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The Mouse *Fused* Locus Encodes Axin, an Inhibitor of the Wnt Signaling Pathway That Regulates Embryonic Axis Formation

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Summary

Mutations at the mouse *Fused* locus have pleiotropic developmental effects, including the formation of axial duplications in homozygous embryos. The product of the *Fused* locus, Axin, displays similarities to RGS (Regulators of G-Protein Signaling) and Dishevelled proteins. Mutant *Fused* alleles that cause axial duplications disrupt the major mRNA, suggesting that Axin negatively regulates the response to an axis-inducing signal. Injection of *Axin* mRNA into Xenopus embryos inhibits dorsal axis formation by interfering with signaling through the Wnt pathway. Furthermore, ventral injection of an *Axin* mRNA lacking the RGS domain induces an ectopic axis, apparently through a dominant-negative mechanism. Thus, Axin is a novel inhibitor of Wnt signaling and regulates an early step in embryonic axis formation in mammals and amphibians.

Introduction

A fundamental problem in mammalian embryology is the mechanism by which the egg cylinder, an epithelial cup in which only the dorsal–ventral axis is established, gives rise to an embryo with anterior–posterior (A–P) polarity. In the mouse, the earliest morphological manifestation of the A–P axis is the delamination of mesoderm in the primitive streak at ~E6.5. The position of the streak cannot be predicted by earlier morphological asymmetries in the embryo (Gardner et al., 1992), and the regulative abilities of early mouse embryos appear to rule out axis determination by localized determinants from the egg. While a few secreted factors or transcription factors are expressed asymmetrically in the egg cylinder shortly before primitive streak formation, and thus might be involved in induction of the streak, their roles in this process have not been established (reviewed by Bachvarova, 1996; Conlon and Beddington, 1995).

In the amphibian embryo, the dorsal–ventral axis (the second axis to be specified, analogous to the A–P axis of the mouse) is determined by the point of sperm entry and subsequent cortical rotation. This rotation generates the Nieuwkoop Center, a group of dorsal/vegetal blastomeres that induce formation of the Spemann organizer. Transplantation of the Nieuwkoop Center or the organizer to an ectopic position induces the formation of a complete secondary axis, i.e., notochord, somites, neural tube, and head structures (reviewed by Slack, 1994). Recent studies suggest that the formation of the Nieuwkoop Center depends on activation of components of the Wnt signaling pathway (Carnac et al., 1996; Wylie et al., 1996; Fagotto et al., 1997). The Wnts are a family of secreted polypeptides related to Drosophila wingless, whose receptors are believed to be members of the frizzled family (reviewed by Miller and Moon, 1996). The next known component of the signaling pathway is Dishevelled (Dsh), a cytoplasmic protein that, when activated by a Wnt signal, inhibits the activity of glycogen synthase kinase 3 (GSK-3). In the absence of a Wnt signal, GSK-3 activity leads (directly or indirectly) to the phosphorylation and consequent degradation of β-catenin. In the presence of a Wnt signal, GSK-3 is inhibited, increasing the cytosolic level of β-catenin and promoting its interaction with downstream effectors.

A role for the Wnt signaling pathway in development of the amphibian embryonic axis was revealed by the ability of several Wnts, or downstream factors, to induce an ectopic axis when injected into Xenopus embryos (Miller and Moon, 1996). Furthermore, components of this pathway are required for normal axial development because depletion of maternal β-catenin mRNA (Heasman et al., 1994), or sequestration of β-catenin to the plasma membrane (Fagotto et al., 1996), results in ventralized embryos that fail to develop a dorsal axis. However, it is not clear whether a Wnt ligand triggers Nieuwkoop Center formation, or whether downstream components of the Wnt pathway are activated by some other mechanism (Hoppler et al., 1996; Miller and Moon, 1996; Sokol, 1996). The Nieuwkoop Center is thought to induce a Spemann organizer by secreting a (yet-to-be-identified) diffusible signal (Wylie et al., 1996; Fagotto et al., 1997), which may act synergistically with mesoderm-inducing factors, such as Activin and Vg1, to activate the expression of dorsal-specific genes, such as Goosecoid (Watabe et al., 1995). Dorsoventral patterning of the mesoderm is further controlled by opposing signals emanating from the organizer and the ventral mesoderm: a ventral bone morphogenetic protein (BMP) signal represses dorsal genes, while in the dorsal side the secreted factors Noggin, Chordin, and Follistatin directly inhibit BMPs (Hogan, 1996).

While little is known about the molecular control of axis formation in mammalian embryos, a potential

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source of insight is the study of mouse mutants that affect this process (Conlon and Beddington, 1995; St-Jacques and McMahon, 1996), such as *Fused* (*Fu*). Two spontaneous alleles of *Fu*, called *Kinky* (Fu^{Ki}) and *Knobbly* (Fu^{Kb}), and a transgenic insertional allele, Fu^{Tg1} (previously called *Hϵ46*), carry recessive mutations that are lethal at E8–E10 (Gluecksohn-Schoenheimer, 1949; Jacobs-Cohen et al., 1984; Perry et al., 1995). In addition to neuroectodermal and cardiac abnormalities, a remarkable property of many early postimplantation embryos homozygous for these three mutant alleles is a duplication of the embryonic axis. This phenotype, unique among mouse mutants, led nearly 40 years ago to the suggestion that *Fu* may play a role in the specification of the embryonic axis (Gluecksohn-Schoenheimer, 1949). *Kinky*, *Knobbly*, and a third spontaneous allele, *Fused* (Fu^{Fu}), but not Fu^{Tg1}, also have dominant effects that include transient bifurcations of the fetal tailbud, asymmetric fusion of vertebrae leading to tail kinks, deafness, and neurological defects (Lyon et al., 1996).

We previously described the cloning of this locus with the aid of a transgene insertion (Perry et al., 1995). Here, we report the isolation and sequence of cDNA clones, and the genomic structure of the wild-type (WT) and Fu^{Tg1} alleles. Analysis of the Fu^{Fu} and Fu^{Kb} alleles (T. J. V. et al., submitted) has shown that both are caused by retroviral insertions. Because two mutant alleles causing axial duplications in homozygous embryos, Fu^{Tg1} and Fu^{Kb}, disrupt production of the major mRNA, we reasoned that the normal gene product may negatively regulate a critical step in the formation of the embryonic axis. This hypothesis is supported by studies in Xenopus embryos, which demonstrate that dorsal injection of WT *Fused* mRNA blocks axis formation, while ventral injection of a dominant-negative mutant form induces an ectopic axis. Coinjection with factors acting at various steps in axis formation reveals that Fused exerts its effects at a very early stage, by specifically inhibiting signal transduction through the Wnt pathway in the Nieuwkoop Center. Thus, analysis of the *Fu* locus has identified a novel inhibitor of the Wnt signaling pathway and suggests that the same pathway regulates an early step in embryonic axis formation in mammals and amphibians. To avoid confusion with the unrelated Drosophila gene *fused*, we have renamed the *Fu* gene *Axin*, for *ax*is *in*hibition.

Results

Identification of the *Axin* Gene

We previously reported that a genomic probe from the $Axin^{Tg1}$ transgene insertion locus detected a 3.9 kb RNA in wild-type embryonic stem (ES) cells but not in $Axin^{Tg1/Tg1}$ ES cells, representing a strong candidate for the *Axin* mRNA (Perry et al., 1995). To isolate cDNA clones, a probe located within a CpG island upstream from the transgene insertion site (Figure 1) was used to screen a mouse embryo library. One cDNA clone contained a region identical in sequence with the genomic probe, confirming that it was encoded at the *Axin* locus, and this clone was used to isolate additional overlapping cDNA clones. The composite cDNA sequence was 3623 bp long, excluding the poly(A) tail. All the cDNA clones were colinear in their regions of overlap, except for a 108 bp sequence present in a fraction of clones following bp 2579, representing an alternative splicing product (Figure 1). Transcripts lacking this 108 bp segment were termed form 1 and those containing it, form 2.

Genomic Organization of *Axin*

Using *Axin* cDNA probes, a series of overlapping clones was isolated from a WT mouse genomic library, and the locations of exons and introns were determined (see Experimental Procedures). As illustrated in Figure 1, 10 exons were identified, spanning ~56 kb. The extra 108 bp segment in form 2 mRNA results from the use of an alternative 5′ splice site following exon 8 and is designated exon 8A. The longest cDNA clones representing the 3.9 kb mRNA appeared to be missing 25–75 nt from the 5′ end, based on RNase H and S1 nuclease mapping studies (unpublished data). Difficulties in cloning these 5′ terminal sequences may be a consequence of the very high GC content of the CpG island surrounding the apparent promoter region (Figure 1). Based on Southern blot hybridization to genomic DNA (data not shown) and the analysis of multiple cDNA and genomic clones, *Axin* appears to be a single copy gene.

In the $Axin^{Tg1}$ allele, exon 2 and parts of the two flanking introns are deleted. Exons 1 and 3 are separated by an ~600 kb transgene insertion (Figure 1), a disruption that leads to the absence of the major WT 3.9 kb mRNA in homozygotes (Figure 2). As described elsewhere (T. J. V. et al., submitted), the $Axin^{Fu}$ allele contains an endogenous intracisternal A particle (IAP) provirus within intron 6, while $Axin^{Kb}$ contains a similar IAP element interrupting exon 7. The $Axin^{Ki}$ allele is apparently extinct.

Ubiquitous Expression of Wild-Type *Axin* mRNA

On Northern blots, a major band of ~3.9 kb was observed in all WT adult tissues examined, embryos at E10.5–E16.5, and ES cells. A 3.0 kb band was also observed at very low levels in some WT tissues and ES cells. In $Axin^{Tg1/Tg1}$ ES cells, the 3.9 kb RNA was absent, but a 3.0 kb RNA was observed (Figure 2a). Because the 3.0 kb mRNA was observed in both WT and $Axin^{Tg1/Tg1}$ cells, and contains exons 3–10 but not 1 and 2 (data not shown), it is likely to be transcribed from a weak promoter downstream from the 3′ end of the transgene-induced deletion. Thus, $Axin^{Tg1}$ is a loss-of-function allele with respect to the major 3.9 kb mRNA, although it may not be a null allele.

In situ hybridization with WT embryos at E7.5–E9.5 showed that *Axin* mRNA is uniformly distributed throughout embryonic and extraembryonic tissues of the postimplantation embryo (Figure 2b). *Axin* mRNA was also detected by RT–PCR in 1-cell through blastocyst stage embryos (data not shown). Form 1 and 2 mRNAs were both present in all adult tissues examined and in ES cells (Figure 2c).

The Predicted Amino Acid Sequences of Axin and Its Human and Chicken Homologs

The murine *Axin* (*mAxin*) cDNA sequence included an open reading frame (ORF) beginning at base 3, which

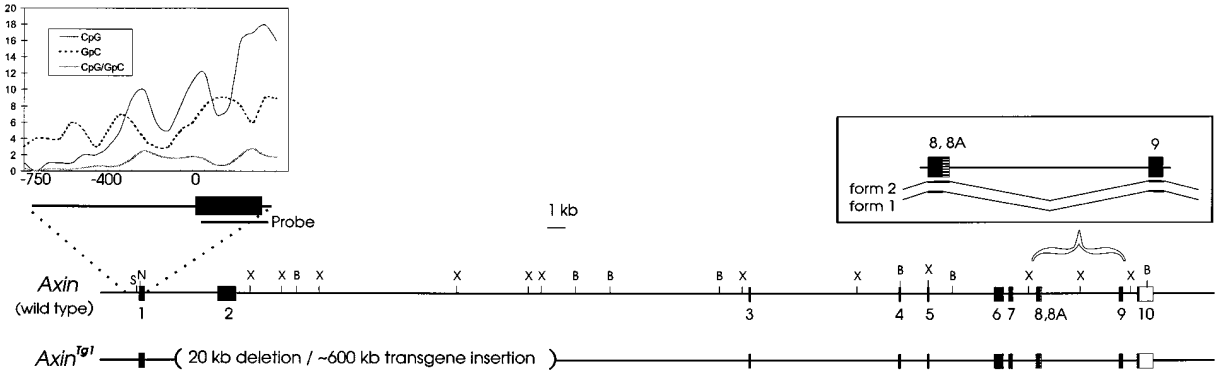

Figure 1. Structure of the WT *Axin* Gene and the Transgenic Allele *Axin*^{Tg1}

In *Axin*^{Tg1}, a random transgene insertion (Perry et al., 1995) was accompanied by a deletion including exon 2. Exon 1 is located in a CpG island, as indicated by the frequency of CpG or GpC dinucleotides per 100 bp, and the ratio of CpG/GpC, calculated at 50 bp intervals (left inset). A genomic probe used to isolate cDNA clones is indicated below the inset. The open box in exon 10 represents the 3′ UTR. The inset at right shows the origin of form 1 and 2 alternatively spliced mRNAs. The cDNA sequences corresponding to exon 1 are 1–308; exon 2, 309–1267; exon 3, 1268–1408; exon 4, 1409–1505; exon 5, 1506–1643; exon 6, 1644–2161; exon 7, 2162–2332; exon 8, 2333–2578; exon 8A, 2579–2686; exon 9, 2687–2854; and exon 10, 2855–3731. The RGS region is encoded in exon 2, and the Dsh homology in exons 9–10. Restriction sites: N, NotI; S, SacII; X, XbaI; B, BamHI.

could encode a protein of up to 956 (form 1) or 992 (form 2) amino acids (Figure 3a). Homology searches identified several ESTs representing a human *Axin* homolog, and additional human cDNA sequences were isolated by 5′ and 3′ RACE (Chenchik et al., 1995). The predicted human and murine Axin amino acid sequences are 87% identical overall. In addition, a cDNA clone representing a chicken homolog was isolated, and its predicted amino acid sequence was 66% identical to mAxin (Figure 3a). The first two AUG codons in the *mAxin* ORF were located at bp 375 and 391 of the cDNA, but neither was surrounded by a consensus initiation site (Kozak, 1986). To determine whether either site could serve as an initiation codon, 293T cells were transfected with *Axin* cDNA expression vectors including a C-terminal epitope tag. The sizes of the in vivo translation products were consistent with initiation at one of the first two AUG codons (data not shown). However, because the murine and human ORFs continue to be conserved upstream of this position, it remains possible that the normal initiation site is further upstream.

The predicted amino acid (aa) sequence contains multiple sites for Ser/Thr phosphorylation and one for Tyr phosphorylation, suggesting that Axin may be a phosphoprotein. It also contains one (form 2) or two (form 1) sequences matching the consensus for a bipartite nuclear localization signal (NLS) (Dingwall and Laskey, 1991). However, detection of epitope-tagged Axin proteins expressed in mammalian cells or Xenopus embryos indicated a perimembrane rather than a nuclear location for both forms (data not shown). Database searches revealed two regions of homology to other known proteins. One of these, aa 213–338, shows 30%–40% identity and 50%–60% similarity to the RGS (Regulation of G-protein Signaling) domain (Dohlman and Thorners, 1997). A second potentially important region of similarity (Figure 3c) is a 51 aa segment near the Axin C terminus, which is ~40% identical and ~60% similar to a conserved sequence near the N terminus of Drosophila Dsh and its vertebrate homologs (Klingensmith et al., 1994; Sussman et al., 1994). Both the RGS and Dsh homology regions are highly conserved among mouse, human, and chick Axin homologs (Figure 3a).

Injection of *Axin* mRNA Inhibits Dorsal Axis Formation in Xenopus Embryos

The observation that the *Axin*^{Tg1} and *Axin*^{Kb} alleles, which cause axial duplications in homozygous mouse embryos, are both unable to produce the major 3.9 kb

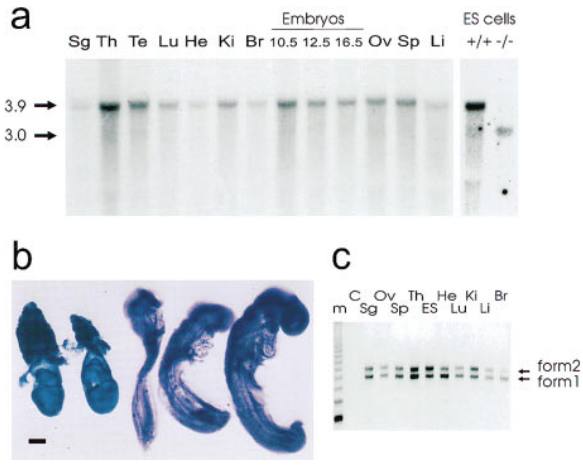

Figure 2. Expression of *Axin* mRNA in Adult Tissues, Embryos, and ES Cells

(a) Northern blot. ES cells are WT (+/+) or *Axin*$^{Tg1/Tg1}$ (−/−). Sg, salivary gland; Th, thymus; Te, testis; Lu, lung; He, heart; Ki, kidney; Br, brain; Ov, ovary; Sp, spleen; Li, liver.

(b) Whole mount in situ hybridization analysis of *Axin* mRNA in (left to right) two E7.5, two E8.5, and one E9.5 WT embryos. Scale bar, 0.2 mm.

(c) Expression of both mRNA isoforms in tissues and ES cells, detected by RT-PCR using primers flanking the 108 bp sequence encoded by exon 8A. The upper band (563 bp) represents form 2 mRNA and the lower band (455 bp) form 1. C, control with no added cDNA; m, 123 bp ladder.

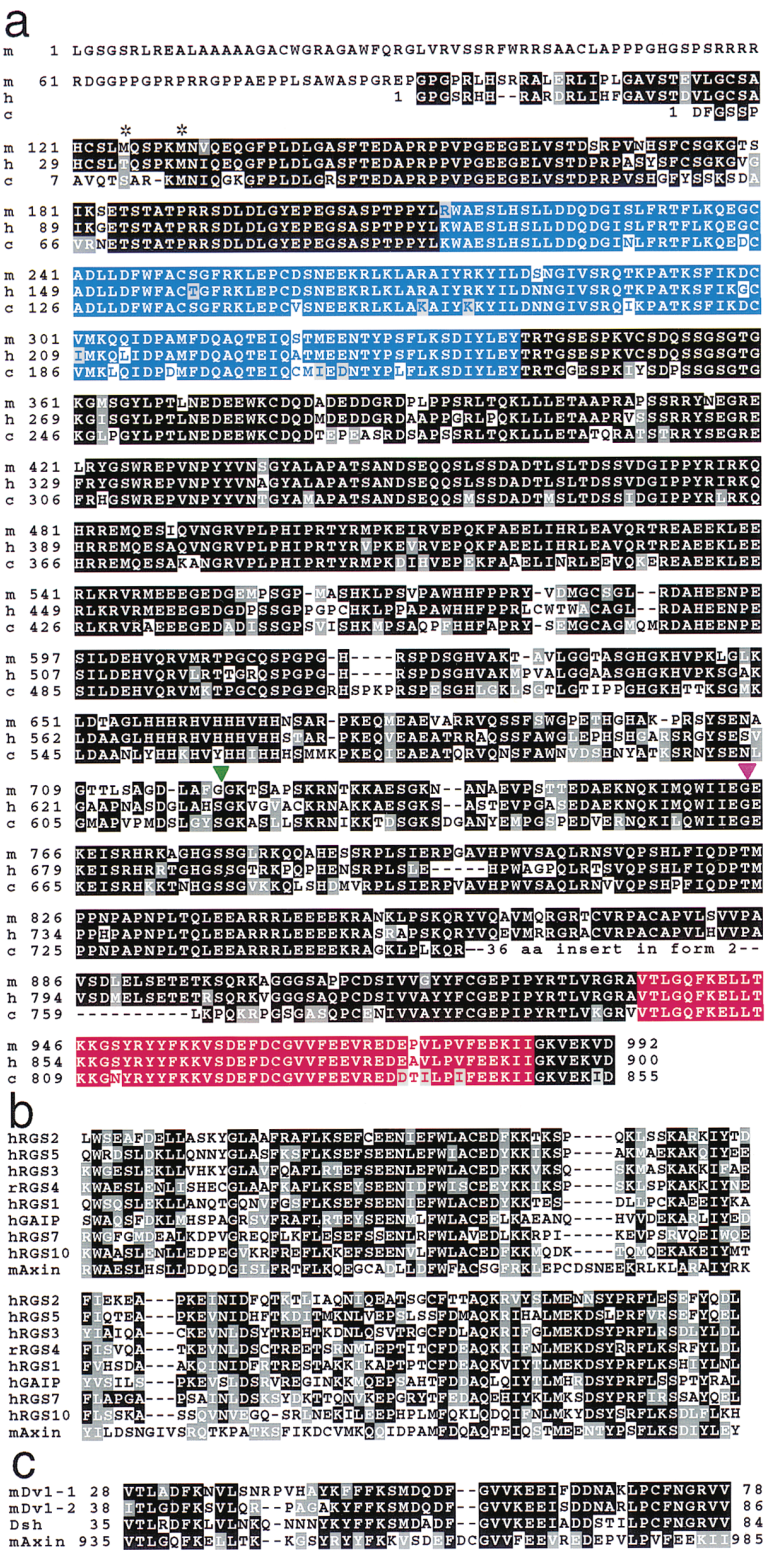

Figure 3. Amino Acid Sequence of Mouse Axin and Its Human and Chicken Homologs, and Similarity to RGS and Dsh Proteins

(a) Mouse (m), human (h), and chick (c) Axin sequences. Identical residues are highlighted in black, blue, or red and conserved residues in gray. RGS and Dsh homologies are highlighted in blue and red, respectively. The mouse sequence begins with the first residue encoded by the cDNA sequence, and the first two Met residues are indicated by asterisks. Also indicated is a 36 aa segment included in murine and human form 2. The mouse and human form 1 and the chicken sequence contain a bipartite NLS consensus at position 749 (K/R, K/R, 10 aa spacer, followed by 3 K/R in the next 5 residues), which is interrupted in form 2. In addition, mAxin includes a second NLS at aa 59. The murine sequence contains one consensus site for tyrosine phosphorylation (aa 192–199), and several for cAMP- and cGMP-dependent protein kinase, protein kinase C, casein kinase II, and GSK-3. The site of intron 6, where *Axin*^{Fu} contains a proviral insertion, is marked by a green triangle, and the site where exon 7 is interrupted in *Axin*^{Kb}, by a magenta triangle.

(b) Alignment of the RGS domains of Axin and 8 human or rat RGS proteins.

(c) Alignment of a 51 aa segment of Axin with a similar region in Drosophila Dsh and two murine homologs.

mRNA suggested that one function of *Axin* is to negatively regulate an early step in axis formation. Because the Axin sequence is highly conserved among amniotes, we reasoned that mAxin might be able to function in amphibian embryos, a system highly amenable to experimental manipulation of early axial development. Therefore, in vitro–synthesized *mAxin* mRNA (encoding aa 13–956, with an N-terminal Myc epitope tag) was injected into the dorsal, subequatorial region of 4-cell Xenopus embryos, which were scored at the tadpole stage for effects on axis formation (Figure 4a and Table 1). Most of these embryos developed with strong axial defects ranging from loss of anterior structures to complete lack of body axis, a phenotype characteristic of

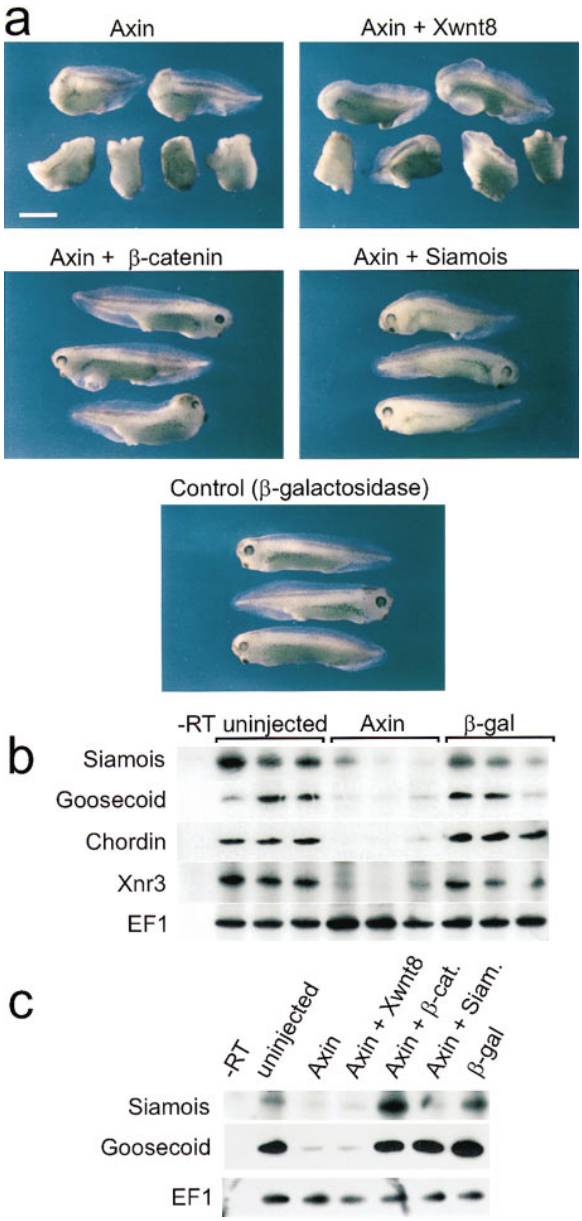

Figure 4. Dorsal Injection of *Axin* mRNA Ventralizes Xenopus Embryos

(a) Ventralization of Xenopus embryos by dorsal injection of *Axin*, and rescue by β-*catenin* or *Siamois* but not *Xwnt8*. Two nanograms of *Axin* mRNA, either alone or together with the other mRNA indicated, was injected into each of two dorsal blastomeres at the 4-cell stage. Embryos were evaluated at the tadpole stage (Table 1), and examples are shown. The amount of *Xwnt8* (20 pg), β-catenin (300 pg) or *Siamois* (100 pg) mRNA used was the minimal amount required to obtain full axis induction when each was injected alone in one ventral blastomere (see Figures 5a and 5b). Scale bar, 1 mm.
(b) Dorsal injection of *Axin* reduces expression of dorsal markers *Siamois*, *Goosecoid*, *Chordin*, and *Xnr3*, but not the ubiquitously expressed elongation factor *EF1*. Each column shows the RT-PCR analysis of a pool of uninjected embryos or embryos injected at the 4-cell stage with *Axin* or control β-*gal* mRNA (2 ng), and grown to early gastrulae. −RT, control experiments in which RNA from uninjected embryos was processed without reverse transcriptase.
(c) Dorsal coinjection of β-*catenin* with *Axin* restores expression of *Siamois* and *Goosecoid*, and coinjection of *Siamois* restores *Goosecoid* expression, while coinjection of *Xwnt8* has no effect. Note that the injected *Siamois* (not detected with the primers used in this assay) does not induce expression of endogenous *Siamois*.

completely ventralized embryos (Kao and Elinson, 1988). Control injections of β-*gal* mRNA had no effect. Embryos injected dorsally with *Axin* also showed a markedly reduced expression of the dorsal markers *Siamois*, *Goosecoid*, *Chordin*, and *Xnr3*, consistent with the observed ventralizing effects (Figure 4b). Forms 1 and 2 *Axin* mRNAs were equally active, and α-Myc staining showed that both proteins were similarly localized in a punctate pattern near the plasma membrane (data not shown).

Inhibition of Dorsal Axis Formation by Axin Is Mediated by the Wnt Signaling Pathway

The ventralizing effect of Axin could be due either to inhibition of Nieuwkoop Center activity, which requires the activation of the Wnt signaling pathway, or to perturbation of further downstream inductive processes, i.e., establishment of the Spemann organizer or BMP-dependent mesodermal patterning. To test whether Axin might exert its effects via the Wnt pathway, mRNA encoding Xwnt8, β-catenin, or Siamois was coinjected with *Axin* mRNA into the dorsal, subequatorial region. *Siamois* is a homeobox gene whose expression is specifically activated by Wnt signaling and which appears to mediate the effects of the Wnt pathway on axis formation (Lemaire et al., 1995; Carnac et al., 1996; Fagotto et al., 1997). Coinjection of *Siamois* or β-*catenin*, but not *Xwnt8*, overcame the ventralizing effect of *Axin*, rescuing normal axis formation in a large proportion of embryos (Figure 4a and Table 1) and restoring expression of dorsal markers (Figure 4c).

As *Xwnt8* or several downstream factors can induce a secondary dorsal axis when injected into the ventral side of the embryo, the ability of *Axin* to inhibit secondary axis formation was examined. Coexpression of Axin completely inhibited the axis-inducing activity of Xwnt8, Xdsh (a Xenopus Dsh homolog), and dominant-negative GSK-3, while it did not affect secondary axis formation by β-catenin or Siamois (Figures 5a and 5b). Thus, injection of *Axin* mRNA can block either normal or secondary dorsal axis formation in Xenopus embryos, apparently by interfering with signaling through the Wnt pathway at a level downstream of Wnt, Dsh, and GSK-3, and upstream of β-catenin and Siamois.

Expression of Axin Does Not Affect Other Downstream Pathways Involved in Axis Formation

Induction of the Spemann organizer can also be mimicked by Activin, a potent mesodermal inducer, which at high concentrations induces dorsal mesoderm. Axin did not inhibit the induction of *Goosecoid* by Activin in the ventral region of early gastrula embryos (Figure 5c) and had no effect on the formation of an ectopic blastopore lip or a partial secondary axis in Activin-injected embryos (Figure 5a). These results are consistent with the conclusion that Activin acts downstream of, or in parallel to, the Wnt pathway (Carnac et al., 1996; Wylie et al., 1996; Fagotto et al., 1997).

Axial patterning is also regulated further downstream by the antagonistic activity of factors secreted by the organizer (Noggin, Chordin, Follistatin) and the ventral mesoderm (BMPs). For instance, ventral expression of

Table 1. Frequency and Extent of Ventralization by Dorsal Injection of *Axin* mRNA, and rescue by β-*catenin* or *Siamois* but not *Xwnt8*

mRNAs injected	Ventralized (%)[a]	Average Dorso-Anterior Index	Number Analyzed
β-*galactosidase* (2 ng, control)	3	4.8	31
Axin (2 ng)	78	1.7	118
Axin + *Xwnt8* (10–20 pg)	90	1.9	67
Axin + β-*catenin* (300 pg)	29	4.3	35
Axin + *Siamois* (50–100 pg)	2	5.1	96

Both dorsal blastomeres of 4-cell embryos were injected in the subequatorial region. Dorso-Anterior Index (DAI) is a measure of axial development, where 5 is normal, 0 is completely ventralized, and >5 is hyperdorsalized (Kao and Elinson, 1988).
[a] DAI < 4 was considered significant ventralization.

Noggin, a natural inhibitor of BMPs, or a dominant-negative truncated BMP receptor (ΔBMPR) causes formation of an ectopic axis (Hogan, 1996). However, *Axin* failed to block the induction of a secondary axis, or the ectopic expression of the dorsal marker *Goosecoid*, by ventral injection of *Noggin* or Δ*BMPR* (Figures 5a and 5c). These results confirm that Axin acts specifically on the Wnt signaling pathway and does not perturb other pathways involved in early axial patterning.

Deletion of the *Axin* RGS Homology Region Creates a Dominant-Negative Mutant

To test the importance of the RGS domain, we injected Xenopus embryos with mRNA encoding Δ*RGS*, a mutant form of *Axin* in which the sequences encoding aa 252–351 were deleted. Dorsal injection of Δ*RGS* revealed that it had lost the ability to ventralize (only 4/44 embryos ventralized, average Dorso-Anterior Index ~5). Surprisingly, Δ*RGS* acted as a potent dorsalizer when injected ventrally, producing secondary axes (usually complete, including the most anterior head structures) in 87% of embryos (Figures 6a–6c). Δ*RGS* induced ectopic expression of several dorsal markers, including *Siamois*, consistent with an activation of the Nieuwkoop Center signaling pathway (Figure 6d). Ventral injection of wild-type *Axin* mRNA had no effect on development and did not induce ectopic expression of dorsal markers (Figures 6c and 6d). However, coinjection of *Axin* blocked the axis-inducing activity of Δ*RGS*, as did coinjection of *C-cadherin*, which binds to and inhibits signaling through β-catenin (Fagotto et al., 1996) (Figure 6c). Thus, ΔRGS has an effect opposite to that of Axin and appears to act through a dominant-negative mechanism to inhibit an endogenous Axin activity. The axial duplications induced by ΔRGS are reminiscent of those caused by loss-of-function *Axin* alleles in the mouse embryo, two examples of which are shown in Figures 6e–6g.

Discussion

Mutations at the *Fused* locus have been a subject of interest since the early days of mouse genetics because of their pleiotropic effects on a variety of developmental processes. The most remarkable abnormality seen in early postimplantation embryos homozygous for *Fused* alleles was the formation of ectopic axial structures, which led to the suggestion that this locus played a role in the determination of the embryonic axis (Gluecksohn-Schoenheimer, 1949). We have cloned and characterized the structure of the wild-type *Axin* gene (formerly called *Fused*), and a transgenic insertional mutant allele, $Axin^{Tg1}$. Two of the old mutant alleles, $Axin^{Fu}$ and $Axin^{Kb}$, have also been characterized (T. J. V. et al., submitted). The observation that the major *Axin* mRNA is disrupted in two different alleles that cause axial duplications suggested that the normal gene product plays a negative regulatory role at some step in axis formation. This hypothesis is supported by the observation that dorsal injection of *Axin* mRNA blocks axis formation in Xenopus embryos, while ventral injection of a dominant-negative form of *Axin* induces a complete secondary axis. Furthermore, coinjection of *Axin* with factors in the Wnt signal transduction pathway shows that Axin exerts its effects on axis formation by inhibiting the Wnt pathway. These studies reveal that Axin is a novel regulatory protein for a signaling pathway known to trigger an early step in embryonic axis formation in amphibians. Our results, together with the phenotype of *Axin* mutant embryos, also imply that the Wnt signaling pathway plays an early and critical role in axis formation in mammalian embryos.

The *Axin* Gene

The *Axin* gene encodes a major mRNA of 3.9 kb, which is expressed ubiquitously in embryos and adult tissues. In the $Axin^{Tg1}$ allele, exon 2 is replaced with ~600 kb of transgene DNA, preventing expression of the major mRNA. Two spontaneous *Axin* alleles, $Axin^{Fu}$ and $Axin^{Kb}$, are each caused by the insertion of an IAP provirus, within intron 6 or exon 7, respectively (T. J. V. et al., submitted). Many of the similarities and differences between the phenotypic effects of *Axin* alleles can be explained by the nature of these mutations. While the provirus in the $Axin^{Fu}$ intron is efficiently spliced out, resulting in near-normal levels of the 3.9 kb mRNA, the provirus in $Axin^{Kb}$ precludes the production of the normal mRNA. Therefore, the similar recessive defects and embryonic lethality seen in $Axin^{Tg1}$ and $Axin^{Kb}$ (but not $Axin^{Fu}$) embryos can be attributed to the inability of either allele to encode the major *Axin* mRNA. On the other hand, the dominant effects of $Axin^{Fu}$ and $Axin^{Kb}$, which are not seen in $Axin^{Tg1}$ mice, appear to be a specific consequence of abnormal transcripts associated with the proviral insertions in these alleles (T. J. V. et al., submitted).

Two genomic cosmids encoding part of a human *Axin* homolog map to chromosome 16p13.3 (Accession nos. Z69667 and Z81450). Examination of the human genetic map did not reveal any genetic traits (e.g., developmental or neurological defects) that seem likely to be associated with *Axin* mutations.

Figure 5. Ability of *Axin* to Block Ectopic Axis Formation

(a) Ventral coinjection of *Axin* mRNA inhibits ectopic axis formation by upstream components of the Wnt pathway (Xwnt8, Xdsh, and dnGSK-3), but not by β-*catenin* or *Siamois*, nor by *Activin*, *Noggin* or Δ*BMPR*. mRNA encoding the indicated dorsalizing factor was injected subequatorially in one ventral blastomere at the 4–8 cell stage, with or without 1 ng *Axin*, and embryos were examined for axial duplications at the late neurula–tailbud stage. The fraction of embryos with duplicated axes is indicated above each bar. mRNAs were injected in the minimal amounts needed to induce ectopic axes at high frequency: 10–20 pg *Xwnt8*, 1.5 ng *Xdsh*, 2 ng *dnGSK-3*, 300 pg β-*catenin*, 100 pg *Siamois*, 7.5 pg *Activin*, 200 pg *noggin*, or 1 ng Δ*BMPR* (Fagotto et al., 1997). *Activin*-induced secondary axes were generally very incomplete. Higher amounts of *Activin* mRNA lead to uninterpretable phenotypes.

(b) Examples of injected embryos. Scale bar, 2 mm.

(c) Coinjection of *Axin* mRNA inhibits induction of the dorsal marker *Goosecoid* by *Xwnt8*, but not by *Activin*, *Noggin*, or Δ*BMPR*. Ectopic expression of *Goosecoid* in the ventral half of early gastrulae (stage 10 1/2) was analyzed by RT-PCR. Dorsal (D) and ventral (V) halves of uninjected embryos served as positive and negative controls (ctrl) for normal expression of *Goosecoid*.

The predicted Axin protein includes regions of similarity to two families of proteins involved in signal transduction, RGS and Dsh. Several proteins containing an RGS domain (De Vries et al., 1995; Druey et al., 1996; Koelle and Horvitz, 1996) bind G_α subunits (De Vries et al., 1995; Dohlman et al., 1996) and serve as GTPase-activating proteins (GAPs) for the G_i subfamily of G_α subunits, thus inhibiting signal transduction by accelerating the rate of the intrinsic GTPase (Berman et al., 1996; Hunt et al., 1996; Watson et al., 1996). At least 17 mammalian RGS proteins have been identified, and it is not yet clear if they all serve as GAPs for members of the $G_{i\alpha}$ subfamily, or if some serve as GAPs for other G_α subfamilies or perform other functions (Dohlman and Thorners, 1997). While the Axin RGS domain contains similar residues at many positions of amino acid conservation among RGS proteins, it differs at other conserved positions and contains two short inserts not present in other RGS domains (Figure 3b). Thus, whether the Axin RGS is a G_α GAP remains to be determined. Axin also displays homology to a 50 aa sequence within a conserved N-terminal region of Drosophila and vertebrate Dsh proteins. The importance of this sequence is unknown, although deletion of a 165 aa segment including this sequence rendered the Drosophila protein inactive (Yanagawa et al., 1995).

Axin and Wnt Signaling in Establishment of the Amphibian Nieuwkoop Center

Based on its ability to block ectopic axis formation in Xenopus embryos by Xwnt8, Dsh or dnGSK-3, Axin appears to negatively regulate signaling through the Wnt pathway, either at the level of GSK-3 or further downstream. Furthermore, its inability to block the effects of β-catenin or Siamois suggests that Axin acts upstream of β-catenin (Figure 7). GSK-3 is a Ser/Thr protein kinase whose activity results in the phosphorylation of β-catenin and its consequent degradation. GSK-3 may directly phosphorylate β-catenin (Yost et al., 1996), or its effects on β-catenin may be mediated by the phosphorylation of adenomatous polyposis coli (APC), which associates with β-catenin and GSK-3 (Miller and Moon, 1996). When GSK-3 activity is inhibited, either naturally through the activity of Wnt and Dsh, or experimentally by dnGSK-3, the level of cytosolic β-catenin is increased, downstream effectors are activated, and Nieuwkoop Center activity results. Thus, both Axin and GSK-3 negatively regulate formation of the Nieuwkoop Center by inhibiting the same signaling pathway.

One hypothetical mechanism for the similar action of Axin and GSK-3 (Figure 7) is suggested by the observation that Axin can bind to the Ser/Thr protein phosphatase PP2A (unpublished data). If Axin were to inhibit PP2A activity, and if PP2A dephosphorylated the GSK-3 substrate(s) involved in Wnt signaling, then the overexpression of Axin would increase the level of phosphorylation of this substrate. Thus, even if GSK-3 activity were reduced by Dsh or dnGSK-3, the substrate would remain

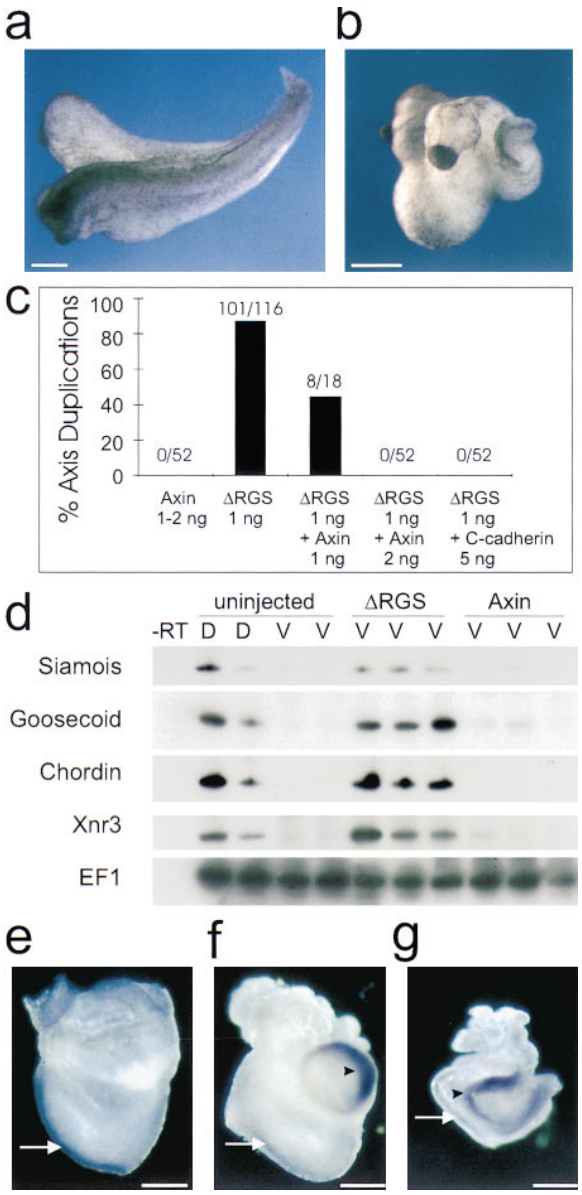

Figure 6. Axis Duplications in Xenopus Embryos Injected Ventrally with Δ*RGS* and in Mouse Embryos Homozygous for the Loss-of-Function *Axin*^{Tg1} Allele

(a and b) Xenopus embryos with axis duplications caused by injection of 2 ng Δ*RGS* in one ventral blastomere (a) or 1 ng Δ*RGS* in two ventral blastomeres (b). The embryo in (b) is also strongly dorsalized. Scale bars, 0.5 mm.

(c) Frequency of axis duplications in embryos injected with *Axin*, Δ*RGS*, or Δ*RGS* together with *Axin* or *C-cadherin*.

(d) Ectopic expression of dorsal markers in embryos injected ventrally with Δ*RGS*. Each column shows the RT-PCR analysis of the dorsal (D) or ventral (V) halves of a pool of embryos. In uninjected embryos, *Siamois*, *Goosecoid*, *Chordin*, and *Xnr3* are expressed dorsally. Ventral injection of Δ*RGS*, but not *Axin*, induces ectopic expression of the four dorsal markers.

(e–g) Lateral view of a normal E7.5 mouse embryo (e) and two E8.5 *Axin*$^{Tg1/Tg1}$ embryos with axial duplications (f and g), visualized by in situ hybridization to *HNF-3*β, a marker of anterior axial mesoderm (Sasaki and Hogan, 1994). White arrows, primary axes; black arrowheads, ectopic axes. Scale bars, 0.2 mm.

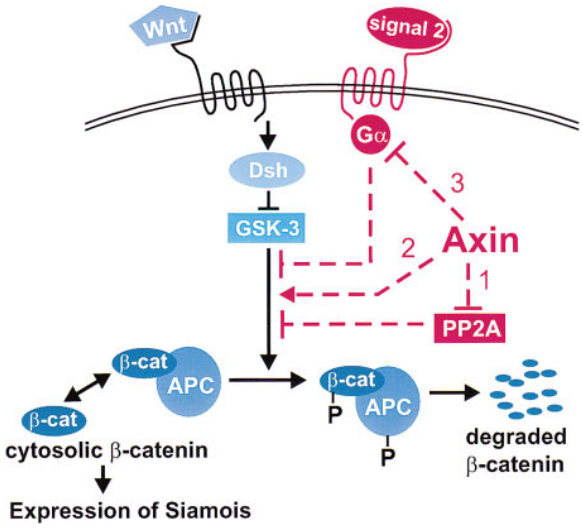

Figure 7. Model for the Inhibitory Effect of Axin on Wnt Signal Transduction

Established components of the Wnt pathway in the Nieuwkoop Center are indicated by blue symbols and solid black arrows, and positions where Axin might inhibit the pathway are indicated by red symbols and dashed arrows. GSK-3 promotes the degradation of β-catenin, while Wnt signals inhibit GSK-3 (via Dsh) and lead to accumulation of cytosolic β-catenin and expression of Siamois. Axin blocks the stimulation of this pathway by Wnt, Dsh, or dominant-negative GSK-3 but not by overexpression of β-catenin or Siamois. Three alternative hypotheses are illustrated: (1) Axin might inhibit a protein phosphatase (PP2A) that may otherwise dephosphorylate substrates of GSK-3; (2) Axin might stimulate the activity of GSK-3 through an unknown mechanism; (3) Axin might inhibit, via its RGS domain, the transmission of a second signal (signal 2) involving a G-protein-coupled receptor, which would otherwise stimulate the Wnt pathway downstream of GSK-3. See text for further details.

highly phosphorylated, which could explain why Axin appears epistatic to these two proteins. Alternatively, Axin might stimulate GSK-3 activity by another mechanism that can overcome its inhibition by Dsh or dnGSK-3. A third possibility is suggested by the demonstrated importance of the RGS domain for the ability of Axin to inhibit Wnt signaling and to ventralize the frog embryo. There is no evidence for a G_α protein in the Wnt pathway, and the evidence that Axin functions downstream of Dsh and GSK-3 would argue against an activity at the level of a hypothetical G-protein coupled to a Wnt receptor. However, the Axin RGS domain, if it is a functional G_α GAP, might inhibit a second signaling pathway involving a G_α protein, which converges with and stimulates the Wnt pathway at a level downstream of GSK-3. In order to explain the ability of high levels of Axin to block the Wnt pathway, this second signal would have to be required for some step in the transmission of the Wnt signal (e.g., cytosolic accumulation of β-catenin) in the early frog embryo.

Not only does deletion of the RGS region abolish the axis-inhibiting properties of Axin, but it creates a dominant-negative form that can induce an ectopic dorsal axis. An obvious implication is that the amphibian embryo contains a protein homologous to Axin, which normally serves to inhibit ectopic axis formation, and whose

activity is blocked by ΔRGS. This conclusion is consistent with the observation that loss-of-function *Axin* mutations in the mouse cause the development of ectopic axial structures. Thus, not only is Axin *capable* of inhibiting signaling through the Wnt pathway when it is overexpressed, but this appears to be a natural function of the protein. Presumably, the levels of endogenous Axin in the embryo are high enough to prevent signaling in the absence of a strong upstream signal (e.g., a Wnt ligand), but low enough to allow signaling when the pathway is activated by a natural or experimental stimulus. While the dominant-negative mechanism of ΔRGS remains to be determined, one possibility is that it competes for binding to a protein to which Axin normally binds, but fails to perform the function carried out by the RGS domain.

Axin, Wnts, and Axis Formation in Mammalian Embryos

The ability of Axin to regulate an early step in Xenopus axis formation mediated by the Wnt signaling pathway, together with the occurrence of axial duplications in *Axin* mutant mouse embryos, argues strongly that an evolutionarily conserved mechanism involving the Wnt pathway plays a critical role in embryonic axis formation in mammals as well as amphibians. In Xenopus, signaling through components of the Wnt pathway is thought to confer Nieuwkoop Center activity to a group of dorsal–vegetal blastomeres, which consequently secrete factors that induce formation of the organizer by the adjacent dorsal mesoderm. In the mouse, the equivalent of the organizer is the node, a group of cells at the anterior end of the primitive streak (Beddington, 1994). Fate mapping of the prestreak embryo indicates that the node derives from epiblast cells at the future posterior pole (Lawson et al., 1991). While the location of the Nieuwkoop Center equivalent (i.e., the cells that induce formation of the organizer) is unknown, the most likely locations are the posterior extraembryonic or embryonic ectoderm proximal to the cells fated to form the node, or the overlying visceral endoderm (Conlon and Beddington, 1995; Bachvarova, 1996).

We propose that during normal mouse embryogenesis, the Wnt signaling pathway is activated in a discrete region of the early egg cylinder, by the localized production of a Wnt or another stimulus that activates downstream components of the Wnt pathway. This localized signal establishes the A–P axis of the embryo, and the responding cells constitute a Nieuwkoop Center equivalent (NCE). According to this model, the ubiquitously expressed Axin serves to attenuate the response to this signal, so that cells in regions of the embryo not exposed to the signal, or exposed to low levels, do not respond, and only a single NCE is formed at the appropriate developmental stage. In mutant embryos lacking Axin, the Wnt pathway could be inappropriately activated and multiple NCE would result. The presence of multiple, discrete axes in mutant embryos could be explained by lateral inhibition mechanism, whereby once an NCE or organizer is formed, it restricts axis formation in adjacent regions of the embryo (Cooke, 1972; Khaner and Eyal-Giladi, 1989; Ziv et al., 1992).

The hypothesis that the Wnt pathway is important for mammalian axis formation is supported by the observation that ubiquitous expression of Cwnt-8C in transgenic embryos causes axial duplications (R. Beddington, personal communication). Also consistent is the failure of β-*catenin* null mutant mouse embryos to undergo gastrulation (Haegel et al., 1995). No *Wnt* mutants produced so far have affected early events in axis formation, and the expression of Wnts in prestreak mouse embryos has not been reported (Parr and McMahon, 1994; Moon et al., 1997), leaving open the question of what signal triggers this pathway in the normal embryo. In the prestreak chick embryo, on the other hand, *Cwnt-8c* is expressed in the posterior marginal zone, a region possibly equivalent to the Nieuwkoop Center (Hume and Dodd, 1993).

While the earliest stage at which axial duplications originate in *Axin* mutant embryos has not been defined, they appear to occur by the stage at which NCE activity would be expected, i.e., the early egg cylinder. Some of earliest $Axin^{Ki/Ki}$ embryos examined (E7.0–E7.5) contained a bifurcated epiblast with two discrete amniotic cavities or two primitive streaks (Gluecksohn-Schoenheimer, 1949; Tilghman, 1996). At later stages, in addition to duplication of anterior axial mesoderm (e.g., Figures 7f and 7g), duplication of the allantois (derived from posterior streak) has been observed in both $Axin^{Tg1}$ and $Axin^{Ki}$ embryos (Gluecksohn-Schoenheimer, 1949; Perry et al., 1995). In contrast, transplantation of the node to an ectopic site resulted in formation of ectopic notochord, neural tube, and somites, but not allantois (Beddington, 1994). Therefore, structures derived from the most anterior and posterior portions of the primitive streak are duplicated in *Axin* mutant embryos, suggesting that the duplications precede the formation of the streak. The extent of anterior neuroectodermal development of the ectopic axes in *Axin* mutant embryos remains to be further studied using molecular markers: "complete twinning" as well as partial duplications were reported in $Axin^{Ki}$ embryos, but ectopic forebrain structures have not yet been documented in $Axin^{Tg1}$ or $Axin^{Kb}$ embryos.

In addition to their effects on axis formation, *Axin* mutations cause neuroectodermal defects (incomplete closure, malformation or truncation of the head folds), cardiac defects and embryonic lethality in homozygotes. It remains to be determined whether these abnormalities are also due to defective regulation of Wnt signaling pathways. Anterior truncations have been observed in transgenic mouse embryos that ubiquitously expressed Cwnt-8C (R. Beddington, personal communication) and in frog embryos ectopically expressing Xwnt-8 after the midblastula transition (Christian and Moon, 1993). Therefore, inappropriate Wnt signaling may also account for the neuroectodermal defects in *Axin* mutant embryos. Another interesting question that can now be addressed is the molecular basis of the dominant defects seen in $Axin^{Fu}$, $Axin^{Kb}$, and $Axin^{Ki}$ heterozygotes, which were attributed to gain-of-function mutations (Greenspan and O'Brien, 1986). Analysis of $Axin^{Fu}$ and $Axin^{Kb}$ suggests that their similar dominant effects may be mediated by C-terminally truncated Axin proteins that are potentially encoded by abnormally spliced transcripts (T. J. V. et al., submitted). It is possible that these abnormal Axin proteins perturb Wnt signaling pathways involved in brain and skeletal development.

Experimental Procedures

Isolation and Characterization of Mouse *Axin* cDNA and Genomic Clones

Primers for *mAxin* are named for the position of their 5′ terminus in the cDNA (+ numbers) or in upstream genomic DNA (− numbers). F indicates a forward and R a reverse primer. Sequences are listed 5′ to 3′. A 315 bp NotI-StuI genomic probe at the left of the transgene insert (Perry et al., 1995) was used to screen an E8.5 mouse embryo cDNA library, yielding one clone (N7) that was partially colinear with the probe. Using a fragment of N7, 12 more clones were isolated from a WEHI-3 cDNA library (Stratagene). Additional *Axin* cDNA clones were obtained from various libraries using probes from clone N7, but none extended as far 5′ as N7. 5′ RACE was performed using kidney cDNA, AP1 primer (Advantage cDNA PCR kit, Clonetech) and mAxin primer +98R (caccagccctctctggaacc). The RACE product extending farthest 5′ was colinear with clone N7 and contained 4 more bp at the 5′ end. To estimate the ratio of forms 1 and 2 mRNA, total RNA was reverse transcribed using oligo-dT primer, and the cDNA was amplified using +2289F (gagggagagaaggagatcag) and +2744R (gtagctccccttcttggttag).

Intron/exon structure was determined by restriction mapping and sequencing of genomic subclones and products of long template PCR using primers in adjacent exons. Previously isolated clones (Perry et al., 1995) included exons 1–3, and clones including exons 6–10 were isolated from a strain 129 library. The remaining region was isolated by long template PCR (Boehringer Mannheim) using primers at different positions in the cDNA.

Isolation of Human and Chick Axin cDNA Clones

Database searches revealed ESTs (T07178, R75687, T30966, T32063, T15895, and T72547) representing the 3′ region of a human *Axin* homolog (*hAxin*). Additional clones were isolated by 5′ and 3′ RACE using human placenta RNA (Clonetech Marathon RACE kit). A stage 12–15 chick embryo cDNA library (a gift of Dr. D. Wilkinson) was screened with a *mAxin* probe and four clones containing the same insert in both orientations were isolated. The 3131 bp *cAxin* cDNA sequence contains a polyadenylation signal near the 3′ end, but is shorter than the *cAxin* mRNA (~3.6 kb), even after accounting for a poly(A) tail, and thus may lack part of the 5′ UTR.

Sequence Analysis

Database searches were conducted using BLAST, and sequence alignments using ClustalW and BOXSHADE. Other RGS sequences are: Q08116 (hRGS1), P41220 (hRGS2), U27768 (hRGS3), U27768 (rRGS4), D31257 and R35272 (hRGS5), U32328 (hRGS7), H87415 (hRGS10), X91809 (hGAIP).

Northern Blot and *In Situ* Hybridization

Twenty micrograms of total RNA from embryos, adult tissues, or ES cells was run on a formaldehyde-agarose (1.2%) gel, blotted to Genescreen plus (NEN-Dupont), and hybridized with a ^{32}P-DNA probe containing the entire *mAxin* cDNA sequence, as described (Perry et al., 1995). For *in situ* hybridization (Wilkinson, 1992), an anti-sense probe was produced by T7 transcription of a HindIII-SacI fragment of *mAxin* cDNA (bp 765–1065, within exon 2) in pBluescript. A sense probe did not produce a significant signal (data not shown). The *HNF-3*β probe was produced from clone c21 (Sasaki and Hogan, 1993).

Constructs for Xenopus Injection

Axin cDNAs were cloned into the XhoI site of pCS2+MT (Rupp et al., 1994). The experiments shown employed vector MTPA2, which includes *Axin* form 2 (bp 37 to 3310) and encodes aa 13 to the normal C terminus. Translation initiates in the N-terminal Myc tag. Three other *Axin* vectors (MTPA1, MTFU1, and MTFU2) were similarly active at ventralization: MTPA1 was identical to MTPA2 except it was derived from a form 1 cDNA. MTFU1 and MTFU2 were identical to MTPA1 and MTPA2, except they contained a longer 3′ UTR (bp 3311–3731). ΔRGS was derived from MTFU1 by deleting cDNA bp 754–1053. *Siamois* was cloned in pCS2+MT (Fagotto et al., 1997) and β-*catenin* (C-terminally HA-tagged) in pSP36 (Funayama et al., 1995). Other expression vectors were: *Xwnt8* and *Noggin* (gift of Dr. R. Harland), *Activin* (Dr. D. Melton), Δ*BMPR* (Dr. A. Susuki), *dnGSK-3* (GSK-3-K→R, Dr. D. Kimelman) and *Xdsh* (Dr. U. Rothbacher). GSK-3-K→R is a mutant of Xenopus GSK-3β without kinase activity (Pierce and Kimelman, 1995). ΔBMPR is a truncated BMPR lacking the kinase domain (Suzuki et al., 1994).

Xenopus Injections and Analysis of Phenotypes

mRNAs were synthesized and injected as previously described (Fagotto et al., 1996, 1997). For RT–PCR, mRNA was extracted from whole early gastrulae (stage 10 1/2) or dissected dorsal and ventral halves, and specific mRNAs were detected as described (Fagotto et al., 1997). *Siamois* primers were: 5′ ttgggagacagacatga (corresponds to part of the 5′ UTR, present in the endogenous mRNA but not the injected synthetic *Siamois* mRNA) and 3′: tcctgttgactgcagact. Other primers were as described (Fagotto et al., 1997). For immunofluorescence, Myc-tagged Axin was detected in frozen sections of early gastrulae using anti-Myc antibody 9E10.2, as described (Fagotto et al., 1996; Fagotto and Gumbiner, 1994).

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GenBank Accession Numbers

The GenBank accession numbers for the protein sequences reported here are as follows: mouse Axin, AF009011; chick Axin, AF009012; human Axin, AF009674.

Note Added in Proof

The observations cited above as R. Beddington, personal communication, are now in press: Pöpperl, H., Schmidt, C.W., Wilson, V., Hume, C., Krumlauf, R., and Beddington, R.S.P. (1997). Misexpression of Cwnt8C in the mouse induces an ectopic embryonic axis and causes a truncation of the anterior neuroectoderm. Development, in press.