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Unexpectedly robust assembly of the Axin destruction complex regulates Wnt/Wg signaling in *Drosophila* as revealed by analysis *in vivo*

Wynne Peterson-Nedry [1], Naz Erdeniz [1], Susan Kremer, Jessica Yu, Shahana Baig-Lewis, Marcel Wehrli *

Department of Cell and Developmental Biology, Oregon Health and Science University, 3181 SW Sam Jackson Park Road/L215, Portland, OR 97239-3098, USA

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ABSTRACT

Secreted proteins in the Wnt family regulate gene expression in target cells by causing the accumulation of the transcriptional activator β-catenin. In the absence of Wnt, a protein complex assembled around the scaffold protein Axin targets β-catenin for destruction, thereby preventing it from transducing inappropriate signals. Loss of Axin or its binding partners APC and GSK3 results in aberrant activation of the Wnt signaling response. We have analyzed the effects of mutant forms of *Drosophila* Axin with large internal deletions when expressed at physiological levels *in vivo*, either in the presence or absence of wild type Axin. Surprisingly, even deletions that completely remove the binding sites for fly APC, GSK3 or β-catenin, though they fail to rescue to viability, these mutant forms of Axin cause only mild developmental defects, indicating largely retained Axin function. Furthermore, two lethal Axin deletion constructs, AxinΔRGS and AxinΔβcat (ΔArm), can complement each other and restore viability. Our findings support a model in which the Axin complex is assembled through cooperative tripartite interactions among the binding partners, making the assembly of functional complexes surprisingly robust.

Introduction

Canonical Wnt signaling is critically important for many aspects of embryonic development and for the maintenance of functional stem cells in adults. Besides causing severe developmental defects, dysregulation of Wnt signaling also results in a variety of disorders associated with misregulation of cell proliferation and differentiation, including abnormal changes in bone mass and osteoporosis, colorectal cancer, and medulloblastoma (reviewed in Logan and Nusse, 2004; Reya and Clevers, 2005). Wnts are secreted glycoproteins that function as morphogens during embryogenesis. Activation of the Wnt signaling cascade in target cells requires the binding of Wnt ligands to receptor complexes that consist of Frizzleds plus members of the *L*DL-receptor-related *p*rotein (LRP) family of transmembrane receptors (Arrow in *Drosophila;* LRP5 and LRP6 in vertebrates). This receptor complex can apparently activate a number of signaling components, the most critical of which is Dishevelled. In turn, Dishevelled inhibits a constitutively active protein complex, termed the destruction complex, which is responsible for maintaining the "off" state of the Wnt signaling pathway by targeting β-catenin for degradation. This destruction complex consists of the scaffold protein Axin, which binds two other key components, Adenomatous Polyposis Coli (APC) and Glycogen Synthase Kinase-3 (GSK-3). While active, the Axin complex is thought to promote the phosphorylation of β-catenin by GSK-3, triggering the subsequent ubiquitination and degradation of β-catenin by the proteasome. Thus, despite the constitutive production of β-catenin, its levels in the cytoplasm are kept low by the destruction complex. When the destruction complex is inhibited by Dishevelled, however, β-catenin accumulates in the cytoplasm and enters the nucleus, where it binds TCF/Lef transcription factors and activates the expression of genes required for the Wnt signaling response.

In vertebrates, two genes each encode Axin, APC and GSK3 (Polakis, 2007), while only a single Axin gene and one GSK-3 orthologue Shaggy (Sgg)/Zeste-white3 (Zw3) are present in *Drosophila*, although two partially redundant APC genes have been identified. Complete loss of function of Axin or Shaggy, or loss of both APC genes in flies, results in identical phenotypes that mimic a fully activated Wnt pathway. These results demonstrate that a functional Axin destruction complex is critically important for the normal control of Wnt signaling. However, our understanding of the Axin destruction complex derives largely from *in vitro* biochemical analyses, which have shown that each combination of Axin, APC, GSK3 and β-catenin can be co-immunoprecipitated (reviewed in Luo and Lin, 2004); Axin itself is also capable of forming homodimers (Hedgepeth et al., 1999; Sakanaka and Williams, 1999; Julius et al.,

* Corresponding author.
E-mail address: wehrlim@ohsu.edu (M. Wehrli).
[1] Joint first authors.

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2000; Luo et al., 2005). In several instances, overexpression of mutated forms of Axin lacking the binding sites for these other components was shown to disrupt the function of the complex (Nakamura et al., 1998; Fagotto et al., 1999; Hedgepeth et al., 1999; Hinoi et al., 2000; Kishida et al., 1998; Sakanaka and Williams, 1999; Willert et al., 1999a; Zeng et al., 1997; Yanagawa et al., 2000) although this strategy has also produced contradictory results. For example, overexpressing a mutant Axin lacking its APC binding (RGS) domain produced the same effects as wild type Axin in the developing *Drosophila* wing, while overexpressing a similar mutant form of the Axin in *Xenopus* embryos or tissue culture dominantly interfered with the function of the destruction complex (Cliffe et al., 2003; Nakamura et al., 1998; Willert et al., 1999a; Fagotto et al., 1999; Hedgepeth et al., 1999; Yanagawa et al., 2000; Cliffe et al., 2003; Tolwinski et al., 2003). Such paradoxical effects raise concerns about cell-type and species-specific differences in the dynamics of the destruction complex, negative interference with endogenous wild type protein, and artifacts associated with the overexpression of proteins involved in signal transduction.

An important issue that remains unknown concerns the stochiometric composition of the destruction complex. In particular, it is still not known which complement of Axin binding partners is essential, whether dimeric Axin is required *in vivo* for fully functional destruction complexes, and whether an Axin protein complex can still function without full occupancy of its binding sites. Given the fact that 44 different configurations of the Axin complex are formally possible (if only Axin, APC, GSK3 and β-catenin are considered), an *in vivo* analysis of its functional stochiometry would provide critical information about how physiological interactions among its different components may modulate Wnt signaling. To date, an analysis of how binding site-deficient isoforms of Axin affect the function of the destruction complex has yet to be performed with physiological concentrations of the proteins in their normal cellular context. To address this issue in this study, we expressed either wild type Axin or mutant forms of the protein (lacking the specific binding sites for different components of the destruction complex) at near-physiological levels in flies lacking endogenous Axin. Contrary to our expectation that many of these mutant proteins would result in *axin*^{null} phenotypes, all of the deletion constructs were able to rescue the *axin*^{null} phenotype, to varying degrees. This *in vivo* analysis indicates a highly cooperative assembly of the destruction complex, involving the recruitment of the different components via indirect interactions with other bound partners. As a result, the Axin-based destruction complex behaves in a surprisingly robust manner, ensuring the proper regulation of β-catenin levels in the context of Wnt signaling.

Results

Full rescue of the axin^{null} mutant by ubiquitously expressed Axin

To validate our strategy for analyzing the structure–function relationships of Axin *in vivo*, we first used the tubulin promoter to drive ubiquitous expression of full-length (FL) FLAG epitope-tagged Axin (here referred to as FLAxin or tub>FLAxin; Fig. 1. All constructs in this study are FLAG-tagged). Transgenic flies expressing tub>FLAxin in addition to endogenous wild type Axin showed no visible defects (see Figs. 1E–G, and data not shown). In Western blots, ectopic Axin could be readily distinguished from endogenous protein by its 6xFLAG tag and higher mobility: 98 kDa versus ~88 kDa for the native protein consistent with the slower mobility of Axin observed previously (Behrens et al., 1998; Itoh et al., 1998; Willert et al., 1999a). When we examined the relative levels of endogenous and ectopic Axin in immunoprecipitates of extracts from wild type and FLAxin expressing wild type embryos, we found that the expression levels of the FLAG-tagged FLAxin were ~4.3 fold higher than endogenous Axin (Figs. 1B, C; Suppl. Fig. 1). As described below, tub>FLAxin expression rescued both the viability and fertility of *axin*^{null} mutant flies, demonstrating that this FLAG-tagged construct of full-length Axin is functional and expressed at sufficient levels to support all stages of development.

Importantly, no defects were observed in adult structures, including the wing (Suppl. Fig. 2). Wing margin bristles are particularly sensitive indicators for proper levels of Wg signaling, since they only form where maximal levels of Wg signal are present. Excess signaling allows ectopic bristles to differentiate; conversely, lower levels of signaling results in the loss of bristles, while severe reductions in Wg signaling causes the loss of wing tissue, manifested by notching in the wing (Couso et al., 1994; Axelrod et al., 1996; Cadigan et al., 1998; Baig-Lewis et al., 2007). Notably, when we examined the wing margin in tub>FLAxin *axin*^{null} flies, we observed none of these defects (Figs. 1G, G′, and data not shown). To determine whether with our constructs higher levels of Axin could also induce wing defects, we created a UAS construct of Axin and drove its expression with the ptc-Gal4 promoter construct. Previous studies have shown that ptc-Gal4 drives high levels of expression in a stripe anterior to the anteroposterior compartment boundary within the developing wing, a pattern that intersects the future wing margin (Figs. 1F–G). However, the wings of these flies showed no defects (Fig. 1G). Therefore, to increase the expression of FLAG-Axin still further and also provide an internal reference to distinguish ectopic from endogenous Axin, we expressed ptc-Gal4/UAS-Axin in the tub>-FLAxin flies. Whereas our data indicated that tub>FLAxin levels are ~4.3 fold higher than endogenous Axin (assuming a linear increase in fluorescence associated with anti-FLAG(Axin) immunoreactivity (Fig. 1B), we estimated that maximal levels of Axin in the ptc-Gal4 stripe of these flies were ~2 fold higher than induced by tub>FLAxin alone (Fig. 1F, Suppl. Fig. 3D), ~8.6 fold above endogenous Axin levels. Nevertheless, adult wings showed no defects (Figs. 1G, G′). Similarly, we detected no visible defects in the embryos of any of these lines, although the survival rate of the tub>FLAxin animals was ~20% lower than wild type (Fig. 2A). Despite this modest reduction in viability, these results indicate that the level of Axin induced by tub>FLAxin expression is within the physiological range required for normal development. Moreover, these studies also show that Axin-dependent signaling is well-regulated *in vivo*, allowing cells to functionally compensate for fluctuations in Axin protein concentration.

Only some mutant Axin proteins interfere with wild type Axin function

Having demonstrated that full-length FLAG-tagged Axin is capable of rescuing *axin* null mutants, we next generated a series of constructs lacking each of the binding sites for other proteins in the destruction complex, as previously determined in immunoprecipitation assays (Behrens et al., 1998; Ikeda et al., 1998; Nakamura et al., 1998; Kishida et al., 1998; Sakanaka and Williams, 1999; Yamamoto et al., 1998; Fagotto et al., 1999; Hamada et al., 1999). Axin domains that would be considered most critical for Axin function are the binding sites for APC (the Axin RGS domain), Sgg kinase (fly GSK3), Armadillo (fly β-catenin), and the DIX domain. The DIX domain is thought to bind Dsh and also is part of an Axin homodimerization domain that extends into the adjacent PP2A domain (Sakanaka and Williams, 1999; Hedgepeth et al., 1999; Julius et al., 2000). In addition, we also designed constructs with deletions of large *i*ntervening sequence between the RGS and Sgg-binding domains, termed the "I" domain, and the large domain between Arm-binding domain and DIX domain; this latter domain also contains a binding site for the catalytic subunit of protein phosphatase 2A (PP2A). Transgenic flies were generated that expressed each construct under control of the tubulin promoter, plus an inducible flip-out cassette to prevent uncontrolled or leaky expression (for details, see Materials and methods and Supplementary Fig. 4). As shown in Fig. 1C, Western blot analysis revealed that all of these constructs could be readily detected in embryonic lysates at