Protein Encoded by the Axin\(^{Fu}\) Allele Effectively Down-regulates Wnt Signaling but Exerts a Dominant Negative Effect on c-Jun N-terminal Kinase Signaling* 

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Axin plays an architectural role in many important signaling pathways that control various aspects of development and tumorigenesis, including the Wnt, transforming growth factor-\(\beta\), MAP kinase pathways, as well as p53 activation cascades. It is encoded by the mouse Fused (Fu) locus; the Axin\(^{Fu}\) allele is caused by insertion of an IAP transposon. Axin\(^{Fu/Fu}\) mice display varying phenotypes ranging from embryonic lethality to relatively normal adulthood with kinky tails. However, the protein product(s) has not been identified or characterized. In the present study, we conducted immunoprecipitation using brain extracts from the Axin\(^{Fu}\) mice with specific antibodies against different regions of Axin and found that a truncated Axin containing amino acids 1–596 (designated as Axin\(^{Fu-NT}\)) and the full-length complement of Axin (Axin\(^{WT}\)) can both be generated from the Axin\(^{Fu}\) allele. When tested for functionality changes, Axin\(^{Fu-NT}\) was found to abolish Axin-mediated activation of JNK, which plays a critical role in dorsoventral patterning. Together with a proteomics approach, we found that Axin\(^{Fu}\) contains a previously uncharacterized dimerization domain and can form a heterodimeric interaction with Axin\(^{WT}\). The Axin\(^{Fu-NT}/Axin^{WT}\) is not conducive to JNK activation, providing a molecular explanation for the dominant negative effect of Axin\(^{Fu}\) on JNK activation by wild-type Axin. Importantly, Axin\(^{Fu}\) exhibits no difference in the inhibition of Wnt signaling compared with Axin\(^{WT}\) as determined by reporter gene assays, interaction with key Wnt regulators, and expression of Wnt marker genes in zebrafish embryos, suggesting that altered JNK signaling contributes, at least in part, to the developmental defects seen in Axin\(^{Fu}\) mice.

Axin is a multifunctional protein that controls many important regulatory pathways, including \(\beta\)-catenin-mediated canonical Wnt signaling, transforming growth factor-\(\beta\), mitogen-activated protein (MAP) kinase, and p53 pathways (1–7). In Wnt signaling, Axin provides a platform for the assembly of the \(\beta\)-catenin degradation complex to keep the activity of \(\beta\)-catenin in check, which otherwise would lead to developmental defects such as axis duplication and outgrowth of tissues or tumorigenesis. It also forms complexes with the upstream MAP kinases MEKK1 or MEKK4 to activate JNK and p38 MAP kinases (8, 9). A remarkable aspect of Axin activation of MAPKs is that Axin has to form a homodimer or a higher order of complex formation (6, 9). In addition, binding of GSK-3\(\beta\), casein kinases, or deshevelled can attenuate Axin activation of JNK by competing against MEKK binding or disrupting Axin homodimerization, suggesting that Axin activation of the MAPKs is highly conformation-dependent (10, 11). We recently showed that Axin-mediated JNK activation plays a critical role in dorsoventral patterning, in that Axin exerts two opposing roles, one to down-regulate \(\beta\)-catenin and cause ventralization and the other to activate JNK leading to dorsalized embryos when overexpressed (12).

Axin is encoded by the Fused (Fu) locus (12, 13, 14). Mutant alleles of the Fused locus include Fu\(^{fused}\) (later termed as Axy\(^{Fu}\), Ref. 14), Fu\(^{Knobblly}\), Fu\(^{Knobby}\), and Fu\(^{fused}\) (13, 15). The Fu\(^{fused}\) and Fu\(^{Knobblly}\) alleles are each caused by insertion of intracisternal A-particle (IAP), one kind of transposons. While the IAP insertion occurs at intron 6 in the Fu\(^{fused}\) allele, IAP is inserted into exon 7 in Fu\(^{Knobblly}\), Fu\(^{Knobby}\), Fu\(^{Knobblly}\), and Fu\(^{fused}\) are dominant mutations, which are presumably caused by their truncated proteins resulting from the IAP insertion. Several studies have examined the nature of the mRNA transcripts from the Fu\(^{fused}\) allele. Vasiček et al. (19) showed that a 3.9-kb mRNA corresponding to wild-type Axin RNA could be detected in both wild-type and Fu\(^{fused}\) homozygous mice by

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\(^{3}\) The abbreviations used are: MAP, mitogen-activated protein; HA, hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; MERK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase; GST, glutathione S-transferase; GSK-3\(\beta\), glycogen synthase kinase-3\(\beta\); JNK, c-Jun N-terminal kinase; IAP, intracisternal A-particle; GFP, green fluorescent protein; WT, wild-type; HEK, human embryonic kidney cells; NT, N-terminal region; CT, C-terminal region; gsc, goosecoid.
Northern blotting, suggesting that the IAP insertion does not entirely prevent the generation and processing of the FuFused allele into a normal mature Axin transcript. However, transcripts that terminate immediately after exon 6 were also detected, suggesting the existence of other Axin transcripts that could potentially create a premature stop codon caused by the aberrant splicing between exons 6 and 7 (19, 20). It is also believed that the inserted IAP may provide an internal promoter that may yield transcripts encoding a C-terminal portion of Axin.

Some AxinFu/Fu mice die in utero, and others are viable with a kinked tail as well as deafness and defects in walking behavior (16, 17, 21, 23). It is important to note that the phenotypic penetrance of the Fused allele is highly epigenetic, i.e. some AxinFu/Fu homozygotes die prenatally, and some can develop into adulthood with kinked tails. In heterozygotes, some adult mice display kinky tails, and some do not. These variations in phenotypes among animals with the same genotype are likely a reflection of epigenetically regulated expression of the mutated Axin gene. It was reported that the presence or absence of the characteristic phenotype, a kinky tail, correlates with different expression patterns of brain extracts from 

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EXPERIMENTAL PROCEDURES

AxinFu Mice and Genotyping—Inbred 129P4/RrKk AxinFu/+ mice were purchased from the Jackson Laboratory. Genomic DNA was isolated from the mouse ear. Mice were genotyped for the AxinFu and wild-type alleles by multiplex PCR using the following primers: P23: 5′-cgccagctctccccgacgacg-3′, G245: 5′-gcggagtcctctgagccag-3′ and IAP forward: 5′-gcccagctctccccgacgacg-3′ according to protocols from Dr. Frank Costantini (Columbia University).

Preparation of Mouse Brain Extract—For brain protein extract preparation, the mouse brain was homogenized in 2 ml of radioimmune precipitation assay buffer (5) containing 0.1% SDS with Polytron centrifuged at 1700 × g for 30 s on ice. Samples were sonicated three times for 10 s each and centrifuged at 18,000 × g rpm for 30 min at 4°C. The supernatant was collected, and the protein concentration was determined using the Bio-Rad Protein Array. Equal amounts of protein were subjected to immunoprecipitation with C2b or CT36 antibody raised against Axin.

Plasmid Constructions—Expression vectors for HA- or Myc-tagged wild-type mouse Axin (short form), HA-glycogen synthase kinase-3β (GSK-3β), HA-β-catenin, HA-casein kinase Iα (CKIα), Wnt-1, HA-MEKK1-CT, HA-MEKK4-CT, and FLAG-JNK, were constructed as previously described (8, 9, 25). AxinFu-NT and AxinFu-CT were generated by PCR amplification using the following primers: AxinFu-NT: 5′-cattatcagccgggatcctcccag-3′ (forward), and 5′-cttcccccgaacctgttccctgctctc-3′ (reverse); AxinFu-CT: 5′-cttcccccgaacctgttccctgctctc-3′ (forward), and 5′-cttcccccgaacctgttccctgctctc-3′ (reverse). The fragments were then subcloned into pCMV5- HA, Myc, or FLAG, and, into pX7 for in vitro transcription. All PCR products were verified by sequencing.

Fish and Microinjection—Zebrafish embryos of the Tubingen strain were incubated in Holtfreter’s solution at 28.5°C. Capped mRNAs were synthesized using T7 Cap Scribe (Roche Applied Science) according to the protocol described previously (26). Digoxigenin-UTP labeled antisense RNA probes were generated by in vitro transcription and used for whole mount in situ hybridization (27, 28).

Cell Culture and Transient Transfection—HEK293T, HEK293 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, antibiotics, and 2 mM l-glutamine, and maintained at 37°C, 5% CO2, in a humidified incubator. Transfection was performed using PEI. For transfection in 60-mm dishes; transfection mixture was prepared by adding DNA into 240 μl of HBS (150 mM sodium chloride, 20 mM HEPES, pH 7.4), and 180 μl of 10 μM PEI (Cat. 23966, Polysciences, dissolved in HBS), immediately followed by mixing. The mixture was left at room temperature for about 30 min before dropping into cells. During the 30-min incubation, the cell medium was changed with 1.0 ml of DMEM (fetal bovine serum (FBS)-free). After 6–12 h of transfection, the FBS-free DMEM medium was changed with complete DMEM medium.

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Preparation of Antibodies—Mouse anti-HA (F-7), anti-Myc (9E10), rabbit anti-MEKK1 (C-22), and goat anti-Axin (S-20, R-20) were purchased from Santa Cruz Biotechnology. Mouse anti-FLAG (M2) and mouse anti-β-tubulin were purchased from Sigma. Rabbit anti-phospho-c-Jun (Ser-63) was purchased from Cell Signaling. The polyclonal antibody against Axin (C2b) was described previously. For the rabbit polyclonal anti-Axin (CT36) antibody, the GST fusion protein containing the amino acid sequence from amino acids 676–832 was produced in BL21 bacterial cells and injected into rabbits following purification using glutathione-agarose beads.

Immunoprecipitation, Immunokinase Assay, and Western Blotting—Cells were lysed with lysis buffer (9) at 36-h post-transfection, and the lysates were immunoprecipitated with the indicated antibodies for about 3 h. Then beads were spun down and washed with lysis buffer three times. The proteins were eluted with 2× SDS sample buffer and along with the total cell lysates were analyzed by Western blotting with different antibodies. The immunokinase assay on JNK activation was performed as previously described (9).

GST Pull-down Assay—GST-AxinNT400, which contains the Axin N-terminal-half (amino acids 1–400), was expressed in BL21 bacterial cells transformed with pGEX-AxinNT400 bacterial expression plasmid and was purified with glutathione-agarose beads (Sigma). Expression was induced with 0.1 mM isopropyl-1-thio-β-d-galactopyranoside for 4 h at 37°C. Approximately 0.2 mg of the GST fusion protein bound to agarose beads was added to each brain extract from adult Axin+/+ and AxinFu/Fu mice, and incubated for 3 h with gentle rotation, followed by washing three times with lysis buffer. The proteins were eluted with SDS sample buffer.

Transcription Reporter Assay—For the LEF-1 reporter assay, 0.5 μg of LEF-1 reporter gene and 0.1 μg of LEF-1 were co-transfected with HA-Axin or HA-AxinFu-NT or both of them into HEK293T cells. Wnt1 (0.1 μg) was also transfected as indicated. At 30-h post-transfection, cells were lysed, divided into two portions, and each measured for the activities of luciferase and β-galactosidase. The ratio of luciferase activity to β-galactosidase activity varied less than 10% among samples. Data are presented as means plus standard deviation from three separate experiments performed in duplicate.

RESULTS

Detection of a Truncated N-terminal Fragment of Axin in AxinFu Mice—In some of the heterozygous (AxinFu+/−) mice (36/134), the tails appeared normal, whereas all of the homozygotes showed kinked tails with some of the kinked tails also having bifurcation at the distal end. These observations are consistent with previous reports showing that, AxinFu+/− and AxinFu/Fu adult mice display variable phenotypes characterized by kinked tails (13, 16, 17). Based on previous work on the IAP insertion position and the detection of normal mature Axin mRNA transcript as well as aberrant transcripts, we outlined putative protein products resulting from the IAP insertion in the AxinFu allele (Fig. 1A). Normal mature Axin mRNA must be produced by splicing out the IAP sequence between the donor sequence of exon 6 and the acceptor site in exon 7. It was also speculated that the IAP-disrupted intron 6 may not be properly spliced out, and as a consequence the translation is extended into the intron sequence and prematurely terminated immediately after exon 6 junction as diagrammed in Fig. 1A. In addition, it was hypothesized that the long terminal repeat sequence in the transposon may serve as a promoter to drive transcription of the Axin sequence downstream of exon 6 to the 3′ terminus, resulting in a potential truncated protein containing amino acids 597–832 of Axin (19, 23). However, none of the putative AxinFu protein species has been identified or characterized.

We set out to identify the naturally occurring Axin truncates in the AxinFu mice. For convenience of characterization of the AxinFu truncates, we first generated cDNA fragments corresponding to the predicted AxinFu-NT and AxinFu-CT respectively, as diagrammed in Fig. 1A. In parallel, we generated an additional polyclonal antibody against a C-terminal region (amino acids 676–832) of Axin, which was designated as Axin antibody CT36. The antibody against the N-terminal Axin (antibody C2b) had been previously reported (5). The C2b and CT36 antibodies specifically react with AxinFu-NT and AxinFu-CT that were expressed in HEK293T cells (Fig. 1B). C2b recognized HA-Axin and HA-AxinFu-NT, but not AxinFu-CT; conversely, CT36 only reacted with the full-length Axin and HA-AxinFu-CT, but not AxinFu-NT.
We carried out immunoprecipitation experiments with total brain protein extracts from adult Axin\textsuperscript{+/-}, Axin\textsuperscript{fu/+} (with normal tail appearance), Axin\textsuperscript{fu/-} (kinked tail), and Axin\textsuperscript{fu/fu} mice. After immunoprecipitation with C2b and CT36 antibodies, the precipitated protein samples were separated on PAGE gels along with total lysates of HEK293T cells expressing untagged Axin\textsuperscript{WT} (full-length, the first lanes, Fig. 2A), Axin\textsuperscript{fu-NT} and Axin\textsuperscript{fu-CT} (the last two lanes, on the right of Fig. 2A), which were constructed as diagrammed in Fig. 1A and were used as molecular weight references. The separated proteins were then subjected to immunoblotting with two commercial antibodies from Santa Cruz Biotechnology, S-20 (against the N-terminal region) and R-20 (against the C-terminal region). The antibodies are indicated on the left of each panel (Fig. 2A). In the wild-type mice, a protein with a molecular mass of \(\sim 110\) kDa that corresponds exactly to the control full-length Axin protein (lane 1 of Fig. 2A) expressed in HEK293T cells was detected in immunoprecipitates of C2b. In Axin\textsuperscript{fu/-} mice, heterozygous or homozygous, a second specific protein species that co-migrated with the constructed Axin\textsuperscript{Fu-NT} was also detected by S-20 that was raised against the N-terminal region of Axin. These results showed that the proposed Axin\textsuperscript{Fu-NT} indeed exists in mice containing the Axin\textsuperscript{fu} allele.

We also carried out immunoprecipitations with the CT36 antibody, followed by Western blotting with the different antibodies indicated. In the immunoprecipitates, only the full-length Axin protein was specifically detected by all the antibodies; no specific Axin species corresponding to the putative Axin\textsuperscript{fu-CT} was detected (Fig. 2A). However, it remains possible that the failure to detect the Axin\textsuperscript{Fu-CT} is due to the high background of the Western blot. Protein levels of tubulin in the mouse brain extracts were detected by immunoblotting and served as an internal loading control.

**Axin\textsuperscript{fu-NT} Effectively Attenuates Wnt Signaling**—To characterize the functional properties of the Axin\textsuperscript{fu-NT} protein, we first tested whether it affects Wnt signaling. The Axin\textsuperscript{fu-NT} and TOPFLASH reporter were co-transfected with or without Wnt1, into HEK293T cells, and the luciferase activities at 30-h post-transfection were measured. As shown in Fig. 3B, Axin\textsuperscript{fu-NT} attenuated Wnt signaling almost as effectively as the wild-type Axin. Similar results were obtained using a similar reporter, the LEF-1 luciferase plasmid (Fig. 3A). Consistently, the Axin\textsuperscript{Fu-NT} protein interacted with GSK-3\(\beta\), \(\beta\)-catenin, and APC with similar affinities compared with wild-type Axin (Fig. 3, C–E). We also examined the interaction between Axin\textsuperscript{fu-NT} and CK\(\alpha\), and the results demonstrated that Axin\textsuperscript{fu-NT} also effectively interacts with CK\(\alpha\), albeit with slightly lesser affinity (Fig. 3F). These results indicate that Axin\textsuperscript{fu-NT} is fully capable of forming the degradation complex and promoting the GSK3\(\beta\)-mediated, phosphorylation-dependent, \(\beta\)-catenin degradation. When injected into zebrafish embryos, Axin\textsuperscript{fu-NT} could down-regulate the expression of dharma/boz that is a specific maternal \(\beta\)-catenin target (30, 31), as effectively as Axin\textsuperscript{WT} (Fig. 3G, upper panel). At the shield stage, the zygotic \(\beta\)-catenin target \(tbx6\) (32) in zebrafish was also impaired by Axin\textsuperscript{fu-NT} to the same extent as Axin\textsuperscript{WT} (Fig. 3G, lower panel). The statistics of the phenotypic changes in these differently injected embryos is shown in Fig. 3H.

**Identification of an N Terminus Proximal Dimerization Domain of Axin**—The Axin\textsuperscript{fu} allele is a gain-of-function mutation. We therefore tested whether Axin\textsuperscript{Fu-NT} can form a dimeric interaction with the wild-type Axin. We performed a co-immunoprecipitation assay in HEK293T cells transfected with HA-tagged wild-type Axin and Myc-tagged Axin\textsuperscript{Fu-NT}. When co-expressed, HA–Axin was detected in the immunoprecipitate by anti-Myc (Fig. 4A), suggesting that Axin\textsuperscript{Fu-NT} forms a heterodimer with wild-type Axin. To verify that, we also transfected differentially tagged Axin\textsuperscript{fu-NT} into HEK293T and carried out immunoprecipitation. HA–Axin\textsuperscript{Fu-NT} was detected in the immunoprecipitate by anti-Myc antibody (Fig. 4B).
These results indicate that AxinFu-NT could strongly interact with wild-type Axin, unexpectedly revealing a previously unidentified dimerization domain in the N-terminal region of Axin. To further confirm that the N-terminal of Axin could form a homodimer, we carried out a GST pull-down assay with GST-AxinNT400 corresponding to the N-terminal-half (amino acids 1–400) and the brain extracts separately from adult Axin+/− and AxinFu/Fu mice. Consistently, pull-down with GST-AxinNT400 and the brain extract of Axin+/− mice produced two Axin protein species corresponding to the full-length wild-type Axin and AxinFu-NT, but only the wild-type Axin was detected from the pull-down using the extract of Axin+/− mice (Fig. 4C). The Axin proteins were not seen in the pull-down using GST alone.

**Dominant Negative Effect of AxinFu-NT on Axin-induced JNK Activation**—Next, we tested if AxinFu-NT has an effect on JNK activation. Whereas wild-type Axin robustly activated JNK in HEK293T cells, AxinFu-NT failed to activate JNK, in agreement with our observations that Axin strictly requires its C-terminal regions for JNK activation (11). When Axin and AxinFu-NT were co-expressed, JNK activation was almost completely abolished (Fig. 5A), indicating that AxinFu-NT has a dominant negative effect on JNK activation by the wild-type Axin. One possible means for AxinFu-NT to exert its inhibitory effect is to sequester MEKK1. Indeed, when assayed for its ability to bind MEKK1 by immunoprecipitation experiments, AxinFu-NT was found to strongly interact with MEKK1 (Fig. 5B). We previously found that Axin can utilize MEKK4, in addition to MEKK1, to activate JNK (8, 9). We therefore tested whether AxinFu-NT interacts with MEKK4, and whether overexpressed AxinFu-NT sequesters MEKK1 and MEKK4 against their binding to the wild-type Axin.

In the presence of increasing amounts of AxinFu-NT, levels of AxinFu-NT co-precipitated with MEKK1-CT or MEKK4-CT were gradually increased, whereas lesser wild-type Axin was co-immunoprecipitated with MEKK1-CT or MEKK4-CT were gradually increased, whereas lesser wild-type Axin was co-immunoprecipitated with MEKK1-CT or MEKK4-CT (Fig. 5C). These results indicated that AxinFu-NT indeed prevents MEKK1 or MEKK4 from binding to the AxinWT, but that MEKK4 forms a physical interaction with AxinFu-NT. When AxinFu-NT was co-expressed with MEKK4-CT, we detected a strong direct interaction of MEKK4-CT with AxinFu-NT (Fig. 5D). This new observation suggested that the previous Axin deletion mutants that failed to interact with MEKK4 (8) might not actually lose the domain for MEKK4 interaction, but rather that the domains deleted comprised conformations that disallow MEKK4 binding.

**FIGURE 3.** AxinFu-NT effectively down-regulates Wnt signaling. A, AxinFu-NT inhibits LEF-1 transcriptional activity. The LEF-1 reporter was co-transfected with HA-Axin and HA-AxinFu-NT into HEK293T cells in the presence or absence of Wnt1 as indicated. At 30 h post-transfection, cells were harvested, and the luciferase activity was measured as described previously. AxinFu-NT could inhibit LEF-1 transcriptional activity as effectively as Axin. B, similar experiments were performed with the TOPFLASH reporter. C–F, AxinFu-NT interacts with GSK3β (C), β-catenin (D), APC (E), and CKIα (F). HEK293T cells were transfected as indicated and incubated for 36 h. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody and followed by Western blot with the antibodies indicated. AxinFu-NT binds to GSK3β, APC, and β-catenin as efficiently as wild-type Axin, whereas it shows less affinity for the interaction with CKIα. G, AxinFu-NT inhibits the expression of maternal and zygotic-specific β-catenin target genes in zebrafish. The upper panel is the animal pole views of maternal β-catenin target dharma/boz expression at 30% epiboly stage in GFP control mRNA, AxinWT mRNA, and AxinFu-NT mRNA, respectively. The lower panel is the animal pole views of the expressions of zygotic β-catenin target tbx6 at shield stage in GFP control mRNA, AxinWT mRNA, and AxinFu-NT mRNA, respectively. H, statistical data for AxinFu-NT. Down-regulated β-catenin targets dharma/boz and tbx6 in G. AxinFu-NT could down-regulate maternal β-catenin target dharma/boz as effectively as wild-type Axin in zebrafish embryos at 30% epiboly stage (left panel). AxinFu-NT could down-regulate zygotic β-catenin target tbx6 as effectively as wild-type Axin in zebrafish embryos at the shield stage (right panel). Each number (n) represents total embryos from two independent experiments, and 600 pg of each mRNA were injected for each embryo.
Axin\textsuperscript{Fu-NT} Causes Ventralized Phenotypes in Zebrafish Embryos—Our recent study indicated that Axin, besides ventralizing activity by facilitating β-catenin degradation, displays a dorsalizing activity that is mediated by Axin-induced JNK activation (12). To understand the biological function of Axin\textsuperscript{Fu-NT} \textit{in vivo}, we injected Axin\textsuperscript{Fu-NT} mRNA into zebrafish embryos and observed the effect of its ectopic expression on embryonic development. In embryos each injected with 400 pg of Axin\textsuperscript{Fu-NT} mRNA, 68.2% of all embryos (n = 44) at middle gastrulation (shield stage) displayed reduced expression of the dorsal mesoderm marker goosecoid (gsc), while embryos injected with the control GFP mRNA expressed goosecoid properly on the dorsal side (Fig. 6A). When injected alone, 400 pg of β-catenin induced an expanded expression of the dorsal marker gsc, co-injection of Axin\textsuperscript{Fu-NT} mRNA (400 pg) strongly abrogated the dorsalizing effect of β-catenin. Similarly, JNK mRNA injection (300 pg) induced dorsalization, which was also reversed by Axin\textsuperscript{Fu-NT} mRNA. However, in the embryos co-injected with JNK and Axin\textsuperscript{Fu-NT}, it is evident that the dorsal marker gsc was expressed at much higher levels than those in embryos injected with Axin\textsuperscript{Fu-NT} alone. These results indicate that Axin\textsuperscript{Fu-NT} could effectively attenuate β-catenin signaling and that JNK could on its own cause an expanded expression of the dorsal marker, which is consistent with our previous observations (12, 33, 34).

In contrast, in embryos injected with Axin\textsuperscript{Fu-NT} mRNA, expression of the ventral margin-specific gene eve1 at the shield stage was increased, expanding to the whole blastomere margin in 88.9% (Fig. 6B). Injection of β-catenin or JNK mRNA reduced the expression of the ventral marker, which was reversed by co-injection of Axin\textsuperscript{Fu-NT} mRNA (Fig. 6B). Of particular note, Axin\textsuperscript{Fu-NT} mRNA induced expanded expression of this marker around the whole blastomere can be rescued only by JNK, conforming to the notion that JNK and β-catenin are two independent inducers for dorsalization. Based on the zebrafish experiments, JNK assays, as well as Wnt reporter gene assays, we summarized the functions of Axin\textsuperscript{Fu-NT} in Fig. 6C, showing that Axin\textsuperscript{Fu-NT} can exert a dual negative effect on dor-salization, one aspect of which is to effectively down-regulate β-catenin function and the other to dominant negatively attenuate JNK signaling mediated by the wild-type Axin.

**DISCUSSION**

In the present study, we have identified the protein product of the Axin\textsuperscript{Fu/Fu} allele, Axin\textsuperscript{Fu-NT}, and have characterized the
molecular nature of the truncated Axin. Consistent with the previous observation that the Fused allele can give rise to a normal 3.9-kb mRNA for wild-type Axin, in addition to the aberrant fused-specific mRNA species, we also detected Axin of normal size in both heterozygous and homozygous AxinFu mice. We showed that AxinFu-NT is virtually intact in the inhibition of Wnt signaling as determined by LEF reporter assay and based on biochemical properties of AxinFu-NT including its ability to bind to β-catenin, GSK3β, APC, and CKIα. Moreover, zebrafish embryos injected with AxinFu-NT mRNA displayed defects characteristic of impaired Wnt signaling, including reduced expression of the dorsal marker goosecoid, and increased expression of the ventral marker eve1. Moreover, the expressions of dharma/boz and tbx6, specific target genes for β-catenin, in the AxinFu-NT mRNA-injected embryos were also found decreased to the same degree seen in embryos injected with AxinWT mRNA. These observations are consistent with previous studies using various Axin deletion mutants (35). However, it is difficult to reconcile with the report that the most notable phenotypic change in AxinFu/Fu embryos is the formation of an ectopic axis (14). We therefore cannot rule out the possibility that AxinFu-NT may in some way inadequately regulate Wnt signaling; in particular, the missing C-terminal region is critical for interaction with regulatory factors such as Dschevell (36, 37). On the other hand, as Axin has been shown to play pleiotropic roles in signaling pathways that control various aspects of development, we speculate that the defects seen in the AxinFu/Fu mice may be caused by perturbation of more than one signaling pathway. Another possibility is that in mice, JNK signaling may exert a negative role in dorsal axis formation as reported in Xenopus (38), such that the deficiency of Axin-mediated JNK signaling in mutant mice may lead to axis duplication.

We found that AxinFu-NT drastically inhibits JNK activation, which is in accordance with our previous studies showing that an intact C-terminal of Axin is required for JNK activation. Mechanistically, AxinFu-NT was unexpectedly found to possess an additional dimerization domain, through which it forms a dimeric complex with the wild-type Axin, rendering the existing wild-type Axin unable to activate JNK. Moreover, AxinFu-NT sequesters MEKK1 and MEKK4 to prevent their interaction with wild-type Axin, thereby exerting a dominant negative effect on Axin-mediated JNK activation.
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JNK signaling has been implicated in numerous biological processes ranging from stress responses to the formation of planar cell polarity during development (39–41). Compound mutant mice lacking JNK1 and JNK2 genes die on embryonic day 10.5 (E10.5) because of defective closure of the neural tube in the hindbrain (41–43). Overactivity of JNK signaling induced by ectopic expression of Axin in zebrafish embryos can lead to enlarged head and other dorsalized phenotypes, demonstrating that the Axin-mediated JNK signaling plays an important role in doroventral patterning including head formation (12). These findings emphasize the importance of JNK signaling during early development. It is therefore reasonable for us to speculate that the developmental abnormalities seen in homozygotes of the Axinfu mice, including neurological defects and embryonic lethality, may be caused by improper Axin-mediated JNK signaling, at least to a certain extent. It is important to note that the dominant negative nature of AxinFu-NT in JNK signaling, through forming AxinFu-NT/AxinWT, conforms to the structure and function of Axin that is a master scaffold for many important signaling pathways.

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