	9 (24%)	23 (60%)

Morphological abnormalities observed in embryos injected at the 2-cell stage in the vegetal pole of both blastomeres with 1-2 ng of Xrgs4a RNA. Values in parentheses are the percentage of total survivors after each injection.

Histological analysis of Xrgs4a overexpressing embryos revealed a shortened trunk with a marked decrease in skeletal muscle compared with that of control embryos (data not shown). Immunohistochemical analysis of Xrgs4a overexpressing embryos with the 12/101 monoclonal antibody confirmed the decrease and relative disorganization of skeletal muscle in the rRGS4 overexpressing embryos (Fig. 7A,B). To evaluate alterations in gene expression in Xrgs4a overexpressing embryos, whole-mount in situ hybridization experiments were performed on gastrula-stage embryos. Xvent2 and gsc expression were unaffected in RGS4-injected embryos (Fig. 7C-F), demonstrating that Xrgs4a did not affect signaling by the BMP pathway and did not affect Spemann organizer formation (Cho et al., 1991; Onititchchouk et al., 1996).

To determine whether Xrgs4a could block the early effect of misexpressed Xwnt-8, Xrgs4a RNA was coinjected into a single ventral-vegetal blastomere of 16-cell embryos with Xwnt-8 RNA (Fig. 8). In embryos injected with 10 pg Xwnt-8, axis duplication was noted in 48% of embryos (Fig. 8A,D). In embryos injected with 10 pg Xwnt-8 plus 1 ng Xrgs4a RNA, axis duplication was markedly reduced to 3% embryos; and the inhibition of Xwnt-8 activity by Xrgs4a was dose-dependent (Fig. 8C,D). Injection of a single ventral-vegetal blastomere of a 16-cell embryo with 10 pg Xwnt-8 plus 1 ng of the catalytically inactive point mutant form of rRGS4 (RGS4-

N128A) RNA resulted in axis duplication in 55% of embryos (Fig. 8B,D).

Developmental expression pattern of Xrgs4a

Whole-mount *in situ* hybridization was used to examine the spatial expression pattern of Xrgs4a in the early embryonic development (Fig. 9A-H). In early gastrula-stage embryos, Xrgs4a was highly expressed in the dorsal lip of the blastopore, with expression extending anteriorly along the dorsal aspect of the embryo (Fig. 9A). In neurula-stage embryos, Xrgs4a was highly expressed in the developing neural folds from the anterior to the posterior poles (Fig. 9C,E). In tailbud-stage embryos, Xrgs4a was highly expressed in the brain, retina, heart, and spinal cord (Fig. 9G). Xrgs4a was not detected in the posteroventral portion of the embryo at any stage of development. To determine the timing of Xrgs4a expression in embryonic development, we evaluated the global expression pattern by stage-specific RT-PCR. We found that Xrgs4a was expressed throughout embryonic development, and was most highly expressed during late blastula stages and also during tailbud and tadpole stages (Fig. 9I).

DISCUSSION

The role of heterotrimeric G proteins in early vertebrate development is not well defined. Many of the growth and differentiation factors that are thought to be important in early vertebrate morphogenesis do not signal via heterotrimeric G proteins. In the nematode *C. elegans*, work with GPB-1 has demonstrated that G proteins are essential for early axis formation and late muscle development (Zwaal et al., 1996). Recently, the discovery and characterization of RGS proteins has led to the development of useful tools for the study of G proteins *in vivo* (Koelle and Horvitz, 1996; Druey et al., 1996). RGS proteins are GAPs that promote the deactivation of G proteins of the G_i, G_q and G_{12/13} but not G_s families (Berman et al., 1996; Hepler et al., 1997; Hunt et al., 1996; Watson et al., 1996). Not all RGS proteins have identical biochemical activities: recent work has suggested that RGS2 selectively deactivates G_q family members but has limited activity towards G_i or G_{12/13} family members

(Heximer et al., 1997, 1999). In addition, the RGS family member p115rhoGEF has specific GAP activity towards G_{12/13} (Kozasa et al., 1998). The protein axin contains an RGS-like domain and plays a role in axial patterning in mice (Zeng et al., 1997). The RGS-like domain of axin does not bind to G_α subunits, however, and does not have GAP activity when tested

A

```
Xrgs4a 1 EENIDFWTACENYKKIKTQARLPEQAQKIYEDFISVEATKEVNLDSTREETSN 54
***** ** ***** * * * * * ***** *****
mRGS4 86 EENIDFWISCEYKKIKSPSKLSPKAKKIYNEFISVQATKEVNLDSTREETS 139
*** ** * * * * * * * * * * * * * * * * * * * * * * *
Xrgs4b 1 EENMDFWLACENYKKIKTPAKLPEQAQKIYEDFISVEATREVNLDSTREETS 54

Xrgs4a 55 NILQPTYSTFDEAQHKIFILMEKDSYR 81
* ** * * * * * * * * * * * * * *
mRGS4 140 NMLQPTITCFDEAQKKIFNLMERDSYR 166
* ** * * * * * * * * * * * * *
Xrgs4b 55 NILQPTFSTFDEAQHRIFILMEQDTYS 81
```

B

```
Xrgs4a 1 EENIDFWTACENYKKIK----TQARLPEQAQKIYEDFI----SVEATKEVNLDSTREETS 54
* * * * * * * * * * * * * * * * * *
hAxin 149 ADLLDFWFACTGFRKLEPCDSNEEKRLKLRARAIYRKYLDNNGIVSRQTKPATKSFIKGCIMK 211
* * * * * * * * * * * * * * * * * *
Xrgs4b 1 EENMDFWLACENYKKIK----TPAKLPEQAQKIYEDFI----SVEATREVNLDSTREETS 54

Xrgs4a 55 NILQPTYSTFDEAQHKIFILMEKDSYR 81
* * * * * * * * * * * *
hAxin 212 QLIDP--AMFDQAQTIEIQATMEENTYP 236
* * * * * * * * * * *
Xrgs4b 55 NILQPTFSTFDEAQHRIFILMEQDTYS 81
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C

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Xenopus MCKGLAGLVYTFPAATCLKSAKDVKHRLGLFLLQKADGCEHSP 40
Rat MCKGLAGL---PASCLRSAKDMKHRLGLFLLQKSDSCHEHS 37
Mouse MCKGLAGL---PASCLRSAKDMKHRLGLFLLQKSDSCHEHS 37
Human MCKGLAGL---PASCLRSAKDMKHRLGLFLLQKSDSCHEHS 37

Xenopus GVKKPGS---QRVRRQEEVKKWAESENLENIINNECGLAAPR 76
Rat SHSKKDKVVTFCQRVSQEEVKKWAESENLENIINHECGLAAPFK 77
Mouse SHSKKDKVVTFCQRVSQEEVKKWAESENLENIINHECGLAAPFK 77
Human SHNKKDKVVTFCQRVSQEEVKKWAESENLENIINSHHECGLAAPFK 77

Xenopus SFLLQSEYSEENIDFWTACEINYKKIKTQARLPEQAQKIYED 116
Rat AFLKSEYSEENIDFWISCEEYKKIKSPSKLSPKAKKIYNE 117
Mouse AFLKSEYSEENIDFWISCEEYKKIKSPSKLSPKAKKIYNE 117
Human AFLKSEYSEENIDFWISCEEYKKIKSPSKLSPKAKKIYNE 117

Xenopus FISVQATKEVNLDSTREETSNNILQPTYSTFDEAQHKIF 156
Rat FISVQATKEVNLDSTREETS SRNMLEPTITCFDEAQKKIF 157
Mouse FISVQATKEVNLDSTREETS SRNMLEPTITCFDEAQKKIF 157
Human FISVQATKEVNLDSTREETS SRNMLEPTITCFDEAQKKIF 157

Xenopus ILM EKDSYR RFLKSKF YLDIVNLS SSGASTKMKKGLT TDS 196
Rat NLM EKDSYR RFLKSRF YLDL TNPSSCGAEKQKGA KSSAD C 197
Mouse NLM EKDSYR RFLKSRF YLDL TNPSSCGAEKQKGA KSSAD C 197
Human NLM EKDSYR RFLKSRF YLDL VNPSSCGAEKQKGA KSSAD C 197

Xenopus NPFIPQCA . 205
Rat TSLVPQCA . 206
Mouse TSLVSPQCA . 206
Human ASLVPQCA . 206
```

Fig. 6. Deduced amino acid sequence of two *Xenopus* RGS homologs obtained by RT-PCR. (A) Deduced amino acid sequence from the RGS core domains of mouse RGS4, Xrgs4a and Xrgs4b. Amino acid identity between the frog and mouse is indicated by an asterisk. (B) Deduced amino acid sequence from the RGS core domains of Xrgs4a and Xrgs4b and the RGS-like domain of human axin (hAxin). Amino acid identity between the frog and human is indicated by an asterisk. (C) Deduced amino acid sequence of full-length Xrgs4a, rat RGS4, mouse RGS4 and human RGS4. Conserved residues are shaded. Xrgs4a shows 71% amino acid identity with rat RGS4, 70% identity with mouse RGS4 and 70% identity with human RGS4.

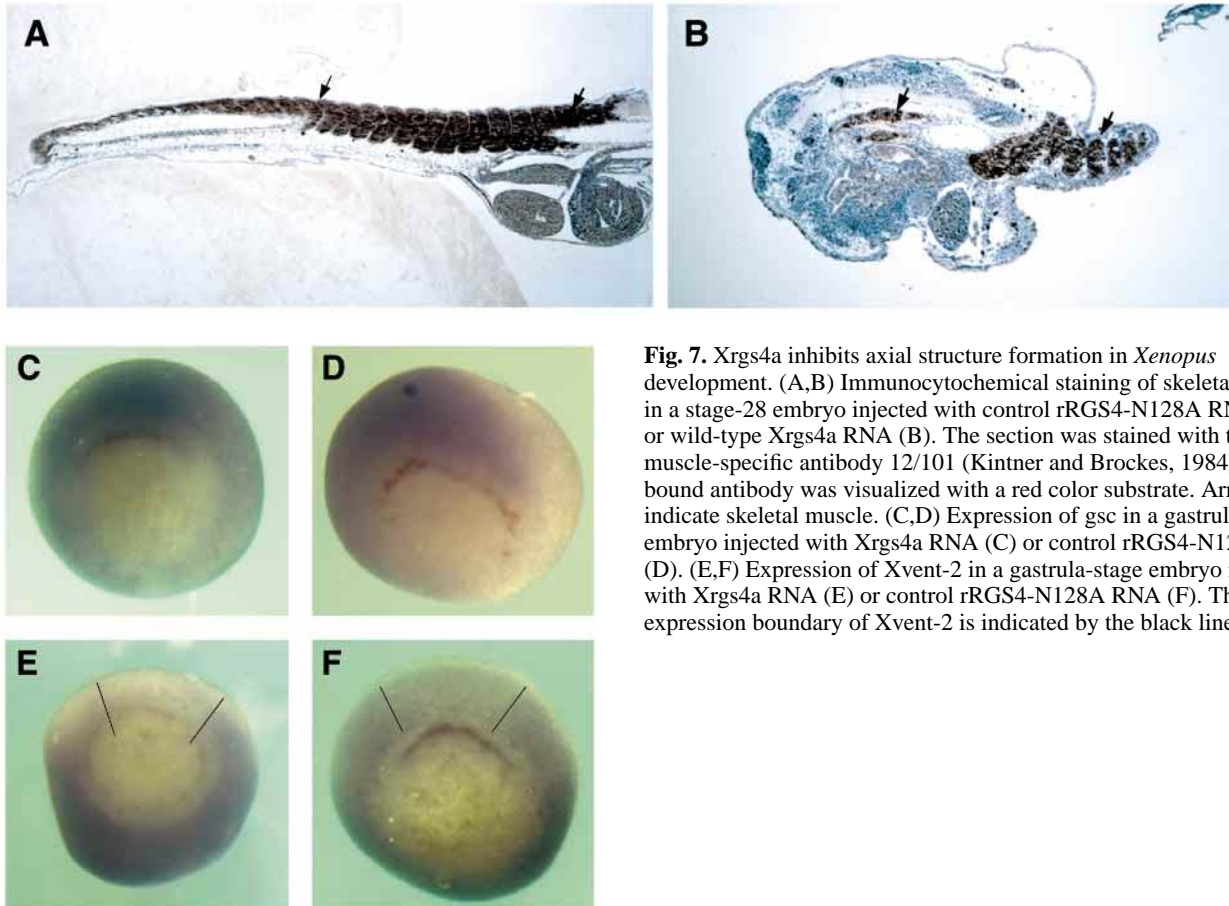


Fig. 7. Xrgs4a inhibits axial structure formation in *Xenopus* development. (A,B) Immunocytochemical staining of skeletal muscle in a stage-28 embryo injected with control rRGS4-N128A RNA (A) or wild-type Xrgs4a RNA (B). The section was stained with the muscle-specific antibody 12/101 (Kintner and Brockes, 1984), and bound antibody was visualized with a red color substrate. Arrows indicate skeletal muscle. (C,D) Expression of *gsc* in a gastrula-stage embryo injected with Xrgs4a RNA (C) or control rRGS4-N128A (D). (E,F) Expression of Xvent-2 in a gastrula-stage embryo injected with Xrgs4a RNA (E) or control rRGS4-N128A RNA (F). The dorsal expression boundary of Xvent-2 is indicated by the black lines.

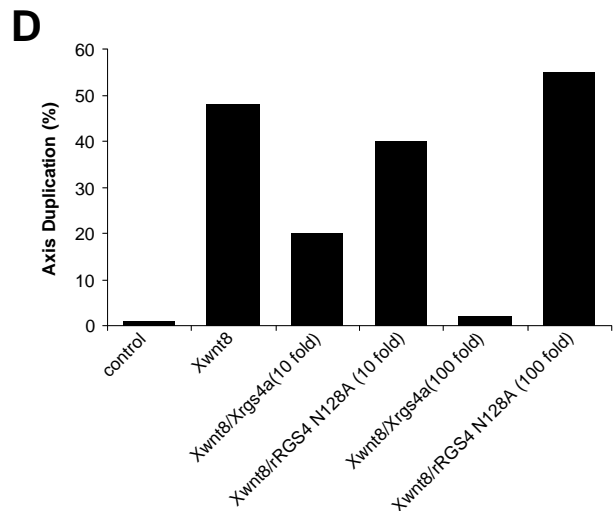
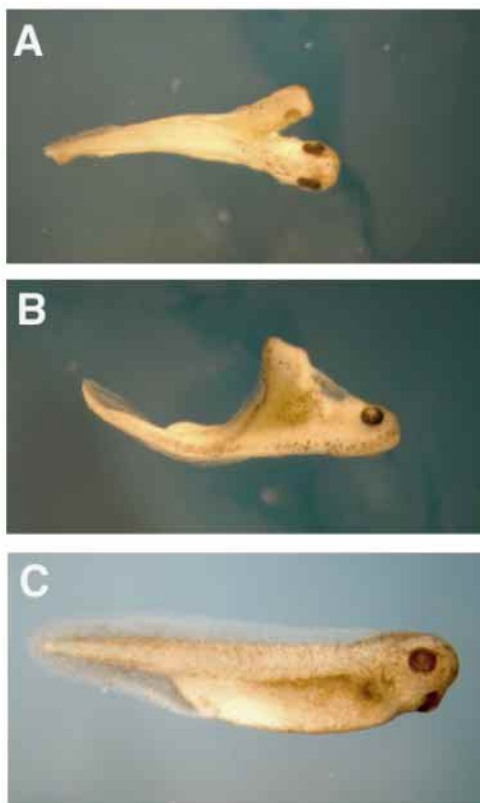


Fig. 8. Xrgs4a blocks Xwnt-8-mediated axis duplication in *Xenopus* embryos. (A) A stage-35 embryo that was injected with 10 pg Xwnt-8 RNA in a ventral-vegetal blastomere at the 16-cell stage. (B) A stage-35 embryo that was coinjected with 10 pg Xwnt-8 RNA and 1 ng rRGS4-N128A RNA in a ventral-vegetal blastomere at the 16-cell stage. (C) A stage-35 embryo that was coinjected with 10 pg Xwnt-8 RNA and 1 ng Xrgs4a RNA in a ventral-vegetal blastomere at the 16-cell stage. (D) Graphic summary of axis duplication experimental results. Embryos were injected with RNA in a ventrovegetal blastomere at the 16-cell stage. Blastomeres were injected with 10 pg Xwnt-8 RNA alone, or with the indicated amount of Xrgs4a or rRGS4-N128A RNA.

in vitro (K. J. Blumer, unpublished data). In addition, a primary role of axin is to assemble a complex that contains GSK-3 β , APC and β -catenin (Behrens et al., 1998; Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Sakanaka et al., 1998; Yamamoto et al., 1998).

When we overexpressed rRGS4, rRGS2 or Xrgs4a in early *Xenopus* embryos, trunk development was markedly inhibited and histological analysis revealed reduced skeletal muscle tissue. Several growth or differentiation factors have been previously demonstrated to play a role in certain aspects of mesodermal differentiation and may be involved in normal trunk development, including Xwnt-8, FGF and BMP-4 (Christian and Moon, 1993; Amaya et al., 1991; Graff et al., 1994). Inhibition of FGF signaling by global expression of a dominant negative FGF receptor results in extreme trunk deficiencies with relatively normal head development (Amaya et al., 1991); inhibition of BMP-4 signaling leads to the conversion of ventral mesoderm to dorsal mesoderm and results in partial secondary axis formation (Graff et al., 1994). Overexpression of frzb, an extracellular inhibitor of Xwnt-8 signaling, also blocks normal trunk development because it reduces notochord and skeletal muscle tissue development (Leyns et al., 1997; Wang et al., 1997). A similar phenotype is observed with expression of a dominant negative form of Xwnt-8 (Hoppler et al., 1996). Since BMP-4 and FGF signal via receptor protein kinases that are not known to activate heterotrimeric G proteins, it seems unlikely that the phenotypic effect of RGS4 expression results from inhibition of signaling by these factors. Xwnt-8 signaling, however, is presumably mediated by a frizzled family member that is thought to be a seven transmembrane receptor (Bhanot et al., 1996; Yang-Snyder et al., 1996).

Downstream signaling molecules that are regulated by Xwnt-8 binding to a frizzled family member include Xdsh, GSK-3, β -catenin and Xtcf-3 (He et al., 1995; Heasman et al., 1994; Miller and Moon, 1996; Molenaar et al., 1996; Sokol et al., 1995). The immediate intracellular effectors of

frizzled are not known. G protein activation by frizzled family members has not been demonstrated, although their predicted topology suggests this possibility. In our experiments, RGS4 or RGS2 overexpression resulted in a phenotype similar to that produced by frzb overexpression, suggesting that RGS proteins may block the ability of endogenous Xwnt-8 to promote trunk development, a developmentally late effect of Xwnt-8 action (Christian et al., 1991; Christian and Moon, 1993).

Embryonic misexpression studies have demonstrated that Xwnt-8 has two distinct biological activities that depend on the timing and localization of exogenously introduced Xwnt-8. First, misexpression of Xwnt-8 in ventral-vegetal blastomeres of early blastula-stage embryos promotes axis duplication (Christian et al., 1991). Second, global overexpression of Xwnt-8 in late blastula- and gastrula-stage embryos leads to ventralization of mesodermal tissues (Christian and Moon, 1993). We examined the ability of RGS proteins to block both of these activities by coinjection of embryos. When RGS4 RNA was coinjected with Xwnt-8 RNA (to give early

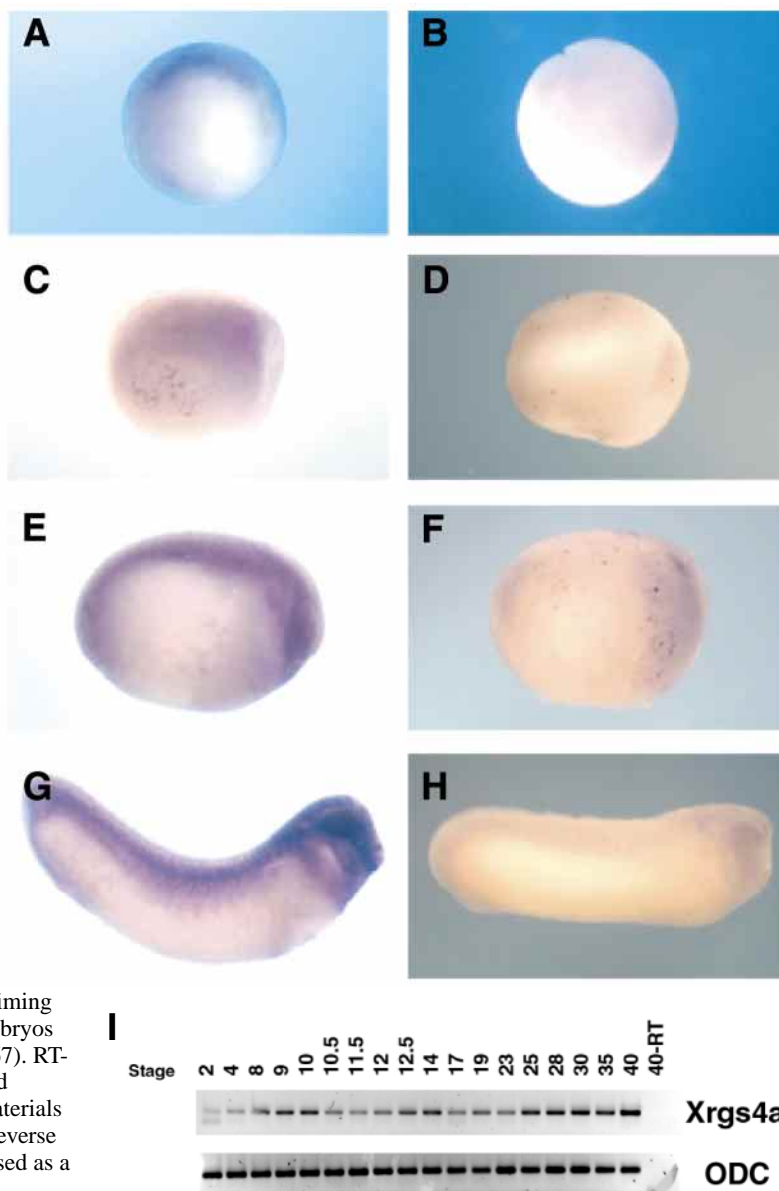


Fig. 9. Developmental expression pattern of Xrgs4a. (A-H) Spatial expression pattern of Xrgs4a. Whole-mount in situ hybridization analysis of gene expression in albino embryos at the gastrula, neurula and tailbud stages.

(A,C,E,G) In situ hybridizations were performed with an antisense Xrgs4a-probe. (B,D,F,H) In situ hybridizations were performed with a sense Xrgs4a-probe. (A,B) Vegetal view of stage-10.25 embryos. (C-H) Lateral view of stage-16 (C,D), stage-25 (E,F) and stage-35 (G,H) embryos. Anterior is right. Xrgs4a is expressed in the dorsal lip of the blastopore, with expression extending over the dorsal aspect of the gastrula-stage embryo (A). Xrgs4a is expressed in the neural folds of neurula-stage embryos (C,E). Xrgs4a is expressed in the head, retina, spinal cord, cement gland and heart of the tailbud-stage embryo (G).

(I) Timing of Xrgs4a gene expression. RNA was purified from whole embryos at the indicated embryonic stages (Nieuwkoop and Faber, 1967). RT-PCR was performed by use of specific primers that recognized Xrgs4a or ornithine decarboxylase (ODC), as described in Materials and Methods. The 40-RT lane contained all reagents except reverse transcriptase, and was used as a negative control; ODC was used as a loading control.

misexpression of Xwnt-8) into a ventral-vegetal blastomere of a 16-cell embryo, axis duplication was markedly inhibited. RGS4 was unable to block axis duplication caused by misexpression of Xdsh, a downstream component of the Xwnt-8 signaling cascade. This result suggests that RGS4 can directly or indirectly inhibit the biological activity of Xwnt-8 upstream of, or parallel to, the activity of Xdsh. When RGS4 RNA was coinjected with CSKA-Xwnt8 plasmid DNA (to give delayed overexpression of Xwnt-8), Xwnt-8-stimulated ventralization of dorsal mesoderm was blocked. Therefore, RGS4 has the capacity to inhibit at least two distinct biological activities of exogenously introduced Xwnt-8.

The inability of pertussis toxin to cause phenotypic abnormalities similar to RGS4 overexpression in early *Xenopus* embryos suggests that G_i family members do not regulate axis formation and do not transduce the Xwnt-8 signal. This finding, coupled with the ability of RGS2 to block axial structure development, suggests that a G_q-related protein may play a role in this process. Furthermore, recent work with mouse F9 teratocarcinoma cells suggests that frizzled-1 signals through G_q and G_o proteins in response to stimulation by conditioned medium containing Xwnt-8 (Liu et al., 1999). On the other hand, work with Zebrafish embryos suggests that a G_i family member regulates calcium efflux mediated by the Xwnt-5a ligand and the rat Frizzled-2 (Rfz-2) receptor (Slusarski et al., 1997). In *Xenopus* embryos, expression of Xwnt-5a and selective Frizzled proteins, including Rfz-2, mouse Frizzled-3, -4 and -6, also stimulated PKC activity in a pertussis toxin-sensitive manner (Sheldahl et al., 1999). Based on the result that individual Frizzleds can differentially stimulate Ca²⁺ and PKC activities in a G-protein dependent manner, distinct Wnt/β-catenin and Wnt/Ca²⁺ pathways have been described (Miller et al., 1999). It is interesting to speculate that different classes of frizzled receptors signal via alternate G proteins. However, previous genetic screens have not established the role of G proteins in the wnt signaling pathway. Biochemical experiments to evaluate whether frizzled family members can directly bind to and activate heterotrimeric G proteins will help to clarify this issue.

The importance of a G protein in early pattern formation raises the question of the identity of G protein effectors. One possibility is that an embryonic G protein directly activates PLC-β or indirectly activates PLC-γ, enzymes that generate IP₃ and diacylglycerol (Wilson et al., 1997). Previous work has demonstrated that IP₃ levels rise sharply at the 64-cell stage of *Xenopus* embryogenesis and that they remain elevated for several hours (Maslanski et al., 1992). Diacylglycerol production can lead to protein kinase C activation, which, in turn, can phosphorylate and inactivate GSK-3β (Goode et al., 1992). Indeed, wingless-induced inactivation of GSK-3β in murine fibroblasts is sensitive to pharmacological inhibitors of PKC (Cook et al., 1996). A second possibility is that a G protein activates phosphatidylinositol-3 kinase, an enzyme that generates phosphatidylinositol-3,4,5-phosphate (PIP₃) and other phosphorylated lipid products (Lopez-Illasaca et al., 1997). The levels of PIP₃ in early embryos have not been previously determined. PIP₃ is an activator of protein kinase B (Akt), which can phosphorylate and inactivate GSK-3β (Klippel et al., 1997; Cross et al., 1995). In these ways, it is possible to

hypothesize how activation of a G protein might lead to inactivation of GSK-3β and stabilization of β-catenin.

We found at least three different RGS homologs present in *Xenopus*. From oocyte RNA, we identified one homolog of human RGS4 using RT-PCR. From adult heart RNA, we identified two homologs of human RGS4: one that was identical to the oocyte clone and one homolog of human RGS4. Xrgs4a was highly expressed in the dorsal lip of the blastopore (Spemann's organizer) in gastrula-stage embryos. Previous work has demonstrated that several inhibitors of growth factor and agonist action are present in the Spemann's organizer, including frzb, inhibin, chordin and noggin (Hemmati-Brivanlou et al., 1994; Leyns et al., 1997; Sasai et al., 1994; Smith and Harland, 1992; Wang et al., 1997). Inhibition of agonist signaling in the Spemann's organizer and in immediately adjacent tissues is thought to be critical to specify dorsal cell fate. Indeed, Xwnt-8 is expressed in all mesoderm cells except those of the organizer field and is thought to promote the ventrolateral mesodermal patterning (Christian and Moon, 1993). It is tempting to speculate that RGS4 expression in the dorsal lip of the blastopore is required to antagonize Xwnt-8 signaling so that dorsal-ventral patterning and mesodermal differentiation can proceed normally.

Our findings suggest that RGS and G proteins play a critical role in early embryonic pattern formation. In particular, our experiments point to the importance of a G protein in the regulation of trunk development. To test the biological function of RGS proteins we have relied on overexpression studies. In the future, loss-of-function experiments may help to confirm the importance of Xrgs4a in early development.

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