Axin: A Master Scaffold for Multiple Signaling Pathways

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Key Words

Axin, biological function · Axin, identification · Axin/JNK signaling pathway · Wnt pathway

Abstract

Axin was originally identified from the characterization of the *Fused* locus, the disruption of which leads to duplication of axis and embryonic lethality. It is a multidomain protein that interacts with multiple proteins and functions as a negative regulator of Wnt signaling by down-regulating the β -catenin levels. Recently, it was demonstrated that Axin also plays an important role in a JNK signaling pathway. Axin utilizes discriminatory domains for its distinct roles in the Wnt pathway and in the Axin/JNK pathway. Here we review the data that show how Axin regulates multiple signaling pathways by serving as a scaffold protein, controlling diverse cellular functions in proliferation, fate determination, and suppression of tumorigenesis.

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Introduction

One fundamental problem in embryonic development is how cell fate determination is achieved which gives rise to cell polarity and dorsoventral and anteroposterior specification. Studies of the Wnt signaling pathways have pro-

vided much information on how such developmental processes are regulated [1, 2]. Scaffold proteins serve as vital mediators for cellular signaling. They play architectural roles in integrating incoming signals to downstream effectors which in turn manifest biological functions. Axin, named for its inhibitory role of axis specification in vertebrates, has emerged as a major scaffold protein for regulating a variety of signaling pathways and biological functions. In Wnt signaling, Axin binds to many components in the pathway, including the Wnt coreceptor LRP (low-density lipoprotein related protein receptor) [3–5], Dishevelled or Dvl [6, 7], tumor suppressor adenomatous polyposis coli (APC), GSK-3 β , β -catenin [8–10], casein kinases [11], protein phosphatase 2A (PP2A) [12], Diver sin [13], Ccd1 [14], and Axam [15]. Interestingly, Axin itself is regulated with its stability being modulated by Wnt receptors [5], Dvl [16], and phosphorylation by GSK-3 β [17]. In addition, Axin also interacts with proteins that have no close relevance to Wnt signaling, including MAP kinase kinase kinases (MEKK) [18, 19], I-MFA [20], DCAP [21], SH2/3 adaptor protein Grb4 [22], and Smad3 [23]. Interaction of Axin with MEKK leads to JNK activation, proceeding through a cascade from Axin, MEKK, and MKK to JNK. The most intriguing aspect of JNK activation by Axin is that multiple seemingly concrete structural elements of Axin are required [18, 19, 24–27]. Our current review will focus on the Axin-interacting proteins and their functional roles in the Wnt and Axin/JNK pathways.

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Table 1. Axin-interacting proteins and their functions in Axin-mediated signaling

Protein	Interaction regions amino acids ^a	Function related to Axin	References
APC	89–216	APC complexes with Axin to downregulate β -catenin	8–10, 39, 40, 68–75
Axam	507–712	Axam downregulates β -catenin by its desumoylation activity	15, 116
Axin	757–820	Axin forms homodimer/oligomer via the DIX domain which is necessary for JNK activation	12, 18
β -Catenin	437–506	Axin serves as a platform for APC, GSK-3 β and β -catenin to enhance β -catenin degradation	38, 81, 90–96
Ccd1	Not determined	Ccd1 acts as a positive regulator for Wnt signaling by interacting with Axin, presumably via its DIX domain	14
CKI	217–352/508–712	CKI ϵ enhances FRAT-GSK-3 β interaction and stabilizes β -catenin by reducing phosphorylation; CKI α enhances β -catenin degradation as a priming kinase for GSK-3 β ; both CKI ϵ and CKI α inhibit Axin-mediated JNK activation by competing against MEKK1 binding	24, 11, 26, 81, 109–112
DCAP	Pro-rich motifs	Axin may regulate DCAP in glucose transport	133
Diversin	353–437	Diversin is reportedly able to recruit CKI ϵ to the Axin/GSK-3 β complex; it is also involved in JNK activation	13
Dvl	530–712/757–820	Dvl inhibits phosphorylation of Axin by GSK-3 β and inhibits Axin-promoted GSK-3 β phosphorylation of β -catenin	6, 7, 110
Grb4	Not determined	Unknown	22
GSK-3 β	353–437	GSK-3 β complexes with Axin to facilitate its phosphorylation of β -catenin, regulates Axin stability via phosphorylation, and inhibits JNK activation of Axin by preventing MEKK1 binding to Axin	24, 17, 38, 77–81
I-mfa	385–468	It inhibits Axin-mediated downregulation of β -catenin and JNK activation	20
LRP	353–437	LRP5,6 recruit Axin to the membrane, leading to degradation of Axin	3–5
MEKK1	217–352	It mediates JNK activation by Axin	18
MEKK4	678–712	It mediates JNK activation by Axin	19
PIAS	687–832	It mediates Axin sumoylation and JNK activation	27
PP2A	298–506/508–712	It interacts with Axin and enhances β -catenin degradation	12, 97–102
Smad3	508–713	Axin facilitates activation of Smad3 by TGF- β receptors for efficient TGF- β signaling	23

^a All amino acid positions are according to the mouse short form of axin (1–832).

Identification of Axin

Axin, the product of the mouse *Fused* (*Fu*) gene, was originally identified as an inhibitor of the Wnt-signaling pathway in that it regulates embryonic axis formation [28]. The mouse *Fused* locus was identified 66 years ago, mutation of which manifests as dominant kinky-tail phenotype. Homozygotes of the original *Fused* allele sometimes display deafness and neurological and urogenital

defects, in addition to the kinked tail [29]. The phenotypic variation among *Fu* mice is a complicated phenomenon due to epigenetic factors inherently determined by the nature of the *Fu* mutation caused by an intracisternal A particle (IAP) element [30–32]. The IAP element is a member of the abundant family of murine transposable elements. The insertion begins 35 bp downstream of exon 6, within intron 6 of the Axin gene. Subsequently, two other *Fu* alleles were identified, *Fu*^{kinky} (*Fu*^{ki}) and *Fu*^{knobby}

Fig. 1. Axin is a multidomain protein and binds to a wide array of proteins. Shown here are the Axin-interacting proteins with known relative binding positions in Axin. Of special note, CKI α/ϵ seems to also require the MEKK1-binding region, in addition to the sequence in the PP2A-binding region, for maximal binding affinity. MEKK1 and MEKK4 bind to two distinct regions, although they share sequence similarity.

(*Fu^{kb}*) [33, 34]. Whereas, the *Fu^{ki}* allele is apparently extinct, the *Fu^{kb}* allele is known to also carry an IAP insertion inside exon 7. Mice with *Fu^{ki}* or *Fu^{kb}* allelic mutation exhibit more severe phenotypes than the original *Fu* mutant mice. While heterozygotes of *Fu^{ki}* and *Fu^{kb}* mice share the kinked-tail phenotype as *Fu* mice, homozygotes die prenatally between 8 and 10 days postcoital. More importantly, these homozygotic embryos show complete or partial duplication of axial structures, neural tube malformation, and outgrowth of ectoderm, suggesting that the gene encoded by the *Fused* locus plays a critical role in axis formation [33, 34]. A fourth allele of *Fu* was more recently identified based on its failure to rescue the embryonic lethality of *Fu^{kb}* which is named *Fu^{Tgl}* as a transgenic insertion into the *Fused* gene [35]. While heterozygotes of *Fu^{Tgl}* display tail kinking, homozygous embryos exhibit phenotypes identical to those of embryos with *Fu^{ki}* or *Fu^{kb}* mutations. Aided by the DNA sequence provided by the transgene insertion, the *Fu* gene was cloned by F. Costantini and his coworkers [28]. It was revealed that the mouse *Fu* gene encodes a protein of 832 amino acids in size and was renamed Axin for *axis inhibition*. Most noticeably, Axin contains an RGS domain conserved in the large protein family regulators of G protein signaling or RGS proteins [36, 37]. It should be pointed out that the RGS domain in Axin is divergent enough not to bind G α subunits [our unpubl. data].

Soon after the cloning of Axin from the analysis of the *Fused* locus, Axin was independently identified as a protein that binds to GSK-3 β , based on a yeast two-hybrid screening of a rat brain cDNA library [38]. In the same screening, an Axin homologue, Axil (Axin-like, also known as Axin2 or conductin), that shares 44% identity with Axin, was also

identified [39] and shown to interact with both GSK-3 β and β -catenin and to inhibit Wnt signaling. Almost simultaneously, Axin2/conductin was also identified by a yeast two-hybrid screen, using β -catenin as bait [40]. The Axin gene is conserved in humans, rats, mice, chickens, *Xenopus*, and *Drosophila* [28, 38, 41, 42]. More recently, an Axin-like protein, termed PRY-1, has also been identified in *Caenorhabditis elegans* and demonstrated to be a functional homologue of Axin [43]. Axin is expressed widespread among tissues, consistent with its diverse roles in controlling development as well as tumorigenesis.

The mouse Axin protein has 832 amino acid residues in size. It must be noted here that there is a confusion in the literature regarding the numbering of amino acids of the mouse Axin due to the initial caution that the cloned cDNA for Axin might not be of full length, and the potential 124 codons in the 5' mRNA region were counted as aa 1–124. However, there is a longer form derived from alternative splicing which is identical to the short form, except for an insertion of 36 amino acids at position 735, encoded by exon 6 [28]. It has now become clear that Axin is a multidomain protein that binds to a great number of proteins, including well-documented components of the Wnt signaling pathways and factors that have no apparent relation to Wnt signaling (see table 1 and fig. 1 and 2).

Biological Functions of Axin

Regulation of Axis Formation

Axin was initially cloned from the analysis of the *Fused* locus, in which mutations cause defects in axis formation [28]. An intriguing feature of many homozygous Axin

Fig. 2. Axin serves as a scaffold for β -catenin degradation in the absence of Wnt signaling. APC, GSK-3 β , CKI α , and PP2A are the major factors that facilitate β -catenin degradation. When Wnt signaling is initiated via Frizzled and LRP, activated Dvl with the help of CKI ϵ increases FRAT binding to GSK-3 β and destabilizes Axin-PP2A affinity, thereby decreasing β -catenin phosphorylation and subsequent degradation. The accumulated β -catenin is then translocated into the nucleus, where it binds and activates other transcription factors such as TCF, regulating expression of a variety of target

genes. In parallel, Axin is also a scaffolding protein for MEKK1/4 in the Axin/JNK pathway. Interaction of MEKK1/4 leads to JNK activation via MKK4/7, in which PIAS binding to the extreme C terminus of Axin plays an integral part. In contrast, Dvl and CKI α/ϵ inhibit JNK activation by Axin, with Dvl interfering with Axin homodimerization and CKI α/ϵ preventing MEKK binding. Overexpression of Axin leads to apoptosis in some cells which depends on Axin's ability to activate JNK.

mutant embryos is a duplication of the embryonic axis [33, 34], suggesting that Axin normally plays a negative regulatory role in the response to an axis-inducing signal. Indeed, when Axin is injected into *Xenopus* embryos, most of them develop with strong axial defects [28]. Coexpression of Axin inhibits the induction of the secondary dorsal axis by Wnt, Dvl, and kinase-negative GSK-3, while it does not affect β -catenin- and Siamois-induced secondary axis formation [28]. Although injection of Noggin or dominant-negative bone morphogenetic protein

receptor also causes secondary axis formation, Axin fails to block this induction [28]. These results indicate that Axin exerts its function on axis formation by specifically inhibiting the Wnt signaling pathway.

Axin as a Tumor Suppressor

As Axin has the ability to downregulate β -catenin protein levels, it can be regarded as a tumor suppressor [44]. A plethora of in vitro and in vivo studies performed in *Xenopus*, *Drosophila*, and cultured mammalian cells has

demonstrated that Axin is central to the downregulation of β -catenin [7, 9, 10, 28, 38, 40, 41, 45–48]. It is not entirely clear how Axin functions, but it has been proposed to facilitate the phosphorylation of β -catenin and APC by GSK-3 β [9, 38]. Thus Axin could be viewed as a tumor suppressor based on its ability to downregulate Wnt signaling, and this has now been verified by documentation of its biallelic inactivation in human hepatocellular cancers and cell lines [49–51]. Importantly, these mutations were identified in those hepatocellular carcinomas that lacked activating mutations in the β -catenin gene (CTNNB1). All of the mutations were predicted to truncate the Axin protein in a manner that eliminated the β -catenin-binding sites [49]. Reintroduction of wild-type Axin into hepatocellular carcinoma cells leads to apoptosis in these cells. The notion of Axin being a tumor suppressor was further underscored by the findings that overexpression of Axin in CHO cells results in apoptosis [52] and that induced overexpression in transgenic mice leads to massive cell death in different organs [53]. It should be noted that Axin-induced apoptosis in CHO cells depends on Axin's ability to downregulate β -catenin [52], again confirming the determining role of β -catenin in promoting cell growth/survival.

Similarly, sequence variants of the Axin gene (*AXIN1*) were found in breast, colon, and other cancers. These variants interfered with Axin and GSK3 binding [54, 55]. Using single-strand conformation polymorphism analysis, many amino acid alterations have been detected in *AXIN1* [56–58]. Therefore, it is evident that Axin mutation is relatively common in cancers. However, in most cases, it remains to be determined whether these cancers contain simultaneous mutations in genes encoding other Wnt components.

Role in Cytoskeleton Rearrangement

Dishevelled (Dvl or Dsh) signals downstream of Wnt receptors and stabilizes β -catenin in Wnt signaling pathway [44]. It was reported that Dvl contributes to cytoskeletal reorganization during gastrulation [59–61] and mitotic spindle orientation during asymmetric cell division [62]. Both Dvl and Axin contain a conserved 85-residue module of unknown structure and biological function called the DIX domain [63]. Recently, it was demonstrated by using nuclear magnetic resonance spectroscopy that the DIX domain mediates targeting Dvl to actin stress fibers and cytoplasmic vesicles in vivo [64]. An attractive hypothesis is that Axin could also mediate cytoskeleton rearrangement via the DIX domain. Furthermore, the recent finding that Axin interacts with

Grb4, an SH2/3 domain adaptor protein that plays a role in Eph/ephrin signaling that controls cell and axon growth-cone movement [22], also suggests that Axin may have a function in cytoskeleton rearrangement.

Mutation of Axin Leads to Neurological Defects

In addition to their effects on axis formation, Axin mutations cause neuroectodermal defects (incomplete closure, malformation or truncation of the head folds). 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 and in frog embryos ectopically expressing Xwnt-8 after midblastula transition [65]. 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 [66]. 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. It is possible that these abnormal Axin proteins perturb Wnt signaling pathways involved in brain and skeletal development.

Axin Interacts with Multiple Components in the Wnt Pathway

Axin possesses multiple functional domains. Of note, it has an RGS domain that is conserved in the protein family of regulators of G protein signaling, near its NH2 terminus. At the C terminus, Axin contains another domain named DIX that is shared by Dishevelled and a recently identified Wnt regulator called Ccd1. In addition, Axin contains numerous domains for interaction with multiple other proteins than APC and Dvl/Ccd1 that bind to the RGS and to the DIX domain, respectively. This section tries to summarize all the Axin-interacting proteins and their interaction positions on Axin, wherever available.

Adenomatous polyposis coli

The RGS domain harbors amino acid residues (aa 89–216) that are sufficient for APC binding [8]; the crystal structure of an Axin-APC complex reveals that APC interacts with the Axin-RGS domain on a face that is distinct

from the G protein interface of classical RGS proteins. Those residues in the Axin-RGS domain that contact with APC are not conserved in $G_{i\alpha}$ -binding RGS proteins [67]. Similarly, many of those residues that form contacts with $G_{i\alpha}$ seen in RGS4- $G_{i\alpha}$ are not conserved in the Axin-RGS domain, consistent with our unpublished observation that Axin does not bind to $G_{i\alpha}$ proteins.

APC is a tumor suppressor genetically identified from the analysis of families that had familial APC. Subsequent studies have demonstrated that APC interacts with β -catenin, downregulating β -catenin signaling by enhancing degradation and exporting it from the nucleus into the cytoplasm [68, 69]. Genetic ablation of the *Drosophila* APC gene (*D-APC*) resulted in upregulation of β -catenin signaling, ultimately confirming that APC is functionally linked with the β -catenin function [70]. The experiments showed that reduction in the *Drosophila* β -catenin, Armadillo, rescues the phenotypic defects in the *D-APC* mutant, while Armadillo overexpression mimics D-APC inactivation.

APC requires Axin to downregulate β -catenin. It was demonstrated that complex formation of APC and Axin facilitates the phosphorylation of β -catenin by GSK-3 β which is followed by degradation of β -catenin [71]. The sites in APC responsible for binding to Axin and conductin/Axil reside in the 20 amino acid repeats region [40, 72]. The region of APC containing these sites is located just downstream of the mutation cluster region, suggesting that the interaction of APC with Axin and conductin/Axil is important to its tumor suppressor function. Indeed, a fragment of APC that induces β -catenin degradation [73] is rendered inactive by disruption of its Axin-binding sites [72]. Also, overexpression of an Axin fragment spanning the RGS domain inhibits APC-mediated β -catenin degradation. An APC fragment with mutated β -catenin-binding sites, but intact Axin-binding sites, also fails to induce degradation of β -catenin. Thus, APC needs to interact directly with both Axin and β -catenin to efficiently induce the degradation of β -catenin. On the other hand, Axin mutants lacking the APC-binding domain are still able to degrade β -catenin when overexpressed [8–10, 39, 74] which can be reconciled by the retained binding sites for GSK-3 β and CKI α that play primary functions in β -catenin degradation [75]. However, the fact that β -catenin is abnormally accumulated in SW480 cells indicates that endogenous expression of Axin is not sufficient to regulate the levels of β -catenin in the absence of APC. Thus, the associated Axin and APC may be more effective in inducing the degradation of β -catenin than Axin alone.

GSK-3 β

From a yeast two-hybrid screen of a rat brain library using GSK-3 β as bait, Axin was identified to be a protein that interacts with GSK-3 [38]. GSK-3 β binds to the Axin region of aa 353–437. The crystal structure of GSK-3 β in complex with a minimal GSK-3 β -binding segment of Axin has been determined [76]. It was revealed that the Axin-binding site is colocalized with FRAT-binding site in the C-terminal domain in GSK-3 β , providing the structural basis for competitive binding of Axin and FRAT to GSK-3 β [76].

The serine/threonine kinase GSK-3 β (glycogen synthase kinase 3 β) is an essential protein kinase that regulates numerous functions within the cell. It was initially isolated as an enzyme that could phosphorylate and inactivate glycogen synthase. The GSK-3 β activity influences cellular events ranging from glycogen metabolism, microtubule dynamics, and modulation of transcription factors (NFAT, CREB, β -catenin, and c-Jun) to axis development [77–79]. In Wnt signaling, it is an instrumental enzyme that phosphorylates β -catenin which is an a priori step for β -catenin degradation or downregulation. Failure of GSK-3 β to bind β -catenin results in dysregulation of β -catenin and leads to cancer. Normally, a small portion of GSK-3 β translocates to the nucleus during the S phase, although the function of this relocation is unknown. However, in the cancers PEL (primary effusion lymphoma) and KS (Kaposi's sarcoma), the latency-associated nuclear antigen (LANA), which possesses sequences with homology to the Axin GSK-3 β -binding domain, traps GSK-3 β into the nucleus and thus results in the depletion of cytoplasmic GSK-3 β , leading to stabilization of β -catenin and downstream TCF- and LEF-mediated transcriptional responses [80]. One unique feature of GSK-3 β phosphorylation of its substrates is that it requires a priming phosphorylation on a separate site by casein kinase I α (CKI α) and hence couple casein kinase for its function to downregulate β -catenin [81].

GSK-3 β , in addition to using Axin as a scaffold to facilitate its phosphorylation of β -catenin, it may well be a kinase that regulates Axin stability via phosphorylation. Axin harbors several possible GSK-3 β phosphorylation sites: SANDSEQQS³³⁰, SDADTLSLT³⁴¹, and SLTDS³⁴³; when these serine or threonine sites are mutated to alanine, the levels of Axin phosphorylation are reduced. Treatment of cells with LiCl, a GSK-3 β inhibitor, decreases the protein stability of Axin, while the phosphatase inhibitor okadaic acid reverses the effect of LiCl, indicating that phosphorylated Axin is more stable than its unphosphorylated form [17].

β-Catenin

β-Catenin was originally identified as a protein that interacts with the cytoplasmic domain of cadherin and links cadherin to α-catenin which in turn mediates the anchorage of the cadherin complex to the cortical actin cytoskeleton [82, 83]. β-Catenin can exist inside the cell in multiple pools: most notably one fraction is bound to the cadherin cytoplasmic tail at the plasma membrane and participates in cell adhesion, the other fraction is in complex with LEF/TCF transcription factors mediating transcriptional regulation [84–86]. Genetic studies in *Drosophila* have demonstrated that β-catenin/Armadillo is a critical element in the Wnt pathway [87]. In fact, gain-of-function or loss-of-function of components in the canonical Wnt pathway results in upregulation or downregulation of β-catenin, respectively, pointing to it as a common denominator for Wnt signaling [88, 89]. β-Catenin is peculiar in that it seems to be both a skeletal factor and a signaling-regulated transcription cofactor. It serves as a relay step to integrate extracellular signals into the transcriptional events.

Axin (aa 437–506) interacts directly with the region containing Armadillo repeats 2–7 of β-catenin [38]. This region of Axin is adjacent to its GSK-3β-binding site. GSK-3β and β-catenin could bind simultaneously to Axin, forming a ternary complex. This complex formation enhances phosphorylation of β-catenin by GSK-3β and CKIα [38, 81]. Phosphorylated β-catenin is recognized by β-TrCP, a component of an E3 ubiquitin ligase, and is degraded via the ubiquitin-mediated proteasome pathway [90]. Activation of Wnt signaling results in displacement of GSK-3β from the complex by Dishevelled and Frat (GSK-3β-binding protein or GBP) and allows accumulation of β-catenin that enters the nucleus. Inside the nucleus, it complexes with a variety of transcription factors/cofactors to regulate a number of cell growth/cell differentiation related genes including *Siamese* [91], *Twin* [92], *Xnr-3* [93], *Ubx* [94], *cyclin D1* [95], and *c-MYC* [96].

Protein Phosphatase 2A

PP2A is an intracellular serine/threonine protein phosphatase. It is a heterotrimeric protein comprising a conserved catalytic subunit (C), a structural subunit (A), and one of variable regulatory (B) subunits. Three unrelated families of PP2A B subunits have been identified, i.e., B, B56, and PR72. These regulatory B subunits regulate the subcellular localization and substrate specificity of PP2A. Distinct PP2A heterotrimers dephosphorylate different substrates or different sites on the same substrate [97].

A domain of Axin (aa 508–712) was found to physically interact with the catalytic subunit of PP2A [12, 98]. However, there seems to be another PP2A-interacting domain on Axin. In a separate study, PP2A was shown to interact with another domain of Axin (aa 298–506), in addition to the aa 508–712 region. Interestingly, the B56 subunit of PP2A interacts with APC, Dvl, in the Axin-GSK3β-β-catenin complex [97, 99–101], while the C subunit interacts with Axin as mentioned above. Increased expression of B56 decreases the abundance of β-catenin, thereby reducing β-catenin signaling. The biochemical role of the interaction between the PP2A C/B heterodimer and the β-catenin degradation complex is unknown. One report shows that PP2A dephosphorylates APC [98]. However, this finding does not seem to address how β-catenin levels are reduced in cells overexpressing PP2A, as it has been reported that phosphorylation of APC is important for β-catenin binding and its subsequent degradation [102]. The role of PP2A became more confusing by another finding that the catalytic subunit of PP2A exerts a positive role in Wnt signal transduction [99].

Casein Kinases

The CKI family of protein kinases is a group of highly related and ubiquitously expressed serine/threonine kinases found in all eukaryotic organisms from Protozoa to man. Recent studies have elucidated diverse roles for CKI in regulating critical processes such as Wnt signaling, circadian rhythm, nuclear import, and Alzheimer's disease progression [103–106]. In Wnt signaling, using expression cloning to identify novel components of the Wnt pathway, two independent groups first identified CKIε, but not CKIα, as a positive factor that can mimic wnt in inducing axis duplication in *Xenopus* [107, 108]. Inhibiting CKIε in *C. elegans* generates worms with a mom phenotype, also indicative of a loss of Wnt signals [107]. CKIε functions downstream of Dvl and upstream of GSK-3β and interacts with Dvl [109]. Biochemically, CKIε was shown to phosphorylate multiple components in the β-catenin degradation complex: APC, Axin, Dvl, and β-catenin [109]. Most recently, the functional significance of CKIε phosphorylation of Dvl was found to enhance the interaction between Dvl and Frat. The enhanced DvlFrat interaction presumably causes a conformational change that leads to dissociation of GSK-3β from Axin and recruits it to Frat [110], reducing phosphorylation of β-catenin by GSK-3β, as previously proposed [111]. However, in a separate study, CKIε was found to interact with an ankyrin repeat protein called diversin and facilitates β-catenin degradation, hence act-

ing as an inhibitor in canonical Wnt signaling. It is unclear how the discrepancy arose. Future work with more genetic evidence should clear the cloud over the interpretation of the actual role of CKI ϵ . Nevertheless, the available data appear to be consistent with the view that CKI ϵ and CKI δ interact directly with Dvl and positively regulate the canonical Wnt pathway by destabilizing the Axin-PP2A complex [109] and stabilizing Frat1 binding to Dvl [110].

CKI α was found also to interact with Axin [24, 26, 81]. Interestingly, unlike CKI ϵ , CKI α enhances β -catenin degradation by serving as a priming kinase that phosphorylates β -catenin on the C-terminal serine residue, for GSK-3 β to phosphorylate N-terminal serine/threonine residues in the proteasome-mediated degradation of β -catenin [81]. CKI α seems to bind to two disparate domains of Axin [26], with one in the MEKK1-binding domain and the other in the PP2A-binding region. CKI α constitutively phosphorylates β -catenin on S⁴⁵ in an unregulated manner, consistent with β -catenin being degraded constantly in the absence of Wnt signaling [112].

Dishevelled and Ccd1

The DIX domain (Dishevelled/Axin homologous domain) in Axin is between aa 757 to 820, twelve residues from the C-terminal end. Axin forms homodimers via its DIX domain [12]. As the name implies, the DIX domain is conserved in Dishevelled (Dvl) [113], and the identity between the DIX domains of Axin and Dvl is 37%. This similarity raises the possibility that Dvl might also associate with Axin through the DIX domain. Indeed, Kishida et al. [6] reported that Dvl-1 forms a complex with Axin and that the DIX domain of Axin is sufficient for the complex formation of Dvl-1. Experiments with various deletion mutants of GST-Axin indicate that aa 530–712 of Axin are also important for its direct interaction with Dvl-1 [6].

Dvl proteins (Dvl-1–3) are intracellular multimodular factors that positively transduce Wnt signals through interaction with the specific Wnt receptor Frizzleds to inactivate GSK-3 β kinase activity. Although the exact mechanism of Dvl inhibition of GSK-3 β is not clear, Dvls are known to interact with CKI [107], CKII [114], and GBP/Frat1 [7, 115]. Dvls interact with Axin via their conserved DIX domain, regulating the β -catenin degradation complex APC/Axin/GSK-3 β / β -catenin/CKI α . It is believed that activated Dvls destabilize GSK-3 β binding to Axin by virtue of enhancing the GBP/FRAT interaction with GSK-3 β [110], attenuating β -catenin phosphorylation and degradation. It has also been reported that an

N-terminal sequence of Axin could interact with the PDZ domain of Dvl [7].

Recently, based on sequence similarity, another DIX-domain-containing protein has been identified, named Ccd1 for coiled-coil DIX1, from both zebrafish and mouse. Ccd1 forms homodimer and heterodimer with Axin and Dvl. Ccd1 acts as a positive regulator for Wnt signaling, in that it activates TCF-dependent transcription, and its dominant-negative form (DN-ccd1) can rescue phenotypes resulting from overexpression of wnt8 [14].

Axam

Axam was identified as a novel Axin-binding protein that inhibits the Wnt signaling pathway by downregulating β -catenin signaling. Expression of various deletion mutants of Axin with Axam in COS cells showed that the region containing residues 507–712 of Axin is mainly responsible for the interaction with Axam [15]. The C-terminal region of Axam has an amino acid sequence similar to that of the catalytic region of SENP1, a SUMO-specific protease. Axam exhibited the activity to remove SUMO from sumoylated proteins in vitro and in intact cells. Neither the Axin-binding domain nor the catalytic domain alone was sufficient for the downregulation of β -catenin. An Axam fragment that contains both domains was able to decrease the level of β -catenin. Upon substitution of Ser for Cys⁵⁴⁷ in the catalytic domain, Axam lost its desumoylation activity and decreased the activity to downregulate β -catenin. These results demonstrated that Axam functions as a desumoylation enzyme to downregulate β -catenin and suggested that sumoylation is involved in the regulation of the Wnt signaling pathway [116]. Most recently, Axam has been shown to reduce TCF-4 sumoylation catalyzed by E3 ligase PIASy [117].

Low-Density Lipoprotein Receptor Related Proteins

The LRPs are structurally similar to those of the LDL receptor gene family that have been considered to be prototypes of cargo receptors for delivery of macromolecules into cells via endocytosis. However, the identification of additional members of the LDL receptor family and the elucidation of their biological functions have revealed that LRPs play diverse biological roles, particularly in cellular signal transduction [118]. LRP5 and LRP6 and their *Drosophila* homologue Arrow have been implicated in the reception of Wnt signals [3, 4, 119, 120]. Arrow acts downstream of Wnt, but upstream of Dvl, and as a membrane protein, it interacts with Frizzleds to initiate signaling [4]. In fact, LRP5,6/Arrow function as coreceptors

with Frizzled proteins. Wnt provides a bridging function in assembling a complex of Frizzled and LRP6, at least for the particular combination of mFz8/mWnt-1/mLRP6 [120, 121].

Axin was shown to interact with the intracellular domain of LRP5, an interaction that is enhanced by Wnt signals [3]. This interaction could cause translocation of Axin to the membrane. Interestingly, the region from the N-terminal to the GSK-3 β -binding domain of Axin seems to prevent binding with LRP. The molecular/biochemical nature of the Wnt-enhanced Axin-LRP interaction remains to be determined. A recent report demonstrated that the consequence of Arrow-mediated relocation of Axin to the membrane leads to Axin degradation, so that β -catenin signaling is enhanced [5].

Diversin

Diversin is a recently reported vertebrate protein that interacts with two components of the canonical Wnt pathway, CK1 ϵ and Axin2/Conductin [13]. Diversin is distantly related to Diego of *Drosophila* which functions in a pathway that controls planar cell polarity. Morpholino-based gene ablation in zebrafish shows that Diversin is crucial for axis formation which depends on β -catenin signaling. It is shown that Diversin acts as a molecular switch that suppresses Wnt signals mediated by the canonical β -catenin pathway and stimulates signaling via JNK. It has also been alleged that Diversin recruits CK1 ϵ to the β -catenin degradation complex that consists of Axin/Conductin and GSK-3 β and allows efficient phosphorylation of β -catenin, inhibiting β -catenin/Tcf signals. However, in another study [81], CK1 α , but not CK1 ϵ , was shown to prime GSK-3 β phosphorylation of β -catenin; it is unclear how the discrepancy arose.

I-mfa

I-mfa is an inhibitor of myogenic basic-helix-loop-helix transcription factors. It was shown that I-mfa interacted in vivo with the Axin complex through its C-terminal I-mfa domain, and this interaction inhibited Axin-mediated downregulation of free levels of cytosolic β -catenin. I-mfa was also shown to decrease Axin-mediated JNK activation [20].

Regulation of Axin

Axin itself seems to be highly regulated, through phosphorylation and intracellular translocation. Wnt signaling induces dephosphorylation of Axin [17, 47]. The dephos-

phorylated Axin binds β -catenin less efficiently than the phosphorylated form. Thus, Wnt-induced dephosphorylation may be important to prevent the phosphorylation of β -catenin by GSK-3 β , so that β -catenin can accumulate to high levels and activate transcription in concert with TCF/LEF. A recent study provided strong evidence, in transgenic flies that express a fusion protein of the cytoplasmic domain of Arrow/LRP5/6 linked to the C-terminal of the Wnt receptor Frizzled-2, that Wnt signaling is regulated by the levels of Axin protein [5]. The physical proximity of LRP to the Wnt receptor recruits Axin to the membrane, where Axin is somehow degraded. As a consequence, β -catenin is no longer bound by Axin, resulting in nuclear signaling by β -catenin. A separate study also reported that Wnt signaling causes a striking relocation of *Drosophila* Axin from the cytoplasm to the plasma membrane [16]. This relocation depends on Dvl and may permit the subsequent inactivation of the Axin complex by Wnt signaling. This LRP-mediated regulation of the Axin stability bypasses GSK-3 β , the kinase normally required for controlling β -catenin levels. This new finding argues for a different regulatory mechanism of Wnt signaling, proceeding through destruction of the negative regulator Axin rather than through the inhibition of the GSK-3 β kinase activity. Consistent with the argument that the Axin stability is regulated are the observations that Axin is phosphorylated by GSK-3 β and that the phosphorylated form is more stable than the unphosphorylated form [17]. However, it remains to be seen whether the regulatory role of phosphorylation by GSK-3 β in Axin stability is actually modulated by LRP5/6.

Axin Acts as a Scaffolding Protein to Coordinate β -Catenin Degradation in Wnt Signaling

Based on yeast two-hybrid screenings, Axin was initially found to interact with GSK-3 β , β -catenin, and APC, forming a core multicomponent complex that governs cellular abundance of β -catenin [8–10, 38, 45, 74]. In the Axin complex, β -catenin and GSK-3 β bind adjacently within the central region of Axin. Axin proteins that lack the β -catenin- or GSK-3 β -binding regions fail to induce downregulation of β -catenin expression when overexpressed in SW480 cells. In PEL cells and KS tissue, LANA, a nuclear expressing protein with sequence homology to Axin GSK-3 β binding site, stimulates the S phase relocalization of GSK-3 β , and this function requires its GSK-3 β -binding domain. Trapping of GSK-3 β

in the nucleus by LANA renders GSK-3 β unable to bind Axin and β -catenin and to degrade β -catenin [80]. Thus, Axin was demonstrated to facilitate the interaction of β -catenin with GSK-3 β and to act as a scaffold upon which related proteins assemble to coordinate the regulation of β -catenin levels for Wnt signaling. Upon Wnt activation, the Axin complex receives signals via LRP and Dvl, and the assembly of the complex is regulated to induce the dissociation of GSK-3 β from the β -catenin degradation complex, presumably augmented by FRAT/GBP and CKI ϵ . Alternatively, Wnt signals lead to Axin translocation to the membrane, where Axin is degraded, so that β -catenin is not occupied by the Axin-based degradation complex and remains stable to manifest Wnt signaling in the nucleus.

Axin in the JNK Pathway

Mitogen-activated protein (MAP) kinases are instrumental in integrating numerous signals into biological processes, including embryogenesis, cell differentiation, cell proliferation, and cell death [122]. It is generally known that a MAP kinase cascade contains at least three components, namely MAP3K, MAPKK, and MAP kinase. A number of scaffold proteins for different MAP3Ks have been identified, including Ste5p, JIPs, and MP1 [123]. It was demonstrated that Axin also exerts a function in the MAP kinase pathway. Overexpression of Axin in HEK293 cells leads to differential activation of MAP kinase, with robust induction for c-Jun NH₂-terminal kinase (JNK)/stress-activated protein kinase, moderate induction for p38, and negligible induction for extracellular signal regulated kinase [18]. Determination of the functional domains of Axin reveals that domains utilized for the Wnt pathway are distinct from those for the JNK pathway. Domains essential in Wnt signaling, i.e., binding sites for APC, GSK-3 β , and β -catenin, are not required for JNK activation by Axin.

Axin Binds to MEKK

Axin seems to differ from those scaffold proteins such as JIP and MP1, in that it does not interact with either MKK4/7 or JNK directly. Rather, it interacts with and activates MEKK directly which in turn somehow activates MKK4/7 and then JNK. One of the most intriguing features of the Axin-mediated JNK activation is that it requires multiple other factors to enable the use of its multiple domains which differ from those used in the Wnt pathway to facilitate the activation of JNK.

Axin forms a complex with MEKK1, but not ASK1 or TAK1, through a domain termed MEKK1-interacting domain (MID) [18]. Axin without the MID domain has a dominant-negative effect on JNK activation by wild-type Axin. Both MKK4 and MKK7, which are downstream of MEKK1, are involved, as their dominant-negative forms can attenuate Axin-mediated JNK activation. The MID domain has been mapped to around aa 244–329, flanked by the binding sites for APC and GSK-3 β [18, 19]. In addition, we have recently identified MEKK4 as a new Axin-binding protein that mediates Axin-induced JNK activation [19]. Dominant-negative MEKK4 diminishes Axin-mediated JNK activation. MEKK4 mediates Axin activation of JNK in the MEKK1^{-/-} embryonic fibroblast cells. Surprisingly, MEKK4 and MEKK1 competes against each other for Axin binding, although MEKK4 binds to a region of aa 678–712 which is inside the PP2A-binding domain and distant from the MEKK1-binding site. Another domain between MEKK1- and MEKK4-binding sites was also identified as being required for both MEKK1 and MEKK4 to activate JNK via Axin. Whereas removal of either the MEKK1-binding site or the MEKK4-binding site did not affect JNK activation by Axin, deletion of a small region encompassing aa 642–673, N-terminal to the MEKK4-binding site, completely abolished Axin activation of JNK, although these mutants are intact in MEKK1 or MEKK4 binding. It remains an interesting issue as to how and why Axin-mediated JNK activation requires this region.

Homodimerization Is Required for Axin-Mediated JNK Activation

Another region of Axin essential for JNK activation is its C-terminal region, aa 507–832, in which the extreme C-terminal segment serves as a dimerization/oligomerization site [12]. It was found that Axin needs to be dimerized for JNK activation, although MEKK is capable of binding C-terminus-deleted monomeric Axin [18]. Consistently, Dvl heterodimeric interaction with Axin can also abolish JNK activation by Axin. Axin with the MEKK1-binding domain deleted could prevent wild-type Axin from activating JNK, indicating that Axin requires MEKK1 to assume a dimeric configuration which may be an important feature of MEKK activation in the Axin/JNK pathway. It is equally possible that Axin dimerization is required for recruitment of other factors to activate MEKK.

Participation of Sumoylating Enzyme PIAS Proteins in the Axin/JNK Pathway

The Axin C-terminal region also exerts its role in JNK activation in another way. It was recently found that Axin interacts with three SUMO-1 (small ubiquitin-related modifier) conjugating enzymes 3 (E3), PIAS1, PIASxb, and PIASy, by a yeast two-hybrid screen using the extreme C-terminal 145-aa region of Axin as bait [27]. The extreme C-terminal six amino acid residues (KVEKVD) of Axin are identified as two contiguous sumoylation sites. Sumoylation represents one of the most important posttranslational modifications, modulating a wide spectrum of proteins that participate in protein translocation, transcriptional regulation, signal transduction, and cell growth control [24, 123–128]. It was discovered that Axin is a heavily sumoylated protein at the C-terminal end. Interestingly, removal of either of the two sites diminished JNK activation by Axin. Removal of both of the sites (Axin Δ C6) rendered Axin virtually to have no JNK-activating activity, but has no effect on its ability to effectively destabilize β -catenin and attenuate LEF1 transcriptional activity. Loss of the JNK-activating ability in Axin Δ C6 is not caused by its failure to form homodimer or to bind to MEKK. It is unclear how sumoylation plays a role in the Axin-induced JNK activation. Possibly, sumoylation helps to regulate protein-protein interactions among the many factors that participate with MEKK to activate JNK. It is interesting to note that both PIAS and Axin are capable of activating JNK and causing apoptosis in a JNK-dependent manner. Remarkably, Axin mutants act dominant negatively on the PIAS-mediated JNK activation. Therefore, PIAS acts either upstream or at a position parallel to Axin [27].

Thus, multiple domains, including MEKK1- and MEKK4-binding sites, DIX domain, the extreme C-terminal six amino acids, and an unnamed domain flanked by the β -catenin-binding site and the MEKK4-binding site, are found at present to be required for JNK activation by Axin. How these factors coordinate to mediate JNK activation via Axin remains unclear.

Inhibition of Axin-Mediated JNK Activation by Wnt Components

In addition to inhibition of Axin activation by Dvl due to disruption of homodimerization, other components in the Wnt pathway also attenuate JNK activation by Axin. These include GSK-3 β , CK1 α , and CK1 ϵ . It was shown that both wild-type GSK-3 β and kinase-dead GSK-3 β -Y216F (capable of binding to Axin), but not GSK-3 β -K85M (incapable of binding to Axin in mammalian cells),

prevented MEKK1 binding to the Axin complex, thereby inhibiting JNK activation [24]. Similarly, CKIs also inhibited Axin-mediated JNK activation by competing against MEKK1 binding [24]. In contrast, β -catenin and APC binding did not affect MEKK1 binding to the same Axin complex, indicating that MEKK1, β -catenin, and APC can be copresent in the same Axin complex. These observations suggest that even when Axin is 'switched' to activate the JNK pathway, it is still capable of sequestering free β -catenin which is a critical aspect for cellular homeostasis. The above studies also indicate that there exist differential molecular assemblies on Axin between the Wnt pathway and the Axin/JNK pathway. The extra-cellular or cellular factors that modulate the distinct complex formations remain to be elucidated. More pressing tasks are to identify the biological function of the Axin/JNK pathway.

Axin in Other Signaling Pathways

The number of Axin-interacting proteins seems to be ever enlarging. Axin also regulates the effects of Smad3 in the transforming growth factor beta (TGF- β) signaling pathway and functions as an adaptor for Smad3, facilitating its activation by TGF- β receptors for efficient TGF- β signaling [23]. In the absence of activated TGF- β receptors, Axin physically interacted with Smad3 through its C-terminal region located between the β -catenin-binding site and DIX domain. Axin2 also interacted with Smad3. In the absence of ligand stimulation, Axin was colocalized with Smad3 in the cytoplasm *in vivo*. Upon receptor activation, Smad3 was strongly phosphorylated by TGF- β type I receptor in the presence of Axin and dissociated from TGF- β type I receptor and Axin. It will be of great interest to elucidate the functional roles of Axin in TGF- β signaling in animals, given that TGF- β signaling is known to play important roles in development [129].

Eph receptors and ephrins exhibit a unique mode of signaling in that each molecule can function as both a ligand capable of sending signals and a receptor capable of transducing signals [130, 131]. In a modified yeast two-hybrid screen that allows for the detection of protein-protein interactions involving phosphotyrosine, using a murine neonatal brain complementary DNA library, the SH2/3 domain adaptor protein Grb4 was identified to interact with the ephrin-B1 cytoplasmic domain in a phosphotyrosine-dependent manner. Grb4 uses its SH2 domain to bind to ephrin-B1 and associates with the reverse signaling of the bidirectional signals that are trans-

duced by Eph receptors and ephrins. Interestingly, the SH3 domain of Grb4 was demonstrated to interact with Axin in another yeast two-hybrid screening [22]. The Eph/ephrin signals are thought to control cell and axon growth-cone movement by repulsion, presumably by inducing changes in the cytoskeleton. The DIX domain that is conserved in Axin, Dvl, and Ccd1 was demonstrated to mediate targeting to actin stress fibers and cytoplasmic vesicles [64]. It remains to be seen whether Axin can link Eph/ephrin signaling to the actin cytoskeleton.

Perhaps the most surprising Axin partner is the CAP protein, a component in the signaling pathway required for insulin-dependent glucose transport [132]. In a yeast two-hybrid screen using the *Drosophila* Axin protein as bait, Axin was found to interact with D-CAP that harbors an SH3 domain at the C terminus. Knockdown of Axin by RNAi in S2 cells results in increased accumulation of glycogen, suggesting that Axin enhances glycogen decomposition and possibly that there is a cross talk between Wnt signaling and insulin signaling [133].

Concluding Remarks

Since its initial cloning, Axin has turned out to be a remarkable protein that exerts a pleiotropic scaffolding role. Clearly, it serves as a central platform for the APC-GSK-3 β - β -catenin-CK- β -catenin degradation complex as well as a major scaffold for the JNK pathway. In the light of the fact that Axin interacts with multiple proteins that participate in different signaling pathways besides the Wnt pathway, it could mediate cross talks among diverse signaling pathways that play critical roles in cell fate decision and suppression of tumorigenesis. In fact, from the evidence we have thus far gathered, Axin controls biological processes ranging from sugar intake, cell proliferation, and organ development to cell death. It would not be surprising that we will find yet more roles of Axin, both biochemical and biological.

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