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Control of β-Catenin Stability: Reconstitution of the Cytoplasmic Steps of the Wnt Pathway in *Xenopus* Egg Extracts

Adrian Salic,† Ethan Lee,†
Leslie Mayer, and Marc W. Kirschner*
Department of Cell Biology
Harvard Medical School
Boston, Massachusetts 02115

Summary

Regulation of β-catenin degradation by intracellular components of the wnt pathway was reconstituted in cytoplasmic extracts of *Xenopus* eggs and embryos. The ubiquitin-dependent β-catenin degradation in extracts displays a biochemical requirement for axin, GSK3, and APC. Axin dramatically accelerates while dishevelled inhibits β-catenin turnover. Through another domain, dishevelled recruits GBP/Frat1 to the APC-axin-GSK3 complex. Our results confirm and extend models in which inhibition of GSK3 has two synergistic effects: (1) reduction of APC phosphorylation and loss of affinity for β-catenin and (2) reduction of β-catenin phosphorylation and consequent loss of its affinity for the SCF ubiquitin ligase complex. Dishevelled thus stabilizes β-catenin, which can dissociate from the APC/axin complex and participate in transcriptional activation.

Introduction

The wnt signaling pathway plays diverse roles in metazoan embryonic development and is a prominent target in tumorigenesis (Wodarz and Nusse, 1998). The components of the pathway have been identified and characterized through a combination of genetics, transfection experiments in cultured cells, and overexpression studies in *Xenopus* embryos. According to the current view, in the absence of the wnt signal, a cytoplasmic complex containing the kinase GSK3, the adenomatous poliposis coli protein (APC), and axin catalyzes the phosphorylation of the transcriptional coactivator β-catenin. Phosphorylated β-catenin is recognized by β-TRCP, an F box protein that is a subunit of a ubiquitin ligase complex (SCF) which ubiquitinates β-catenin and targets it for degradation by the proteasome (Aberle et al., 1997). When the pathway is active, binding of wnt to a frizzled receptor (Bhanot et al., 1996) activates the intracellular protein, dishevelled (dsh), through an as yet unknown mechanism. Activated dsh inhibits β-catenin degradation and increases its steady-state level in the cell. β-catenin is thus free to bind a DNA-binding protein of the Tcf3/Lef1 family (Molenaar et al., 1996), and together they turn on the transcription of target genes. A novel protein called GSK3-binding protein (GBP) has a required role in this pathway (Yost et al., 1998). Despite the progress made in identifying new components, many mechanistic aspects of wnt signaling still remain obscure. Recent reviews (see, for example, Cadigan and Nusse, 1997; Brown and Moon, 1998; Wodarz and Nusse, 1998; Sokol, 1999) present models that differ significantly in describing how the signal is propagated through the pathway and what proteins and interactions are functionally important. An incomplete list of poorly understood aspects in wnt signaling includes: (1) how wnt-bound frizzled activates dsh; (2) how dsh interacts with GSK3/APC/axin/β-catenin complex and stabilizes β-catenin; (3) how β-catenin is recognized by the degradation machinery; (4) how GBP is regulated during wnt signaling; (5) what role is played by the tumor suppressor APC; and (6) what function, if any, is performed by other proteins that bind to known components of the pathway.

A notable obstacle to a better understanding of wnt signaling has been the lack of a biochemically tractable, in vitro system for studying the kinetics and the molecular associations between components of the pathway. Since wnt signaling is active in early *Xenopus* development, we have developed extracts from both *Xenopus* eggs and embryos to study the pathway biochemically. We reconstituted signaling downstream of dsh and examined the regulation and requirements for β-catenin turnover, the mechanism by which dsh and GBP inhibit the degradation of β-catenin, and the role of APC-axin-β-catenin interactions.

Results

Reconstitution and Characteristics of β-Catenin Degradation in Extracts

In *Xenopus* egg extracts, radiolabeled β-catenin is degraded with a half-life of 1 hr (Figure 1a). The degradation rate was half-maximal at 200 nM β-catenin and was inhibited 90% if cold β-catenin was added to a concentration of 1 μM (data not shown). Extracts can effect fast and significant changes in the concentration of β-catenin and are capable, at saturation, of degrading β-catenin at a rate of 50–100 nM/hr.

Degradation proceeded through the ubiquitin–proteasome pathway, as suggested by the conjugates that accumulated in the presence of proteasome inhibitors in low-speed extracts and by the conjugation of radiolabeled β-catenin to His_6-ubiquitin in high-speed supernatants (Figure 1b). As a prelude to identifying novel mechanistic features, we have confirmed biochemically in extracts most of the known properties of wnt signaling. Table 1 summarizes the principal characteristics of our in vitro system. To test whether β-catenin degradation in extracts involves an SCF–ubiquitin ligase complex, we added dominant-negative CDC34 (dnCDC34) protein (Yew and Kirschner, 1997). At a concentration of 2 μM (20 times the concentration of endogenous CDC34), dominant-negative CDC34 inhibited the degradation of β-catenin. Unlike the CDC34-mediated degradation of Xic1 (Yew and Kirschner, 1997), degradation of β-catenin did not require the addition of nuclei (data not shown). The F box protein of the SCF complex mediating

*To whom correspondence should be addressed (e-mail: marc@hms.harvard.edu).
†These authors contributed equally to this work.

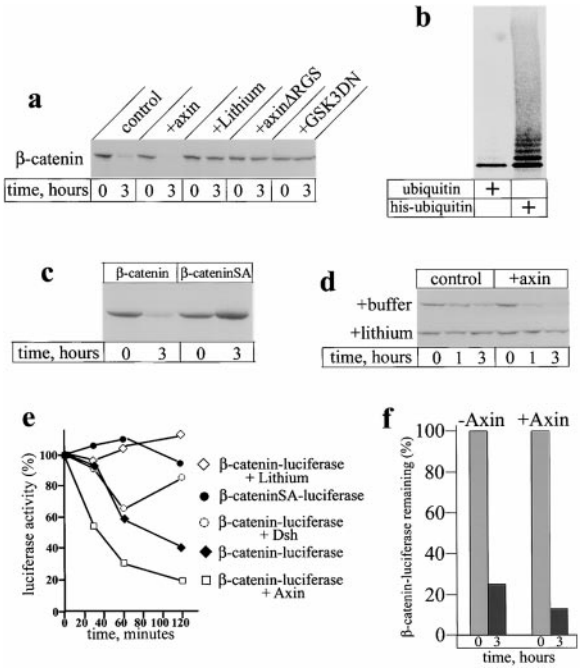


Figure 1. Reconstitution of β-Catenin Degradation in Egg Extracts

(a) Labeled β-catenin was incubated with *Xenopus* egg extract in the presence of axin (10 nM), LiCl (25 mM), axinΔRGS (50 nM), or GSK3DN RNA. (b) β-catenin is ubiquitinated in S100 supernatants. His-ubiquitin conjugates were removed from extract using nickel beads. (c) The nonphosphorylatable β-catenin mutant, β-cateninSA, is stable in extracts. (d) Axin cannot degrade β-catenin in the presence of lithium. β-catenin was incubated in extracts with either LiCl (25 mM) or control buffer with or without 25 nM axin. (e) β-catenin-luciferase fusions are degraded identically to β-catenin in extracts. Recombinant β-catenin-luciferase was incubated in egg extracts supplemented with either LiCl (5 mM), dsh (1 μM), or axin (10 nM). (f) β-catenin-luciferase is degraded in embryos. β-catenin-luciferase (4 ng) was injected into 2-cell stage *Xenopus* embryos with or without purified MBP-axin (5 ng).

β-catenin ubiquitination is slimb/β-TRCP. A slimb mutant that lacks the F box but can still bind to phosphorylated β-catenin acts as a dominant negative (Marikawa and Elinson, 1998; Fuchs et al., 1999; Kitagawa et al., 1999; Liu et al., 1999) and blocked β-catenin degradation in extracts (data not shown).

Glycogen synthase kinase 3 (GSK3) is thought to be central to β-catenin degradation. In extracts, 5–25 mM LiCl (a direct inhibitor of GSK3; Klein and Melton, 1996) completely blocked β-catenin degradation (Figure 1a). We confirmed the importance of GSK3 by translating a kinase-dead GSK3 (GSK3DN) mutant that completely inhibited degradation (Figure 1a). This result is consistent with the phenotype obtained by injection of GSK3DN RNA in *Xenopus* embryos (He et al., 1995; Yost et al., 1998). Since phosphorylation of β-catenin by GSK3 is required for degradation, we examined the stability of a nonphosphorylatable mutant (β-cateninSA, Yost et al., 1996); the protein was completely stable in extracts (see Figure 1c).

Axin, a negative regulator of β-catenin levels (Zeng et al., 1997), is rate limiting for degradation in extracts. As shown in Figures 1a and 1d, addition of axin protein to 5–10 nM above endogenous axin increases the rate of β-catenin degradation 5- to 10-fold (half-life of 15 min or less). Unfortunately, lacking an anti-axin antibody that recognizes *Xenopus* axin on blots, we were unable to measure the endogenous axin concentration. Assuming that the effects are linear, we estimate that the effective endogenous concentration of axin is about 5–10 nM; the total concentration, however, may be higher. Concentrations of added axin above 100–200 nM blocked β-catenin degradation. This was likely due to the titration of endogenous GSK3, as degradation in such cases could be fully rescued by supplementing the extract with recombinant GSK3 to 0.4–2 μM. The GSK3 concentration in extracts by quantitative Western blotting is approximately 50 nM, in good agreement with the axin level at which inhibition is observed. Axin requires GSK3 activity for function. As shown in Figure 1d, lithium blocked stimulation of β-catenin degradation by axin. In addition, axin did not promote the degradation of β-cateninSA at any concentration tested (up to 100 nM). Excess cold β-cateninSA blocked degradation of β-catenin, perhaps by titrating endogenous axin (data not shown). Axin contains an RGS domain, which, if

Table 1. Characteristics of β-Catenin Degradation in *Xenopus* Extracts

	Phenotype in *Xenopus* Embryos	Effect on β-Catenin Degradation in Extracts	Reversed by	Comments
Lithium	Axis duplication	Inhibitory	—	5–25 μM
GSK3	—	Stimulatory	Lithium	100 nM–2 μM
GSK3DN	Axis duplication	Inhibitory	GSK3	—
Axin	Inhibition of axis formation	Stimulatory at <100 nM	Lithium, APC	—
		Inhibitory at >100 nM	GSK3	—
AxinΔRGS	Axis duplication	Inhibitory	GSK3, axin	—
GBP	Axis duplication	Inhibitory	GSK3	100 nM–2 μM
CDC34DN	—	Inhibitory	Not tested	5 μM
DN-Xslimb	Axis duplication	Inhibitory	Not tested	2–5 μM
APC	Axis duplication	Inhibitory	Axin	100 nM–2 μM
β-Catenin	Axis duplication	Inhibitory	Axin	10 μM
β-CateninSA	Axis duplication	Inhibitory	—	Stronger than β-catenin
Dishevelled	Axis duplication	Inhibitory	Axin, GSK3	1 μM
Axin depletion	—	Inhibitory	Axin, not GSK3	—
GSK depletion	—	Inhibitory	GSK3, not axin	—
APC depletion	—	Inhibitory	—	—
Dsh depletion	—	No effect	—	—

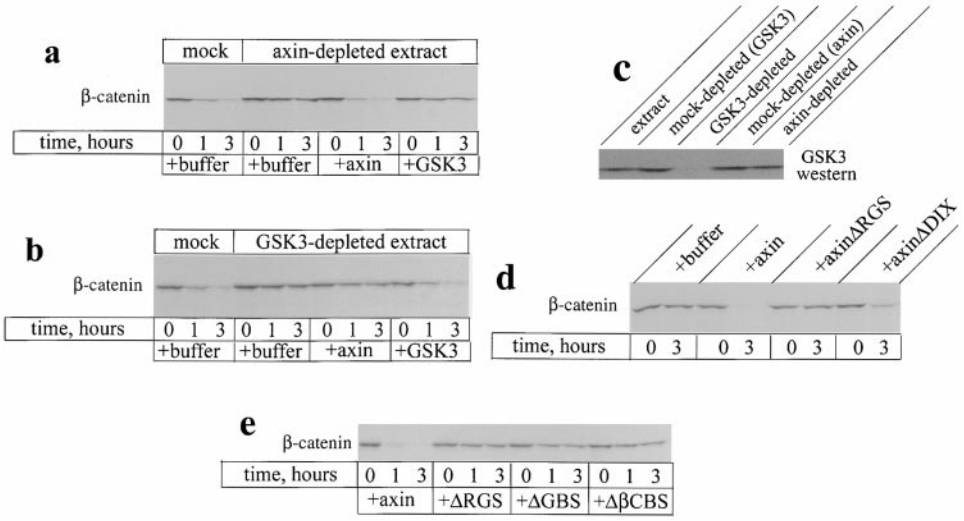


Figure 2. Axin and GSK3 Are Required for β-Catenin Degradation

(a) β-catenin is stable in axin-depleted extracts. Addition of axin (10 nM) rescues degradation. GSK3 (1 μM) cannot rescue β-catenin degradation. (b) β-catenin is stable in GSK3-depleted extracts. GSK3 (100 nM) but not axin (10 nM) rescues degradation. (c) Western blot showing disappearance of GSK3 from extracts incubated with GBP beads. (d) The DIX domain of axin is dispensable for β-catenin degradation. Axin-depleted extracts were incubated with 10 nM axin, axinΔRGS, or axinΔDIX, and the capacity to degrade β-catenin was assessed. (e) The RGS, GSK3-binding, and β-catenin-binding domains of axin are required for β-catenin degradation. Axin-depleted extracts were supplemented with 10 nM axin, axinΔRGS, axinΔGBS, or axinΔCBS.

deleted, results in a dominant-negative protein in *Xenopus* embryos (Zeng et al., 1997). Consistent with this data, axinΔRGS blocked β-catenin degradation in extracts (Figure 1a) at the tested concentrations (100 nM and higher). This effect was reversed by GSK3, suggesting that axinΔRGS perhaps titrates endogenous GSK3. We confirmed this result in embryos by RNA injection. Embryos injected ventrally at the 4-cell stage with axinΔRGS RNA had complete axis duplication (100%, n = 32) while embryos coinjected with axinΔRGS and GSK3 mRNAs developed normally (100%, n = 35). In tissue culture (Behrens et al., 1998; Hart et al., 1998; Kishida et al., 1999; Sakanaka and Williams, 1999; Smalley et al., 1999) and in *Drosophila* embryos (Willert et al., 1999a), axinΔRGS lowers β-catenin levels, in contrast to *Xenopus* embryos and extracts. We did not detect a stimulatory effect on β-catenin degradation even at low concentrations of axinΔRGS (0.1–20 nM).

When we asked if GSK3 was rate-limiting, we found that unlike axin, GSK3 was present near the maximal effective concentration; addition of 1 μM GSK3β to extracts resulted on average in only a 2-fold acceleration in the rate of β-catenin degradation (data not shown).

The experiments above are in general agreement with a large body of work on β-catenin degradation, though they do not establish an accurate quantitative equivalence between the in vitro and in vivo behavior of the system. To compare the half-life of β-catenin in extracts and in vivo, we built and purified a β-catenin-luciferase fusion. In extracts, this fusion was degraded with the same rate as β-catenin and was regulated identically (Figure 1e). The fusion had a half-life of 1.5 hr in embryos (Figure 1f), suggesting that β-catenin degradation kinetics are similar in embryos and extracts. In embryos, coinjection of axin protein accelerated the degradation of β-catenin-luciferase 2-fold (Figure 1f). Embryonic extracts from various stages of *Xenopus* development (7–12) degraded β-catenin 2- to 5-fold slower than egg extracts, but addition of axin dramatically accelerated degradation to levels comparable to those seen in egg extracts (data not shown).

The Nature of the Corequirement for Axin and GSK3 in β-Catenin Degradation

Since extracts recapitulate the most important aspects of wnt signaling described in vivo, we were in a position to examine the biochemical requirements for axin and GSK3 in β-catenin degradation. As shown in Figure 2a, immunodepletion of axin completely blocks β-catenin degradation; degradation rates were normal in mock-depleted extracts. β-catenin degradation in axin-depleted extracts could be fully restored by adding axin to a concentration of 5–10 nM but not by adding GSK3 to 1 μM (Figure 2a).

GSK3 is also absolutely required for β-catenin degradation. To deplete GSK3, we used GBP, a recently described GSK3-binding protein (Yost et al., 1998). Incubating the extract with GBP beads resulted in the removal of more than 95% of GSK3 as judged by Western blotting (Figure 2c). β-catenin was not degraded in GSK3-depleted extracts, and degradation was completely restored by purified GSK3 to 500 nM but not by axin (Figure 1b). Axin and GSK3 are therefore mutually required for β-catenin degradation.

The ability to deplete and replace axin with different mutants allowed a deletion analysis of axin in the absence of the wild-type protein (Figures 2d and 2e). Deleting the RGS domain (amino acids 1–216 of mouse axin), the GSK3-binding domain (amino acids 298–437, axinΔGBS), or the β-catenin-binding domain (amino acids

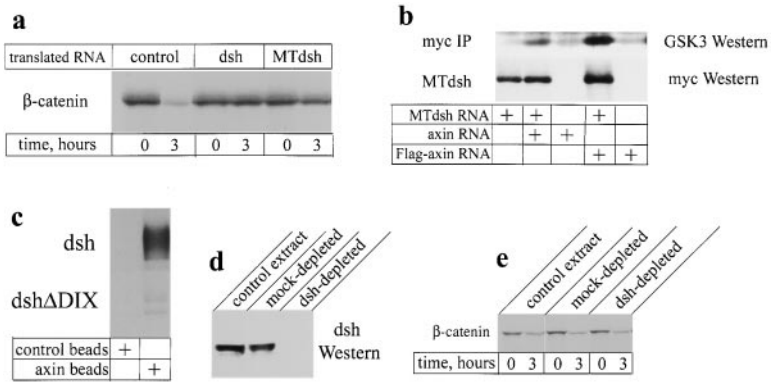


Figure 3. Dsh Inhibits β-Catenin Degradation and Interacts with Axin

(a) Translation of dsh or myc-tagged dsh mRNA in fresh extracts blocks β-catenin degradation. (b) Western blot of myc-dsh immunoprecipitates. Myc-dsh mRNA was translated alone or with two different axin mRNAs. The blot was probed with an anti-GSK3 monoclonal (top panel). The bottom panel is an anti-myc blot of each extract detecting the translated myc-dsh protein. (c) MBP-axin beads bind full-length radiolabeled dsh but not dshΔDIX. (d) Levels of dsh in *Xenopus* egg extracts, mock-depleted extracts, and dsh-depleted extracts. (e) Dsh depletion has no effect on β-catenin degradation.

437–506, axinΔCBS) resulted in proteins that are unable to promote degradation of β-catenin in axin-depleted extracts. By contrast, a C-terminal deletion (from amino acid 625 to the carboxyl terminus of the protein, axinΔDIX) encompassing the DIX domain could rescue the axin-depleted extract, consistent with a deletion analysis in *Xenopus* embryos (Zeng et al., 1997). The DIX domain of axin is therefore not required for β-catenin degradation, though this domain has an important regulatory role, as described below.

The Activity and Function of Dsh

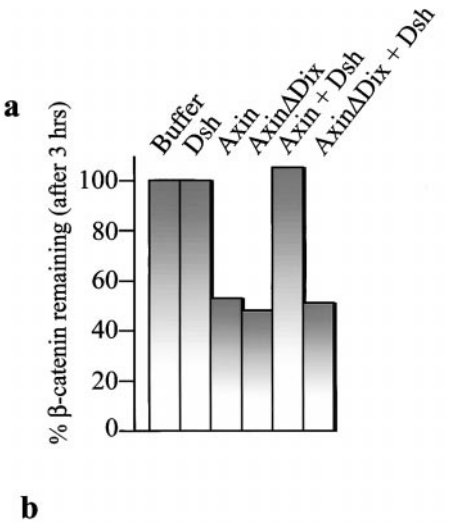
Before we could express recombinant dsh, we employed in vitro translation of dsh mRNA in fresh extracts to examine its effects on β-catenin degradation. As shown in Figure 3a, 100 ng/μl of dsh RNA, translated for 2 hr, blocked β-catenin degradation completely. The amount of dsh produced in extracts from the mRNA is roughly equal to the endogenous levels of dsh protein (measured at 200 nM by immunoblotting). Purified dsh (added at a concentration of 1 μM) also blocked β-catenin degradation; most subsequent studies were performed with recombinant mouse dishevelled 1 (dvl1). Surprisingly, immunodepletion of more than 95% of endogenous dsh (Figure 3d) had no effect on the rate of β-catenin degradation (Figure 3e), consistent with the failure of dominant-negative dsh to block axis formation (Sokol, 1996). In contrast, in early embryogenesis, dsh is enriched on the prospective dorsal side (Miller et al., 1999) and has been suggested to cause the corresponding dorsal accumulation of β-catenin (Larabell et al., 1997). The binding of dsh to axin and the subsequent inhibitory effects of dsh may be limited to the cortex of the dorsal side (which represents only a small fraction of the total volume of early embryos). It should be possible to develop local probes for exchange and degradation that would clarify any region-specific differences in the control of β-catenin levels by dsh.

Dsh Binding to Axin through Their DIX Domains Is Required for Dsh Activity

Dsh might inhibit β-catenin degradation by direct binding to the axin/APC/GSK3/β-catenin complex. We tested this possibility by translating myc-tagged dsh (MTdsh) RNA in extracts with or without axin RNA, followed by precipitation with anti-myc antibodies and immunoblotting for GSK3. MTdsh precipitates contained GSK3 only when axin was coexpressed (Figure 3b), indicating that dsh binds to the axin/APC/GSK3/β-catenin complex, perhaps through axin. In further experiments, axin antibodies brought down labeled dsh while dsh antibodies precipitated labeled axin (data not shown). When purified axin was bound to beads, it bound labeled dsh but not dshΔDIX (see Figure 3c); similarly, purified dsh on beads bound axin but not axinΔDIX (data not shown). These experiments prove that dsh and axin interact directly through their DIX domains in our system, consistent with other studies (Kishida et al., 1999; Li et al., 1999; Smalley et al., 1999). The importance of the dsh–axin interaction for inhibiting β-catenin degradation is demonstrated in extracts depleted of axin and complemented by different axin mutants. Complementation by full-length axin allows dsh to inhibit the degradation of β-catenin. However, there is no inhibition by dsh if complemented by axinΔDIX (Figure 4a). In fact, addition of dsh in the latter case weakly stimulates β-catenin degradation (see below). As an in vivo correlate of these results, the ventralizing effects of axin RNA (injected in both dorsal blastomeres of *Xenopus* 4-cell stage embryos) was reversed by coinjecting dsh RNA, but embryos ventralized to the same extent by injection of axinΔDIX RNA could not be rescued by dsh (Figure 4b). These in vivo and in vitro experiments demonstrate dsh must interact with the DIX domain of axin for activity.

Dsh Inhibits β-Catenin Degradation through GBP

Since dsh uses its DIX domain to bind axin, we asked what other domains of dsh are required for its activity. We fused fragments of dsh to the C terminus of axin, thereby circumventing the need for dsh mutants to bind to axin. As shown in Figure 5a, axin fused to the DEP or DIX domains of dsh or to dshΔPDZ behaved like axin, accelerating β-catenin degradation. In contrast, axin fused to the PDZ domain of dsh or to dshΔDEP or dshΔDIX inhibited β-catenin degradation, suggesting that the PDZ domain might recruit an inhibitor of β-catenin degradation. Deletion of PDZ was reported to have dominant-negative activity in *Xenopus* (Sokol, 1996). Consistent with more recent data (Axelrod et al., 1998), we find that high amounts of dshΔPDZ can block β-catenin degradation in extracts (data not shown); however, it is much less potent than dsh, suggesting that the PDZ domain makes the most significant contribution to the activity of dsh.



b

Rescue of axin-ventralized embyros by Dsh

mRNA Injected	Ventralized (%)	Number Injected
H20	0	25
Axin	100	28
Axin + Dsh	8	27
AxinΔDix	100	25
AxinΔDix + Dsh	100	27

Figure 4. The DIX Domain of Axin Is Required for Dsh Activity

(a) Axin-depleted *Xenopus* extracts were supplemented with 1 nM axin or axinΔDIX in the presence or absence of 200 nM dsh. (b) Dsh RNA rescues axin but not axinΔDIX RNA injections in *Xenopus* embryos. Embryos were injected in both dorsal blastomeres at the 4-cell stage, with 300 pg axin or axinΔDIX mRNAs plus or minus 2.5 ng dsh mRNA. A dorsoanterior index (DAI; Kao and Elinson, 1988) of less than 4 was considered significant.

We tested whether GBP is the inhibitory factor recruited by the PDZ domain of dsh. Figure 5b shows that dsh binds to GBP beads in vitro, while dshΔPDZ does not; recently Li et al. (1999) also showed that GBP binds the PDZ domain of dsh. GBP can bind simultaneously GSK3 and dsh (data not shown). If the GBP-PDZ interaction is important for the activity of dsh, addition of GSK3 should restore β-catenin degradation inhibited by either dsh or by axin fused to PDZ, by titrating endogenous GBP. Indeed, GSK3 at a concentration of 2 μM overcomes the inhibition of β-catenin degradation by 1 μM dsh protein (as shown in Figure 5c) or by the axin-PDZ mRNA (Figure 5d). As mentioned above, in axin-depleted extracts rescued with axinΔDIX, dsh modestly stimulates β-catenin degradation. This is expected if dsh can bind endogenous GBP but not axinΔDIX. If GBP and dsh bind to each other and both inhibit β-catenin degradation, we might expect them to act synergistically. Indeed, levels of GBP and dsh that by themselves do not appreciably inhibit β-catenin degradation induced by axin together inhibit significantly the rate of degradation in extracts (Figure 5e). A more direct measure of the interaction of GBP, GSK3, axin, and β-catenin should be the inhibition of GSK3 in vitro. When these recombinant components are incubated in vitro, one can observe the synergistic inhibition of β-catenin phosphorylation (as well as that of axin; data not shown). Together, GBP and dsh at concentrations of 100 nM and 1 μM, respectively, inhibited the rate of phosphorylation of β-catenin by 75%, whereas neither protein alone showed significant inhibition (Figure 5f).

Evidence for a Critical Biochemical Role of APC

The experiments with axin, dsh, GSK3, GBP, and β-catenin suggest a model for signaling by dsh, where axin recruits GSK3 and β-catenin and where GSK3 is inhibited by dsh binding to axin and recruiting GBP. However, such a model does not include APC, another major component of wnt signaling. The role of APC has been confusing, showing a stimulatory effect on β-catenin degradation in some systems and an inhibitory effect in others, principally *Xenopus*. Evidence for a missing component in the reactions studied above came from looking at the interaction of β-catenin and axin. In the nanomolar range of axin concentration (close to the concentration in extracts), axin binds β-catenin with very low affinity, as measured by coimmunoprecipitation (Figure 6a). The low level of coprecipitation seen in lanes 2 and 3 of Figure 6a increases when axin is present at high concentration on beads (Figure 6b, lane 2 in the minus extract row). β-catenin binding at low concentration of axin is increased 20-fold in the presence of extract (Figure 6a, column 4), suggesting that extract components are required for an efficient interaction. There is no effect of extracts on the affinity of GSK3β for axin (data not shown). The obvious candidate for the missing component was APC (as APC can bind both axin and β-catenin through distinct domains). The RGS domain of axin is required for binding to APC. Addition of GST-RGS to extracts blocked interaction of axin with both APC (data not shown) and with β-catenin (Figure 6a), suggesting that APC mediates the latter interaction. Two additional results (Figure 6b) supported this conclusion: (1) axinΔRGS does not bind β-catenin in extracts although it does in vitro (the lack of binding in extracts is presumably due to competition by endogenous APC and axin), and (2) the RGS domain of axin binds β-catenin in extracts, presumably through APC but not in vitro where APC is absent. APC also enhances the binding of β-catenin to axin in a purified system. A 100 kDa fragment of APC that contains the axin and β-catenin sites (APCm3) increased the rate and level of β-catenin binding to axin beads (Figure 6c). This effect was abolished in an APC mutant lacking the axin-binding site (APCm2). There is also evidence that β-catenin can bridge APC and axin as APCm2 binds axin only in the presence of β-catenin (data not shown). Thus, axin and APC each bind β-catenin independently.

Supporting a role for the axin–APC interaction in β-catenin turnover, addition of GST-RGS to extracts blocked axin-stimulated β-catenin degradation (Figure 6b). This effect could not be rescued by GSK3 (Figure 6d), unlike inhibition caused by axinΔRGS. The requirement for the axin–APC interaction explains the dominant-negative effect obtained with axinΔRGS that can interact with and sequester GSK3 while being unable to recruit β-catenin through APC.

If APC is depleted either by antibodies or RGS beads, β-catenin degradation is significantly inhibited (Figure 6e), demonstrating that APC is required for normal and axin-induced β-catenin turnover in *Xenopus*. β-catenin degradation was not restored in an APC-depleted extract by purified APCm3. We have not yet obtained pure

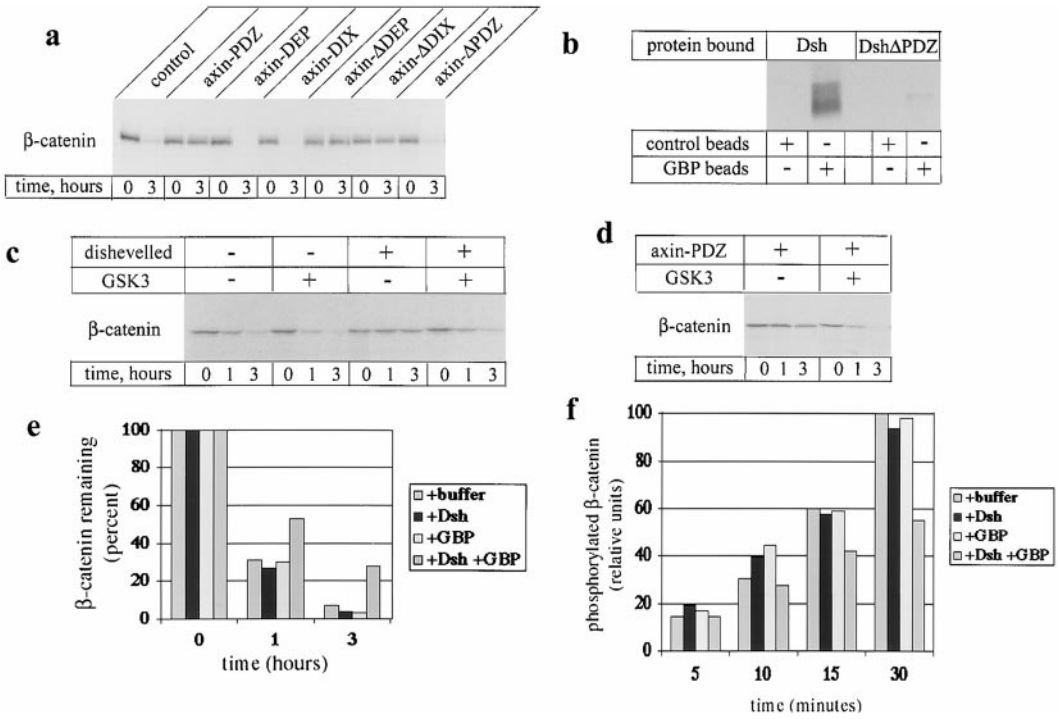


Figure 5. Binding and Functional Synergy of Dsh and GBP

(a) The PDZ domain of dsh blocks β-catenin degradation if fused to axin. mRNAs for different axin fusions to dsh fragments were translated in extracts and stability of β-catenin was assayed. (b) MBP-GBP beads bind dsh but not dshΔPDZ. (c) GSK3 (1 μM) rescues β-catenin degradation inhibited by 0.5–1 μM MBP-dvl1. (d) GSK3 (1 μM) rescues β-catenin degradation inhibited by axin-PDZ RNA. (e) Dsh and GBP synergize to inhibit β-catenin degradation. MBP-dvl1 (200 nM) and MBP-GBP (100 nM) were added alone or together to extracts supplemented with 40 nM MBP-axin (for faster degradation kinetics). (f) GBP and dsh synergize to inhibit phosphorylation of β-catenin in vitro. GSK3 and MBP-axin were preincubated, and then MBP-dvl1, MBP-GBP, and His_6-β-catenin were added, followed by a kinase cocktail. Phosphorylation of β-catenin was measured by densitometry.

full-length APC, which precluded a demonstration that APC can reverse the effects of its depletion from extracts. We were able, however, to rescue axin-induced β-catenin degradation after APC depletion by adding back APC antibody beads preincubated with extracts. Our results therefore allow for the possibility that APC-associated factors play a role in its activity.

Having shown that APC is required for β-catenin degradation and for tight binding to axin, we asked whether dsh modulates this interaction. Previously, we concluded that dsh inhibited GSK3 by mobilizing GBP onto axin. Adding dsh and GBP to extracts weakens the binding of β-catenin to APC and indirectly to axin (Figure 6f). That this is a consequence of the reduced activity of GSK3 and not simply the added dsh was shown by the inhibition of β-catenin binding to APC by lithium. It was previously reported that GSK3-phosphorylated APC binds β-catenin with greater affinity (Rubinfeld et al., 1996). We have repeated these in vitro binding experiments and estimated that GSK3-phosphorylated APC has a 10- to 20-fold higher affinity for β-catenin than unphosphorylated APC (as measured by coimmunoprecipitation experiments; data not shown). In extracts, APC phosphorylation is greatly stimulated by axin but not by axinΔRGS (Figure 6g). Also, the phosphorylation state of APC is highly dynamic, as illustrated by its fast dephosphorylation in extracts (Figure 6h). Given the rapid phosphorylation–dephosphorylation cycles involving APC, it is easy to see how a decrease in GSK3 activity by dsh/GBP or lithium results in a marked loss of β-catenin affinity for the axin/APC/GSK3 complex.

Discussion

The central mechanistic features of β-catenin phosphorylation and ubiquitination have been well described in several systems by genetic approaches and by overexpression in cell lines and embryos. These studies identified dsh, axin, GSK3, APC, and GBP as important regulators of β-catenin levels. However, there is disagreement as to whether APC acts in a positive or negative fashion, whether GBP plays an important role, or whether it just buffers GSK3, how the components interact, what the precise function of axin and APC is in regulating β-catenin phosphorylation, and finally, the role of dsh in this process. To address these issues, we have used cytoplasmic extracts from *Xenopus* eggs to reconstitute the regulated degradation of β-catenin. By measuring β-catenin stability directly rather than steady-state levels of the protein, we avoided the complicating effects of transcription and translation present in other systems. By adding known amounts of purified proteins to extracts and measuring degradation kinetics, a quantitative analysis of wnt signaling can be performed. This

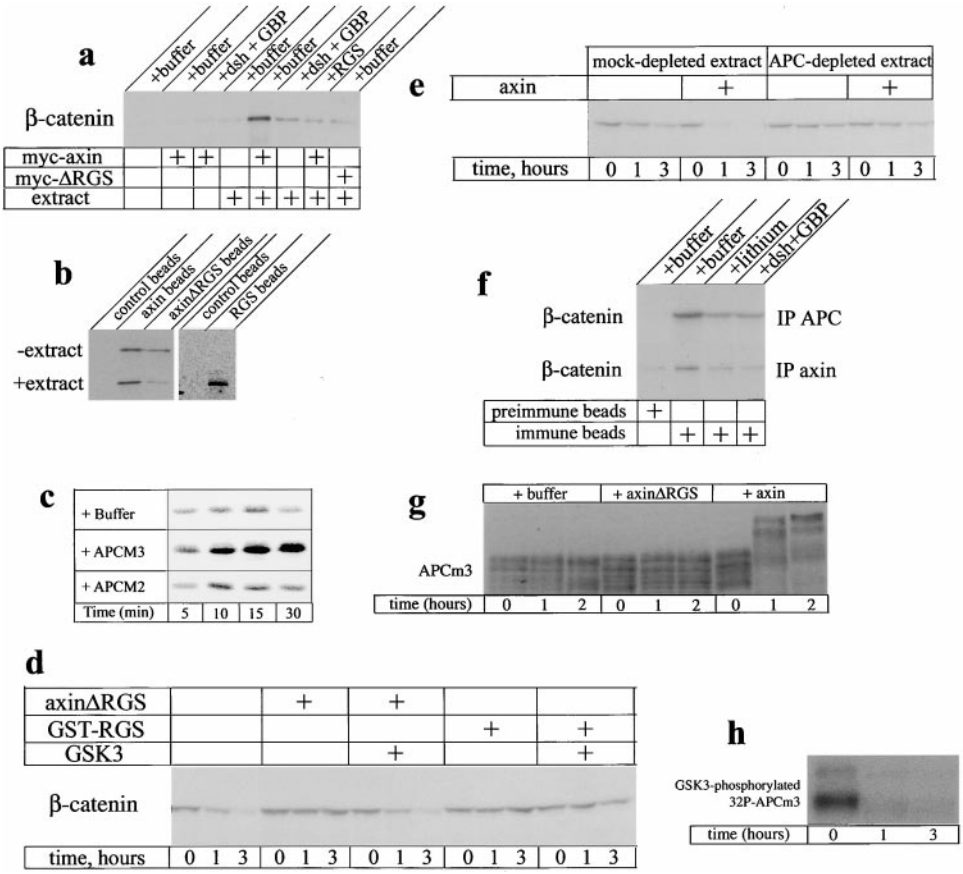


Figure 6. Role of APC in β-Catenin Degradation

(a) Extracts stimulate axin binding to β-catenin. In vitro translated myc-tagged axin was incubated with labeled β-catenin with or without *Xenopus* extracts, followed by anti-myc immunoprecipitation. GST-RGS blocks the increased axin-β-catenin binding elicited by extracts. MBP-dvl1 and MBP-GBP also inhibit axin-β-catenin binding. (b) Binding of labeled β-catenin to MBP-axin, MBP-axinΔRGS, and GST-RGS beads in vitro and in *Xenopus* extracts. (c) APCm3, a fragment of APC that binds β-catenin and axin stimulates binding of hot β-catenin to axin beads in vitro. A fragment of APC (APCm2) that lacks the axin-binding domain has no stimulatory effect. (d) axinΔRGS and RGS (500 nM each) block β-catenin degradation, but only axinΔRGS is rescued by 1–2 μM GSK3. (e) APC is required for β-catenin degradation and axin activity in extracts. MBP-axin at 50 nM stimulates β-catenin degradation in mock-depleted extracts but has no effect in APC-depleted extracts. (f) Lithium chloride (50 mM) or MBP-dvl1 + MBP-GBP (each at 1–2 μM) inhibits the interaction of β-catenin with endogenous APC and axin. (g) MBP-axin (50 nM) but not MBPaxin-ΔRGS promotes phosphorylation of APCm3 in extracts. (h) Rapid dephosphorylation of APCm3 in extracts. APCm3 on nickel beads was ^{32}P-phosphorylated with GSK3 and then incubated with extracts to follow the disappearance of the radioisotope.

system also allows us to examine mutant proteins that might not show phenotypes if expressed in vivo, due to rapid turnover. In extracts, β-catenin was degraded with a half-life of 1 hr, dropping to less than 15 min in the presence of added axin, which suggested that axin is rate limiting for degradation. Addition of dsh, a positive regulator epistatically upstream of GSK3, stabilized β-catenin, suggesting that the wnt pathway from dsh to β-catenin had been recapitulated in extracts.

This in vitro system allowed us to test the biochemical requirement for several different components of the wnt pathway, ruling out the necessary involvement of any machinery upstream of protein translation. β-catenin degradation depends on axin, GSK3, and APC; extracts depleted of any of these proteins cannot degrade β-catenin. Of the four defined domains in axin, only the C-terminal DIX domain was dispensable for activity. The DIX domain of axin binds a related domain in dsh, and this interaction is required for dsh function; hence, binding of axin and dsh through the DIX domains confers signal-dependent regulation of β-catenin. Another domain of dsh required for activity is PDZ, which binds GBP. Dsh and GBP synergize to inhibit β-catenin degradation in extracts and phosphorylation in vitro, which are both axin-dependent processes. The inhibitory effect of GBP and dsh on β-catenin degradation in extracts is reversed by high concentrations of GSK3 due to titration of dsh/GBP on axin. These observations further support a role for dsh, as an adaptor protein recruiting GBP to the axin/GSK3/APC/β-catenin complex and inhibiting locally the enzymatic activity of GSK3. Our recent results suggest that the axin–GSK3 interaction is highly dynamic (unpublished data) and that increasing the local GBP concentration at the level of the complex efficiently promotes the dissociation of GSK3 from its site on axin.

The simplest model would have β-catenin and GSK3

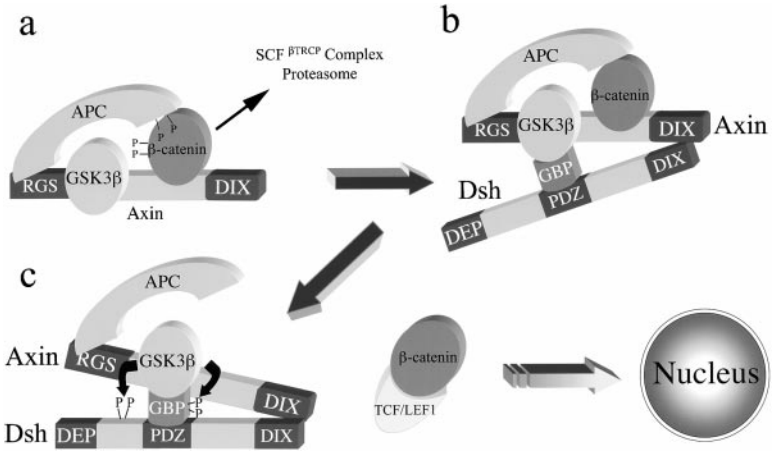


Figure 7. A Mechanism for Dsh Action

In the absence of a wnt signal (a), APC promotes binding of β-catenin to axin. This interaction is driven by phosphorylation of APC by GSK3, which increases the affinity of APC for β-catenin. Axin-bound β-catenin is phosphorylated by GSK3 and targeted for ubiquitination by the SCF-bTRCP ubiquitin ligase complex. Upon activation, dsh and axin form a complex via their DIX domains (b). The recruitment of GBP to the complex by dsh (through its PDZ domain) allows GBP to inhibit axin-bound GSK3. Binding of GBP to GSK3 blocks the phosphorylation of APC and β-catenin. This has two consequences (c): (1) APC has a reduced affinity for β-catenin, and (2) β-catenin is no longer a substrate for the SCF complex. Free β-catenin can form a complex with TCF/Lef1 to drive the transcription of target genes.

binding to their sites on axin and GBP binding to its site on dsh. When dsh and axin bind via their DIX domains, GSK3 would be inhibited. How does APC fit into this model? In addition to the mechanistic confusions about the role of APC, there are suggestions that APC can be an inhibitor of β-catenin degradation in some circumstances or an activator in others. Specifically in *Xenopus*, injection of APC can cause secondary axis formation (Vleminckx et al., 1997), opposite to the ventralizing effects of axin injections. In our in vitro system, there is an absolute requirement for APC in β-catenin degradation, consistent with genetic experiments in *Drosophila* and experiments in cultured cells. We feel the confusion is simply due to the tendency of overexpressed APC to act as a dominant inhibitor. In *Xenopus* extracts, overexpressed APC is a potent inhibitor of β-catenin degradation, although these experiments yield a mixture of truncated and full-length APC proteins. The role of APC in β-catenin degradation is clear from the in vitro experiments. We showed that an interaction of APC with the RGS domain of axin is essential for β-catenin degradation, demonstrating that endogenous APC is in fact a negative regulator of β-catenin levels in *Xenopus*. Although β-catenin binds to axin beads in a purified system, the two proteins show little interaction in the nanomolar range. Extracts contain an activity that promotes the binding of β-catenin to axin at low concentrations. The activity is inhibited by the RGS domain of axin, suggesting that it is either APC or APC complexed to other proteins. Also, axinΔRGS (which cannot bind APC) does not bind β-catenin in extracts. With purified components in vitro, APC accelerates the binding of β-catenin to axin, recapitulating the stimulating effect of extracts on binding between the two proteins. Taken together, these results identify APC as the activity stimulating the axin-β-catenin interaction in extracts.

β-catenin interacts with numerous cellular proteins, some not directly involved in wnt signaling. The requirement for an APC-like molecule makes sense if one realizes that not only the stability of β-catenin is important but also its availability for other interactions, transcriptional and cytoskeletal. Aside from participating in β-catenin degradation, APC also prevents its interaction with Tcf3 (unpublished data), thus inhibiting the transcriptional activation function of β-catenin. Additionally, APC maintains a pool of β-catenin in a state that can either lead to its degradation or release in response to a wnt signal. The phosphorylation state of APC is maintained by rapid futile cycles of opposing phosphorylation and dephosphorylation reactions. Changes in the phosphorylation of APC by GSK3 act as a switch to either trigger β-catenin degradation or its discharge in the cytoplasm. Although Willert et al. (1999b) suggested that phosphorylation of axin modulates binding to β-catenin, we find no evidence for such a regulation in extracts because (1) APC is the principal mediator of the axin-β-catenin interaction and (2) using purified axin and β-catenin, we were unable to detect an increase in the affinity of β-catenin for axin due to phosphorylation of the latter by GSK3 (data not shown).

Our data suggests that the APC/axin/GSK3/β-catenin complex exists in two states. In one state (Figure 7a), β-catenin is bound tightly to axin via APC; β-catenin and APC are both phosphorylated by GSK3. In this situation, phosphorylated β-catenin can only be removed through SCF-dependent degradation while phosphorylated APC continues to bind β-catenin molecules avidly. Upon binding of active dsh to axin, GBP interacts with GSK3 (Figure 7b), removing it from axin competitively. This inhibits phosphorylation on both β-catenin and APC. The former stabilizes β-catenin to ubiquitination, and the latter releases intact β-catenin so that it can interact with other partners (Figure 7c). The β-catenin-binding site on axin is also important because its deletion impairs the function of axin. Although this scenario is written as a set of irreversible steps, it is likely that all binding events are dynamic. The fact that dominant-negative GSK3 blocks degradation of β-catenin in extracts suggests that the interaction between endogenous axin and GSK3 must be dynamic. The dissociation rate for GSK3 bound to axin must be fast enough to allow its displacement by the dominant-negative mutant on a time scale of perhaps less than 1 hr. The same must be true for the axin–APC interaction, as the RGS domain of axin (which interacts with APC) blocks β-catenin degradation.

The stability of β-catenin is subject to a system of weak protein interactions and posttranslational modifications. The ability to dissect this pathway in vitro without altering appreciably its kinetics and without simplifying essential steps should aid our understanding of

these weak interactions and their physiological consequences. Several remaining questions should be clarified by studies in partially purified systems and ultimately by reconstitution from purified components. They include the mechanism of signal transmission from frizzled to dsh and the mechanism of dsh activation. In particular, does the wnt signal regulate the GBP–dsh or the dsh–axin interaction? We also wish to know whether APC suffices by itself to mediate β-catenin degradation or whether associated proteins are also required. Finally, we wish to know whether signaling pathways cross-regulate the wnt pathway and how that might work. In vitro reconstitution should be useful in deciphering the molecular events involved in transducing the wnt signal.

Experimental Procedures

Extract Preparation and In Vitro Translation

Extracts were prepared as described (Murray, 1991), with modifications. After dejellying, eggs were transferred to 2 μl Eppendorf tubes on ice containing 2 mL cytochalasin B (10 mg/ml in DMSO) and packed for 30 s at 30 g. Eggs were then crushed at 21,000 × g in a refrigerated microcentrifuge for 5 min. The cytoplasmic layer was removed and spun twice more at 21,000 × g. Energy mix and protease inhibitors were added before use. In vitro translations were done in fresh extracts. High-speed supernatants were prepared as described (King et al., 1995).

Degradation Assays

Degradation reactions contained 6.5 μl extract plus 0.1 μl of 100 mg/ml cycloheximide, 0.16 μl energy regeneration mix, 0.2 μl bovine ubiquitin at 14 mg/ml in 1× XB (Murray, 1991), and 0.1–0.3 μl of ^{35}S-labeled β-catenin synthesized using the Promega TNT kit. Reactions were incubated at 22°C, and 1 μl samples were removed at different times. Samples were analyzed by SDS-PAGE followed by autoradiography.

Ubiquitination of β-Catenin in High-Speed Supernatants

Labeled β-catenin was incubated 1 hr at 22°C in 30 μl high-speed supernatant in the presence of 1 mg/ml ubiquitin (Ub) or hexahistidine-ubiqutin (His_6-Ub). The mix was diluted 100-fold with XB and applied to 5 μl Ni^{2+}-NTA resin (Qiagen). The resin was washed three times with XB and eluted.

Antibodies

Rabbit anti-axin polyclonals were raised against a fusion of maltose-binding protein (MBP) to a fragment comprising amino acids 298–506 of mouse axin. Anti-dishevelled polyclonals were raised against a mix of three fusions between MBP and the DIX, DEP, and PDZ domains of *Xenopus* dishevelled. Anti-APC polyclonals were raised against a fusion of MBP to the C-terminal 150 amino acids of *Xenopus* APC.

Immunodepletions

Immune and preimmune sera were bound to protein A-affiprep (Bio-Rad). One hundred microliters of extract was incubated with 40 μl of beads at 4°C for 1–2 hr. GSK3 depletion was done using MBP-GBP on amylose beads. For APC depletion using the RGS domain of axin, purified GST-RGS was cross-linked to Ultralink beads (Pierce) at a concentration of 10 mg protein/ml beads. Cross-linked GST-RGS beads were used for depletion identically to that of the protein A antibody beads.

Recombinant Proteins and Expression Constructs

MBP-human dishevelled 1 (MBP-dvl1), MBP-axin, MBP-ΔRGS axin, GST-RGS (amino acids 1–216 of mouse axin), MBP-GBP, and MBP-GSK3 were expressed in bacteria. His-tagged β-catenin, His-tagged β-catenin-luciferase, His-tagged GSK3, His-tagged APCm2 (amino acids 1–1337 of *Xenopus* APC), and His-tagged APCm3 (amino acids 1342–2075) were expressed in Sf9 cells. C-terminal fusions of axin and fragments of dsh were built in pCS2+ by PCR. A linker or 24 amino acids $(GGGSGGGT)_3$ was included between fusion partners. *Xenopus* GBP was cloned by PCR from a *Xenopus* stage 14 library.

mRNA Synthesis and Translation in Extracts

Capped RNA was synthesized from linearized plasmid DNA templates. RNAs at 100–500 ng/μl were mixed with fresh extracts (1:6 to 1:20 ratio) supplemented with RNasin (Promega) (1:50 ratio) and incubated at 22°C for 1–2 hr for translation.

In Vitro Kinase Reactions

GSK3 kinase reactions were done at 22°C in a kinase buffer (20 mM HEPES (pH 7.5), 300 mM NaCl, 2 mM DTT, 1 mM EDTA, 10 mM $MgCl_2$, 0.2% Tween-20, 50 μM ATP, and [γ-^{32}P]ATP). The final concentrations of the components were 100 nM His-GSK3, 75 nM MBP-axin, 1 μM MBP-dvl1, 0.1–1 μM MBP-GBP, and 1 μM His-β-catenin.

Binding

Extracts were diluted 1:5 in XB with 1% Tween and protease inhibitors and incubated with protein beads and labeled proteins at 4°C for 2 hr. The beads were washed with 25 HEPES (pH 7.5), 400 mM NaCl, 1 mM EDTA, 1% Tween and with 25 HEPES (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1% Tween. Bound proteins were released by boiling in SDS-PAGE loading buffer. For binding to GST fusion proteins on glutathione beads, in the presence of *Xenopus* extracts, the extract was first gel filtered through a NAP-5 column (Pharmacia), to remove endogenous glutathione.

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References

Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. EMBO J. *16*, 3797–3804.

Axelrod, J.D., Miller, J.R., Shulman, J.M., Moon, R.T., and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. Genes Dev. *12*, 2610–2622.

Behrens, J., Jerchow, B.A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998). Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science *280*, 596–599.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the frizzled family from *Drosophila* functions as a Wingless receptor. Nature *382*, 225–230.

Brown, J.D., and Moon, R.T. (1998). Wnt signaling: why is everything so negative? Curr. Opin. Cell Biol. *10*, 182–187.

Cadigan, K.M., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. Genes Dev. *11*, 3286–3305.

Fuchs, S.Y., Chen, A., Xiong, Y., Pan, Z.Q., and Ronai, Z. (1999). HOS, a human homolog of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IkappaB and beta-catenin. Oncogene *18*, 2039–2046.

Hart, M.J., de los Santos, R., Albert, I.N., Rubinfeld, B., and Polakis, P. (1998). Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Curr. Biol. *8*, 573–581.

He, X., Saint-Jeannet, J.P., Woodgett, J.R., Varmus, H.E., and Dawid, I.B. (1995). Glycogen synthase kinase-3 and dorsoventral patterning in *Xenopus* embryos. Nature *374*, 617–622.

Kao, K.R., and Elinson, R.P. (1988). The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. Dev. Biol. *127*, 64–77.

King, R.W., Peters, J.M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M.W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell *81*, 279–288.

Kishida, S., Yamamoto, H., Hino, S., Ikeda, S., Kishida, M., and Kikuchi, A. (1999). DIX domains of Dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability. Mol. Cell. Biol. *19*, 4414–4422.

Kitagawa, M., Hatakeyama, S., Shirane, M., Matsumoto, M., Ishida, N., Hattori, K., Nakamichi, I., Kikuchi, A., and Nakayama, K. (1999). An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. EMBO J. *18*, 2401–2410.

Klein, P.S., and Melton, D.A. (1996). A molecular mechanism for the effect of lithium on development. Proc. Natl. Acad. Sci. USA *93*, 8455–8459.

Larabell, C.A., Torres, M., Rowning, B.A., Yost, C., Miller, J.R., Wu, M., Kimelman, D., and Moon, R.T. (1997). Establishment of the dorso-ventral axis in *Xenopus* embryos is presaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. J. Cell Biol. *136*, 1123–1136.

Li, L., Yuan, H., Weaver, C.D., Mao, J., Farr, G.H., III, Sussman, D.J., Jonkers, J., Kimelman, D., and Wu, D. (1999). Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. EMBO J. *18*, 4233–4240.

Liu, C., Kato, Y., Zhang, Z., Do, V.M., Yankner, B.A., and He, X. (1999). beta-Trcp couples beta-catenin phosphorylation-degradation and regulates *Xenopus* axis formation. Proc. Natl. Acad. Sci. USA *96*, 6273–6278.

Marikawa, Y., and Elinson, R.P. (1998). beta-TrCP is a negative regulator of Wnt/beta-catenin signaling pathway and dorsal axis formation in *Xenopus* embryos. Mech. Dev. *77*, 75–80.

Miller, J.R., Rowning, B.A., Larabell, C.A., Yang-Snyder, J.A., Bates, R.L., and Moon, R.T. (1999). Establishment of the dorsal-ventral axis in *Xenopus* embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. J. Cell Biol. *146*, 427–437.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in *Xenopus* embryos. Cell *86*, 391–399.

Murray, A.W. (1991). Cell cycle extracts. Methods Cell Biol. *36*, 581–605.

Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. (1996). Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. Science *272*, 1023–1026.

Sakanaka, C., and Williams, L.T. (1999). Functional domains of axin. Importance of the C terminus as an oligomerization domain. J. Biol. Chem. *274*, 14090–14093.

Smalley, M.J., Sara, E., Paterson, H., Naylor, S., Cook, D., Jayatilake, H., Fryer, L.G., Hutchinson, L., Fry, M.J., and Dale, T.C. (1999). Interaction of axin and Dvl-2 proteins regulates Dvl-2-stimulated TCF-dependent transcription. EMBO J. *18*, 2823–2835.

Sokol, S.Y. (1996). Analysis of Dishevelled signaling pathways during *Xenopus* development. Curr. Biol. *6*, 1456–1467.

Sokol, S.Y. (1999). Wnt signaling and dorso-ventral axis specification in vertebrates. Curr. Opin. Genet. Dev. *9*, 405–410.

Vleminckx, K., Wong, E., Guger, K., Rubinfeld, B., Polakis, P., and Gumbiner, B.M. (1997). Adenomatous polyposis coli tumor suppressor protein has signaling activity in *Xenopus laevis* embryos resulting in the induction of an ectopic dorsoanterior axis. J. Cell Biol. *136*, 411–420.

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. Annu. Rev. Cell Dev. Biol. *14*, 59–88.

Willert, K., Logan, C.Y., Arora, A., Fish, M., and Nusse, R. (1999a). A *Drosophila* Axin homolog, Daxin, inhibits Wnt signaling. Development *126*, 4165–4173.

Willert, K., Shibamoto, S., and Nusse, R. (1999b). Wnt-induced dephosphorylation of axin releases beta-catenin from the axin complex. Genes Dev *13*, 1768–1773.

Yew, P.R., and Kirschner, M.W. (1997). Proteolysis and DNA replication: the CDC34 requirement in the *Xenopus* egg cell cycle. Science *277*, 1672–1676.

Yost, C., Farr, G.H., III, Pierce, S.B., Ferkey, D.M., Chen, M.M., and Kimelman, D. (1998). GBP, an inhibitor of GSK-3, is implicated in *Xenopus* development and oncogenesis. Cell *93*, 1031–1041.

Yost, C., Torres, M., Miller, J.R., Huang, E., Kimelman, D., and Moon, R.T. (1996). The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. Genes Dev. *10*, 1443–1454.

Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T.J., Perry, W.L., III, Lee, J.J., Tilghman, S.M., Gumbiner, B.M., and Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell *90*, 181–192.