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Mouse Axin and Axin2/Conductin Proteins Are Functionally Equivalent In Vivo

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Axin is a central component of the canonical Wnt signal transduction machinery, serving as a scaffold for the β-catenin destruction complex. The related protein Axin2/Conductin, although less extensively studied, is thought to perform similar functions. Loss of *Axin* causes early embryonic lethality, while *Axin2*-null mice are viable but have craniofacial defects. Mutations in either gene contribute to cancer in humans. The lack of redundancy between *Axin* and *Axin2* could be due to their different modes of expression: while Axin is expressed ubiquitously, Axin2 is expressed in tissue- and developmental-stage-specific patterns, and its transcription is induced by canonical Wnt signaling. Alternatively, the two proteins might have partially different functions, a hypothesis supported by the observation that they differ in their subcellular localizations in colon epithelial cells. To test the functional equivalence of Axin and Axin2 in vivo, we generated knockin mice in which the *Axin* gene was replaced with Myc-tagged Axin or Axin2 cDNA. Mice homozygous for the resulting alleles, $Axin^{Ax}$ or $Axin^{Ax2}$, express no endogenous Axin but express either Myc-Axin or Myc-Axin2 under the control of the *Axin* locus. Both $Axin^{Ax/Ax}$ and $Axin^{Ax2/Ax2}$ homozygotes are apparently normal and fertile, demonstrating that the Axin and Axin2 proteins are functionally equivalent.

Axin is a central component of the canonical Wnt signal transduction machinery, serving as a scaffold for the β-catenin destruction complex (23, 31, 36, 45, 52). Axin has specific binding sites for many proteins involved in Wnt signal transduction, including β-catenin, glycogen synthase kinase 3 (GSK3), CKI, adenomatous polyposis coli (APC), Dvl, LRP, and protein phosphatase 2A (Fig. 1A) (23, 31). Its key function in this pathway is to bring together β-catenin and the protein kinases CKI and GSK3, thus promoting the phosphorylation and consequent destruction of β-catenin. In the presence of a Wnt signal, this function is overcome, allowing β-catenin to accumulate and enter the nucleus (9, 13). The mechanism by which a Wnt signal leads to the inactivation of the Axin complex is not entirely clear, but it is thought to involve the binding of Axin to the Wnt coreceptor LRP as well as to Dvl (7, 34, 42, 44, 45). This results in the dephosphorylation of Axin, leading to a decrease in its affinity for β-catenin, and in a decrease in the level of Axin (20, 47, 48). Axin also enters the nucleus and appears to play a role in the nuclear-cytoplasmic shuttling of β-catenin (8, 46).

The major product of the *Axin* gene is a protein of 832 or 868 amino acids (depending on alternative splicing) containing two highly conserved domains (52): the RGS domain, which encompasses the binding site for APC (16, 18), and the DIX domain, a region of homology with Dvl proteins that is implicated in the binding of Axin to Dvl as well as in homodimerization (10, 17, 22, 24, 29, 40). (The open reading frame of Axin cDNA potentially encodes proteins of 956 and 992 amino acids, but proteins of this length have not been detected, and the major protein begins at codon Met-125 of the original sequence.) Mutant embryos lacking Axin die at embryonic day 9.5 (E9.5) with abnormalities including truncation of the forebrain, neural tube defects, and embryonic axis duplications (14, 35). Axin is expressed ubiquitously during embryogenesis, and the presence of axis duplications in its absence is thought to be a consequence of the abnormal accumulation of β-catenin, mimicking a Wnt signal, in the early embryo.

Axin2 (also known as Conductin) is 44% identical to Axin and shares the RGS and DIX domains (Fig. 1A) (5, 39, 49) as well as the binding sites for β-catenin, GSK3, Diversin, and Smad3 (Fig. 1A) (5, 11, 39, 49). While Axin2 has been studied less extensively than Axin, most data suggest that they are similar in function. Thus, when overexpressed in cultured cells, both proteins reduce the levels of β-catenin and the expression of Wnt target genes, and when expressed in frog embryos, both inhibit the development of dorsal structures (5, 19, 37, 49, 52). However, they are not fully redundant in vivo, as Axin2 is clearly unable to compensate for the absence of Axin in *Axin*-null embryos (52). Furthermore, humans with heterozygous germ line *AXIN2* mutations have familial tooth agenesis (25), while mice homozygous for loss of *Axin2* have skull abnormalities (51), indicating that *Axin* does not fully substitute for the lack of *Axin2*. In addition, deletions or mutations in *AXIN* or *AXIN2* have been observed in a number of types of tumors, including colorectal cancer and hepatocellular carcinoma, indicating that each gene is a tumor suppressor and that neither gene can replace the other for this function (30, 38, 43).

One reason for the lack of full redundancy may be the different expression patterns of *Axin* and *Axin2*. While Axin mRNA is ubiquitous (52), Axin2 is expressed in tissue- and developmental-stage-specific patterns, due (at least in part) to its transcriptional upregulation by canonical Wnt signaling (4, 21, 27). Thus, Axin2 potentially constitutes a negative-feed-

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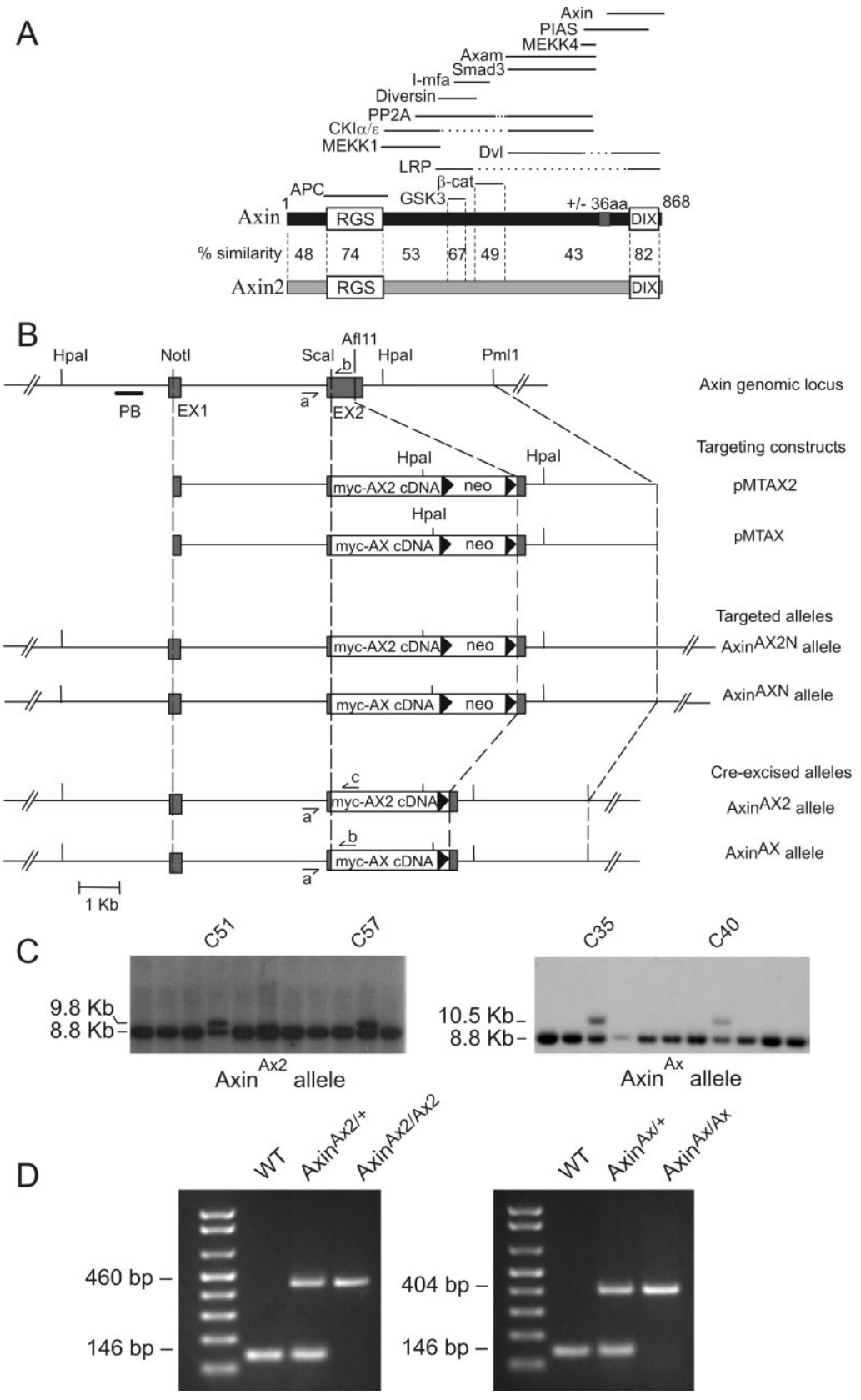
A
Axin
PIAS
MEKK4
Axam
Smad3
I-mfa
Diversin
PP2A
CKIα/ε
MEKK1
Dvl
LRP
β-cat
APC
GSK3
1
Axin
RGS
+/- 36aa
868
DIX
% similarity
48
74
53
67
49
43
82
Axin2
RGS
DIX
B
HpaI
NotI
ScaI
Afl11
b
HpaI
PmlI
PB
EX1
a
EX2
Axin genomic locus
Targeting constructs
HpaI
HpaI
myc-AX2 cDNA
neo
pMTAX2
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myc-AX cDNA
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Targeted alleles
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Axin Ax allele
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AxinAx2/+
AxinAx2/Ax2
460 bp
146 bp
WT
AxinAx/+
AxinAx/Ax
404 bp
146 bp

back mechanism regulating the response to Wnt signals (21, 27, 32, 50). In the mouse embryo, the inability of Axin2 to compensate for the lack of Axin might be explained simply by the fact that Axin2 is not expressed in every cell. However, the two proteins might also differ significantly in function, a possibility supported by their different subcellular localizations in at least some cell types (3). If the functional differences were subtle, they might not be detected using assays that involve overexpression; since the normal level of Axin is extremely low (26), the abnormally high levels of expression in these assays could mask a functional difference.

To address the question of functional redundancy in a more physiological way, we generated knockin mice in which the *Axin* gene was replaced with an Axin2 cDNA sequence. This gene replacement strategy is similar to those used successfully to compare the functions of other paralogous gene pairs (6, 12, 15, 33, 41). The main advantage of this approach over in vitro assays or other in vivo functional assays (e.g., transgenic rescue) is that targeting of a cDNA to the appropriate genetic locus results in a normal pattern and level of expression. In animals homozygous for the resulting allele, $Axin^{Ax2}$, there will be no Axin, but Axin2 will be expressed ubiquitously from the *Axin* locus at a physiological level, as well as in its normal tissue-specific pattern from the unmodified *Axin2* locus. Thus, the total level of Axin plus Axin2 expression in any cell type should be unchanged from that of the wild type. The ability of these mice to develop and survive normally thus tests the capacity of Axin2 to replace Axin in vivo.

MATERIALS AND METHODS

Generation of $Axin^{AX2}$ and $Axin^{AX}$ mice. Targeting vectors pMTAX2 and pMTAX were constructed for the knockin of myc-Axin2 cDNA and myc-Axin cDNA into the *Axin* locus. Using pBluescriptIISK (Stratagene) as the backbone, a series of fragments was subcloned into the polylinker. First, a 4.7-kb KpnI-ScaI fragment of the mouse *Axin* gene (containing intron 1 and parts of exons 1 and 2, including the exon 2 splice acceptor) was inserted into the KpnI site. Next, a 3.2-kb AflII-PmlI fragment (containing the exon 2 splice donor and part of intron 2) was added to the XbaI site, and a floxed PGK-neo cassette was added to the adjacent SpeI site. Finally, mouse Axin and Axin2 cDNAs with N-terminal Myc tags were excised from the vector pCS2-MT (10) using ClaI and NotI, their ends were blunted, and the blunt-ended cDNAs were cloned into the ClaI site of pBluescriptIISK. Axin cDNA sequences started at codon Met^{125} of the published sequence (52), and Axin2 sequences started at Met^1.

The targeting vectors were linearized with NotI and electroporated into CSL3 embryonic stem (ES) cells, which were selected with 0.35 mg/ml G418. DNA was digested with HpaI and screened by Southern blotting with a 479-bp probe, PB (Fig. 1B), generated by PCR of genomic DNA with primers 5′-CTTCTAATG GTATGAGGCTG-3′ and 5′-GCATCTGCACTTGCCATCTAC-3′. The targeting frequencies were 5/150 for pMTAX2 and 7/180 for pMTAX. ES cell clones were microinjected into C57BL/6J host blastocysts, and the PGK-neo cassette was excised by mating chimeric males to a β-actin–Cre transgenic line (28).

PCR genotyping. The following primers were used for genotyping. AXL1 (5′-GGACCACCTTTCCTAATCCTTG-3′) and MTAXR1 (5′-AACCCTGCTC CTGGACATTC-3′) amplify the wild-type (146-bp) and the $Axin^{AX}$ (404-bp) alleles, while AXL1 in combination with MTCONR1 (5′-TGGGATCTGAAG GAGAGTCAC-3′) detects the $Axin^{AX2}$ allele (460 bp) at an annealing temperature of 56.5°C.

Immunoblot analysis of embryonic tissue. Embryos were dissected at E10.5 and homogenized as described previously (10). The anti-myc antibody (Ab-1) was from Calbiochem, and the two anti-Axin polyclonal antibodies were provided by David Virshup (antiserum DV, raised against full-length mouse Axin) and Francois Fagotto (antiserum FF, raised against amino acids 406 to 685 of mouse Axin).

RESULTS AND DISCUSSION

To generate mice in which Axin is replaced by Axin2, we inserted a mouse Axin2 cDNA in place of exon 2 of the *Axin* gene (Fig. 1B). The resulting $Axin^{Ax2}$ allele cannot encode Axin but should express Myc-tagged Axin2 from the *Axin* locus. As a control, we generated a second allele, $Axin^{Ax}$, in which Axin cDNA was inserted into the *Axin* locus. Although *Axin* normally encodes two isoforms that differ by the presence or absence of 36 amino acids (52), a cDNA can encode only a single isoform, and we used form 1 Axin, which lacks the 36 amino acids. There is only one known isoform of Axin2.

Correctly targeted ES cell lines were obtained (Fig. 1B and C) and used to generate germ line chimeric mice. Heterozygotes for both alleles appeared normal and were mated to produce homozygotes, which were identified by Southern blotting and PCR (Fig. 1). $Axin^{Ax2/Ax2}$ and $Axin^{Ax/Ax}$ homozygotes were found in the expected proportions and also appeared normal. Both sexes were fertile and had an apparently normal life span. No premature deaths, obvious behavioral defects, or overt tumors were observed among approximately 50 $Axin^{Ax2}$ homozygotes over a period of 18 months. Histological analysis on a number of organs (liver, lungs, kidney, small and large intestines, stomach, heart, and spleen), including several where Wnt signaling is known to be important for development, revealed no abnormalities (data not shown). Of course, we cannot rule out the possibility that mice expressing only Axin2 have subtle defects yet to be detected.

As a more stringent test, we crossed $Axin^{Ax2}$ mice to those carrying the $Axin^{Tg1}$-null allele (52), thus generating $Axin^{Ax2/Tg1}$ compound heterozygotes, in which the level of Axin2 should be only half that in $Axin^{Ax2/Ax2}$ homozygotes. If Axin2 were less active than Axin at performing their shared functions, the $Axin^{Ax2/Tg1}$ compound heterozygotes might reveal a phenotypic defect not seen in $Axin^{Ax2/Ax2}$ homozygotes. However, these mice were indistinguishable from the $Axin^{Ax2/Ax2}$ or $Axin^{Ax/Ax}$ homozygotes.

FIG. 1. Targeted replacement of the *Axin* gene with myc-tagged Axin2 cDNA or myc-tagged Axin cDNA. (A) Schematic diagram of Axin and Axin2 proteins and binding partners. Percent similarities between the conserved RGS and DIX domains and the GSK3 and β-catenin (β-cat) binding regions of Axin and Axin2 are indicated, as are those of other less-conserved regions. The solid lines at the top indicate the regions of Axin involved in binding to the indicated proteins (31). Of these, only APC, GSK3, β-catenin, Diversin, and Smad3 are known to bind to Axin2. aa, amino acids; PP2A, protein phosphatase 2A. (B) Schematic diagram of the *Axin* genomic locus, targeting constructs, and targeted alleles. Exons 1 and 2 (EX1 and EX2) are depicted as grey boxes, and intron sequences are depicted as solid lines. The positions of the restriction enzyme sites and the probe PB are indicated. Small harpoons (⇀ and ↼) show the PCR primers AXL1 (a), MTAXR1 (b), and MTCONR1 (c). (C) Southern blot analysis of G418-resistant colonies after electroporation of ES cells with targeting constructs. Probing with probe PB following digestion of DNA with Hpa1 detected a band of 9.8 kb for the $Axin^{AX2}$ allele, a band of 10.5 kb for the $Axin^{AX}$ allele, and a band of 8.8 kb for the wild-type allele. (D) Identification of homozygous, heterozygous, and wild-type (WT) $Axin^{AX2}$ and $Axin^{AX}$ mice by PCR.

The only minor defect we detected, both in $Axin^{Ax2/Ax2}$ and $Axin^{Ax/Ax}$ homozygotes, was a reduction of 11 to 14% in birth weight compared to that of wild-type littermates. $Axin^{Ax2/Ax2}$ mice were 11% smaller than wild-type mice at birth ($P = 0.06$), 12% smaller at 14 days ($P = 0.06$), and 12% smaller at 28 days ($P = 0.03$). $Axin^{Ax/Ax}$ mice were 14% smaller than wild-type mice at birth ($P = 0.001$), 12% smaller at 14 days ($P = 0.003$), and 11% smaller at 28 days ($P = 0.06$). However, by 6 weeks of age, the difference was only 5 to 6% and was not statistically significant ($P = 0.55$ for $Axin^{Ax2/Ax2}$ mice and $P = 0.32$ for $Axin^{Ax/Ax}$ mice). As the $Axin^{Ax2}$ and $Axin^{Ax}$ alleles had the same effect, this does not reflect a functional difference between Axin and Axin2 but rather a property of both knockin alleles. One possibility was that these alleles might express too little or too much Axin/Axin2. To compare the amounts of protein encoded by the wild-type and $Axin^{Ax}$ alleles, we performed immunoblotting with $Axin^{Ax/+}$ heterozygous embryos and an anti-Axin antiserum that detects both Myc-tagged and endogenous Axin. Myc-tagged Axin is larger than endogenous Axin, and the two bands are easily resolved (Fig. 2A). This analysis showed that the $Axin^{Ax}$ allele encodes Myc-Axin at the same level as endogenous Axin, ruling out this explanation for the low birth weight. An alternative possibility is that the Myc tag, present in both $Axin^{Ax}$ and $Axin^{Ax2}$ alleles, causes this transient defect.

Although our targeting strategy precluded the expression of any functional Axin from the $Axin^{Ax2}$ locus (since the initiation codon and exon 2, which encodes the essential RGS domain, were deleted), we wanted to test this at the protein level. We therefore performed immunoblotting with anti-myc and anti-Axin antisera on extracts of $Axin^{Ax2}$ embryos (Fig. 2B to D). This confirmed that the $Axin^{Ax2/Ax2}$ homozygotes expressed Myc-tagged Axin2 and lacked endogenous Axin.

Our results show that Axin2, when expressed under the control of *Axin* regulatory sequences, can carry out all the essential functions of Axin during development as well as in adult life. If *Axin* and *Axin2* are functionally identical, why have the two genes been conserved during evolution? One explanation is that the total level of Axin-like protein (Axin plus Axin2) needs to be elevated in certain cells; *Axin* provides a basal level in all cells (52), while *Axin2*, which is induced by Wnt/β-catenin signaling, is regulated to provide elevated levels where needed (4, 21, 27, 32, 50). Additional insight into this question derives from the analysis of a null-mutant allele (*lacZ* insertion) of *Axin2* (32). Mice lacking *Axin2* are viable and fertile but display skull malformations resembling craniosynostosis in humans, due to the premature fusion of cranial sutures (51). Thus, in the presence of a normal complement of *Axin* alleles, *Axin2* is dispensable for most developmental processes, although it is important for postnatal skull development. To further examine the relationship between *Axin* and *Axin2*, mice with various combinations of $Axin^{Tg1}$- and $Axin2^{lacZ}$-null alleles were examined (B. Jerchow and W. Birchmeier, personal communication). Double homozygotes lacking both *Axin* and *Axin2* died much earlier (by E6.5) than those lacking only *Axin*, indicating that endogenous *Axin2* can partially compensate for the absence of *Axin* in early embryogenesis. Furthermore, while mice with only one wild-type *Axin* allele ($Axin^{Tg1/+}$) are normal (52), the further removal of *Axin2* ($Axin^{Tg1/+}$ and $Axin2^{lacZ/lacZ}$) resulted in severe brain and craniofacial abnormalities at birth. Thus, one *Axin* allele is sufficient in the presence of *Axin2* but not in its absence. Overall, these findings argue that when Axin is absent or reduced, Axin2 can partially compensate for its developmental functions. Axin2 does not fully compensate because it is not ubiquitously expressed. However, in the $Axin^{Ax2}$ allele, where the pattern of Axin2 expression is altered to resemble that of Axin, it can replace the functions of Axin.

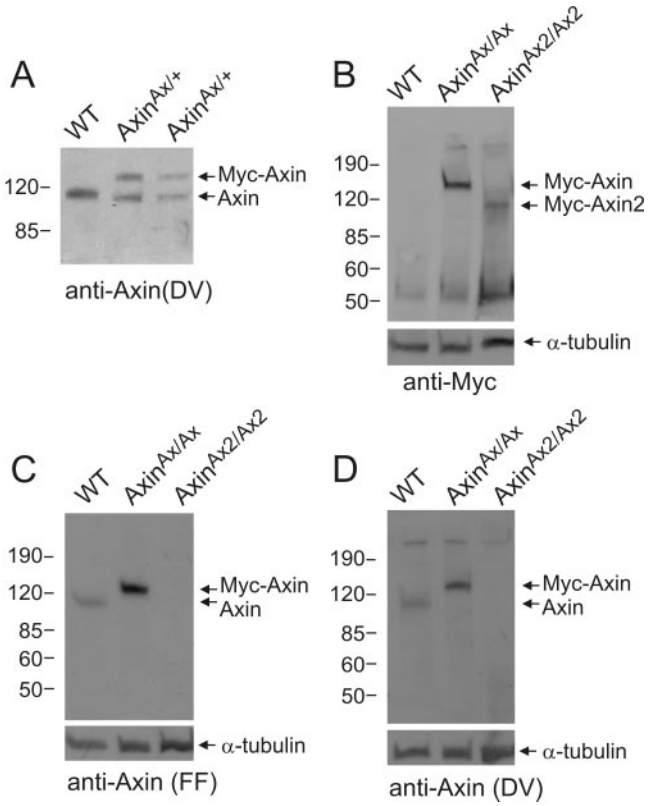

FIG. 2. Expression of Axin2 and Axin in $Axin^{AX2}$ and $Axin^{AX}$ mice. (A) Protein lysates from wild-type (WT) and $Axin^{Ax/+}$ heterozygous embryos were probed with anti-Axin antibodies. The heterozygotes expressed equal amounts of Myc-tagged Axin from the $Axin^{AX}$ allele and wild-type Axin from the endogenous allele, showing that the $Axin^{AX}$ allele is expressed at a normal level. (B, C, and D) Protein lysates from wild-type, $Axin^{AX/AX}$, and $Axin^{AX2/AX2}$ homozygous embryos were probed with anti-Myc antibody (B) or with two different anti-Axin antisera (C and D). In wild-type embryos, the anti-Axin antibodies detected endogenous Axin (~110 kDa), while the anti-Myc antibody detected only background bands. In $Axin^{AX/AX}$ embryos, both the anti-Axin and the anti-Myc antibodies detected Myc-tagged Axin (~130 kDa), while endogenous Axin was absent. In $Axin^{AX2/AX2}$ embryos, the anti-Axin antibodies detected only background bands also seen in wild-type embryos, while the anti-Myc antibody detected Myc-tagged Axin2. Anti-Axin antiserum FF, a gift from François Fagotto, was raised against amino acids 406 to 685 of mouse Axin, while anti-Axin antiserum DV, a gift from David Virshup, was raised against full-length mouse Axin. Molecular size markers (in kilodaltons) are noted at the left of blots.

The functional equivalence of the Axin and Axin2 proteins during mouse development implies that any amino acids that are not conserved between the two proteins are not required for their function. Similarly, any interactions with other proteins that are not shared by Axin and Axin2 are unlikely to be

important. Axin interacts directly with at least 17 other proteins (Fig. 1A) (31), but only a small subset of these has been tested for interaction with Axin2. We suggest that one way to evaluate the importance of these multiple protein-protein interactions for the functions of Axin would be to test if they interact similarly with Axin2; those that fail are unlikely to be required for the developmental functions of Axin.

Our results also call into question the significance of the differences in subcellular locations observed for Axin and Axin2. Anderson et al. (3) previously reported that in normal colon epithelial cells, Axin was found in several locations—diffusely in the nucleus, along cell membranes, and often in the cytoplasm—while Axin2 was uniformly expressed in the nucleus. In adenomatous polyps, Axin was strongly cytoplasmic while Axin2 remained nuclear. Given our results, it is unlikely that these differences in localization reflect important differences in the functions of the two proteins. While other studies have shown previously that Axin shuttles between the nucleus and the cytoplasm (8, 46), it is very unlikely that all of the functions of Axin and Axin2 can be carried out in the nucleus. Therefore, the apparent nuclear localization of Axin2 probably does not reflect its major site of action. Perhaps a low level of the protein in the cytoplasm or at the cell surface, below the level of detection by immunostaining, is sufficient to carry out its essential cytoplasmic functions. The equivalence of Axin and Axin2 proteins, despite their different subcellular localizations, is reminiscent of the finding that APC1 and APC2 are functionally redundant in the fly, although these proteins also display different intracellular locations (1, 2).

The insertion of form 1 Axin cDNA into the *Axin* locus (in the $Axin^{Ax}$ allele), while primarily intended as a control for the $Axin^{Ax2}$ allele, provided some useful information. First, it showed that the long isoform of Axin, form 2, is not required for normal development. On the other hand, the transient growth defects observed in both $Axin^{Ax}$ and $Axin^{Ax2}$ homozygotes might be due to the lack of form 2 Axin or to the absence of the upstream Axin coding sequences. Second, the normal development of $Axin^{Ax/Ax}$ homozygotes suggests that this targeting strategy can be used to efficiently generate new mutant alleles of *Axin*, in which specific domains or amino acids are altered. This approach is currently being used to examine the importance in vivo of several conserved sequences believed to play important roles in the functions of Axin.

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