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A yeast model system for functional analysis of β-catenin signaling

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We have developed a novel *Saccharomyces cerevisiae* model system to dissect the molecular events of β-catenin (β-cat) signaling. Coexpression of mammalian β-cat with TCF4 or LEF1 results in nuclear accumulation of these proteins and a functional complex that activates reporter gene transcription from constructs containing leukocyte enhancer factor (LEF)/T cell factor (TCF) response elements. Reporter transcription is constitutive, requires expression of both β-cat and TCF4 or LEF1, and is not supported by mutated LEF/TCF binding elements or by TCF4 or LEF1 mutants. A cytoplasmic domain of E-cadherin or a functional fragment of adenomatous polyposis coli (APC) protein (APC-25) complexes with β-cat, reduces β-cat binding to TCF4, and leads to increased cytoplasmic localization of β-cat and a reduction in reporter activation. Systematic mutation of putative nuclear export signal sequences in APC-25 decreases APC-25 binding to β-cat and restores reporter gene transcription. Additional β-cat signaling components, Axin and glycogen synthase kinase 3β, form a multisubunit complex similar to that found in mammalian cells. Coexpression of the F-box protein β-transducin repeat-containing protein reduces the stability of β-cat and decreases reporter activation. Thus, we have reconstituted a functional β-cat signal transduction pathway in yeast and show that β-cat signaling can be regulated at multiple levels, including protein subcellular localization, protein complex formation, and protein stability.

Introduction

The β-catenin (β-cat)* signal transduction pathway plays an important role in a variety of developmental and differentiation processes in many organisms (Wodarz and Nusse, 1998) and is aberrantly activated in a majority of human colorectal cancers as well as in other tumor types (Bienz and Clevers, 2000; Peifer and Polakis, 2000; Polakis, 2000). Cell culture and animal studies have validated a key role for active β-cat signal transduction in promoting critical aspects of malignant transformation (Bienz and Clevers, 2000; Peifer and Polakis, 2000).

β-cat protein binds to the cytoplasmic domain of E-cadherin (E-cad) thus linking cadherin to the actin cytoskeleton to promote cell–cell adhesion (Nagafuchi and Takeichi, 1988; Ozawa et al., 1989). In the absence of activating signals, β-cat is targeted for degradation by a complex consisting of Axin, the serine/threonine kinase glycogen synthase kinase 3β (GSK3β), and the tumor suppressor adenomatous polyposis coli (APC) (Peifer and Polakis, 2000). Phosphorylation of β-cat by GSK3β results in its recognition by β-transducin repeat-containing protein (βTRCP), an F-box/WD-40 repeat protein, and part of the ubiquitin apparatus, thus targeting β-cat for ubiquitination and degradation by a proteasome pathway (Yost et al., 1996; Aberle et al., 1997; Easwaran et al., 1999; Hart et al., 1999; Kitagawa et al., 1999; Liu et al., 1999).

Upon signal transduction activated by growth factors, including Wnt proteins, hepatocyte growth factor (HGF), or EGF, β-cat accumulates in the cytoplasm (Bradley et al., 1993; Papkoff et al., 1996; Papkoff and Aikawa, 1998; Muller et al., 1999), translocates into the nucleus, and associates with high mobility group (HMG) box transcription factors of the leukocyte enhancer factor (LEF)/T cell factor (TCF) family (Fagotto et al., 1998; Yokoya et al., 1999) to regulate gene expression (Behrens et al., 1996; Huber et al., 1996; Molenaar et al., 1996). Human tumor cells with mutations in regulatory phosphorylation sites of β-cat or inactivating mutations of APC and Axin also have a stabilized free pool of β-cat and consequently increased β-cat–TCF/LEF–mediated

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*Abbreviations used in this paper: APC, adenomatous polyposis coli; β-cat, β-catenin; β-gal, β-galactosidase; βTRCP, β-transducin repeat-containing protein; E-cad, E-cadherin; GSK3β, glycogen synthase kinase 3β; IF, indirect immunofluorescence microscopy; LEF, leukocyte enhancer factor; NES, nuclear export signal; RGS, regulator of G protein signaling; TCF, T cell factor.

Key words: *Saccharomyces cerevisiae*; active transport; cell nucleus; cytoskeletal proteins; adenomatous polyposis coli

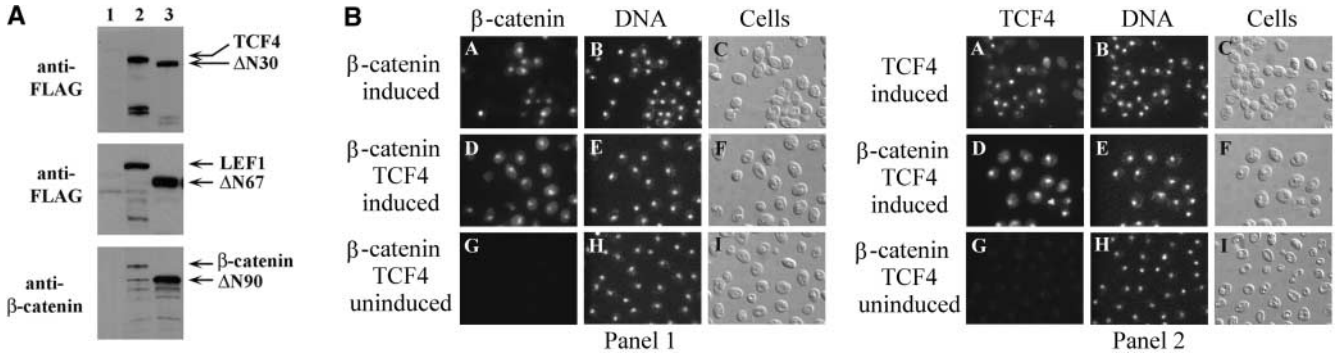


Figure 1. **Expression and localization of β-cat and LEF/TCF proteins in yeast.** (A) Full-length and NH_2-terminal deletion mutants of epitope-tagged human β-cat, LEF1, and TCF4 were expressed in yeast and equivalent amounts of protein from cells containing vector only (lane 1), full-length protein (lane 2), or NH_2-terminal deletion mutant protein (lane 3) were subjected to Western blot analysis using antibodies against the FLAG epitope (top and middle) or β-cat (bottom). (B) Yeast cells containing expression plasmids for β-cat only (panel 1, A–C), TCF4 only (panel 2, A–C), or β-cat together with TCF4 (panels 1 and 2, D–F and G–I) were grown under conditions where protein expression was induced (panels 1 and 2, A–F) or repressed (panels 1 and 2, G–I). Cells were subjected to IF with an antibody against β-cat (panel 1) or the FLAG epitope to detect TCF4 (panel 2). Proteins (A, D, and G), DNA (B, E, and H), and cells (C, F, and I) were photographed.

transcription (Kinzler and Vogelstein, 1996; Morin et al., 1997; Rubinfeld et al., 1997; Sparks et al., 1998; Satoh et al., 2000).

Inhibition of β-cat degradation serves an important role in providing a pool of signaling-competent β-cat. Furthermore, the cytoplasmic and cadherin-bound pools of β-cat may be in equilibrium (Fagotto et al., 1996; Orsulic and Peifer, 1996; Sanson et al., 1996) and the cadherin-associated population of β-cat may serve as a reservoir of β-cat that can be liberated for nuclear translocation and signal transduction (Birchmeier, 1995; Gumbiner, 1995). However, although a free pool of β-cat is a prerequisite for subsequent association with TCF/LEF and transcriptional modulation, it is not sufficient for nuclear localization. This is evidenced by the observed cytoplasmic as well as nuclear distribution of β-cat under circumstances, such as Wnt-1 signaling or APC mutation, where β-cat degradation is inhibited (Munemitsu et al., 1995; Kawahara et al., 2000; Barker and Clevers, 2001; Brabletz et al., 2001). Nuclear accumulation of β-cat and consequently β-cat–TCF/LEF signaling must therefore involve a regulated balance between nuclear import and export.

The mechanisms of β-cat nuclear transport are unclear because β-cat does not have a classic NLS and can enter the nucleus by binding directly to the nuclear pore machinery in the absence of other known factors, in a manner similar to importinβ (Fagotto et al., 1997; Yokoya et al., 1999). Furthermore, β-cat export is not blocked by leptomycin B, a specific inhibitor of nuclear export signal (NES)–mediated export (Eleftheriou et al., 2001; Wiechens and Fagotto, 2001). APC contains two NLS sequences in its central domain (Zhang et al., 2001) and can be found in the nucleus (Neufeld and White, 1997). APC also contains several consensus NES sequences, two at its NH_2 terminus (Neufeld and White, 1997; Henderson, 2000) and at least three within the 20-aa repeat region (Rosin-Arbesfeld et al., 2000). Mutation of these sequences results in accumulation of APC in the nucleus and correlates with increased β-cat nuclear accumulation, leading to the hypothesis that nucleocytoplasmic shuttling of APC participates in the regulation of the nuclear localization of β-cat (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000; Zhang et al., 2001).

As a novel approach to study β-cat signal transduction in a simplified context, we developed a model system where a significant portion of the β-cat signal transduction pathway has been reconstructed in yeast. Individual known components of the mammalian β-cat signal transduction pathway were systematically introduced into yeast cells and protein complex formation, subcellular localization, and function in regulation of β-cat–TCF/LEF–mediated transcription were measured. The data suggest that β-cat signaling can be regulated at multiple levels, including modulation of subcellular localization, complex formation, and stability of key proteins that participate in the β-cat pathway.

Results

Expression of β-cat signaling components in yeast

Plasmids for regulated expression of epitope-tagged human β-cat, LEF1, and TCF4 and corresponding NH_2-terminal deletion mutants were introduced into wild-type yeast as a first step toward reconstituting a basic β-cat signaling network. Full-length recombinant proteins (Fig. 1 A, lane 2) and deletion mutants (Fig. 1 A, lane 3) were specifically detected at their predicted molecular weights by Western blot analysis (Fig. 1 A). β-cat-ΔN90, an NH_2-terminal deletion mutant of β-cat that is stabilized in mammalian cells, reproducibly accumulated to significantly higher levels as compared with the full-length β-cat protein (Fig. 1 A, β-cat, compare lanes 2 and 3).

Yeast cells expressing β-cat and TCF4, alone or in combination, were subjected to indirect immunofluorescence microscopy (IF) to ascertain the subcellular localization of these proteins (Fig. 1 B). Either β-cat or TCF4, when expressed alone, accumulated in the nucleus (Fig. 1 B, panel A). Coexpression of β-cat with TCF4 had no effect on the localization of either protein (Fig. 1 B, panel D). Thus, translocation of either protein into the nucleus occurs independently. LEF1 localization was indistinguishable from that of TCF4, and that of NH_2-terminal deletion mutants of β-cat, TCF4,

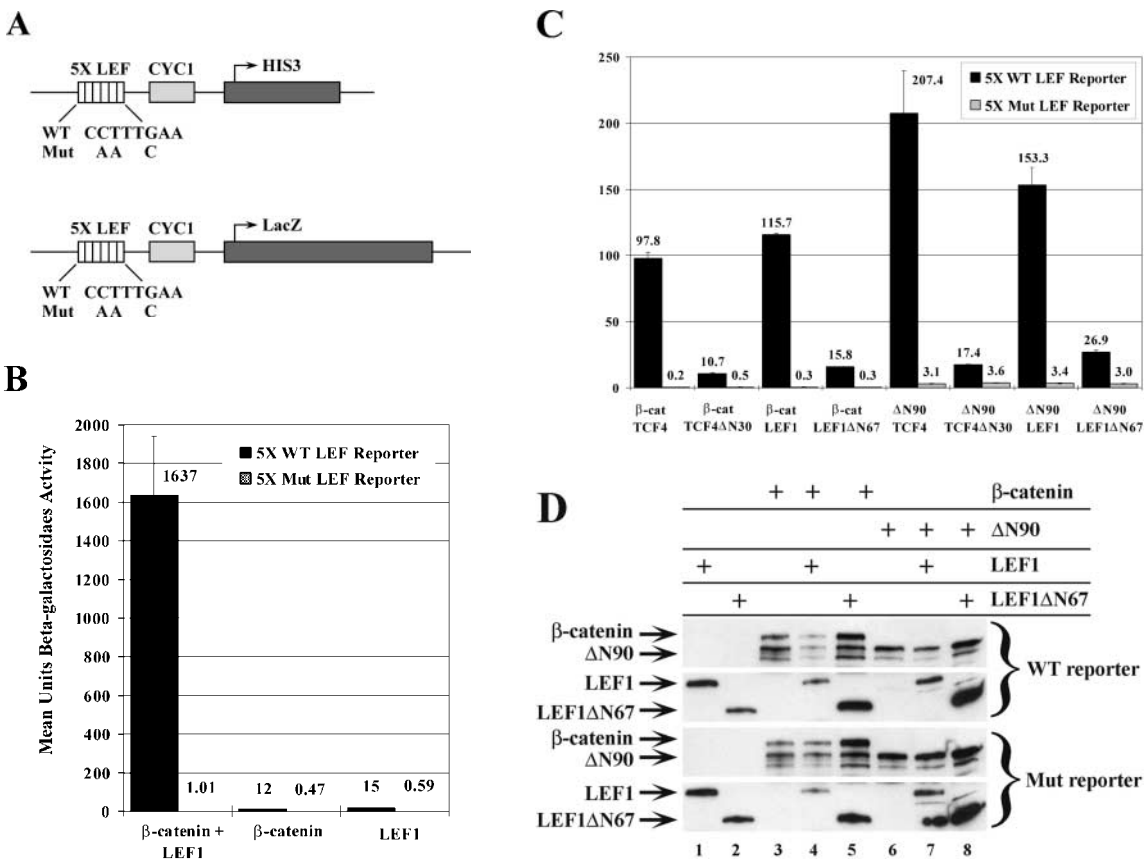


Figure 2. **β-cat cooperates with LEF1 and TCF4 to activate yeast reporter transcription.** (A) Schematic diagram of the LacZ and HIS3 reporter constructs. (B) Cells expressing β-cat and LEF1, β-cat only, or LEF1 only along with either WT or Mut 5X LEF LacZ reporter plasmids were assayed for β-gal activity. (C) Cells with either WT or Mut 5X LEF LacZ reporter plasmids were induced to express either full-length or NH_2-terminal–deleted forms of the indicated proteins and β-gal activity was determined. (D) Equivalent amounts of total protein from cells used for the assay in C expressing β-cat alone (lanes 3), β-cat ΔN90 alone (lanes 6), β-cat with LEF1 or LEF1ΔN67 (lanes 4 and 5, respectively), or β-cat ΔN90 with LEF1 or LEF1ΔN67 (lanes 7 and 8, respectively) from strains also containing WT 5X LEF LacZ (top two panels) or Mut 5X LEF LacZ (bottom two panels) were subjected to Western blot analysis.

and LEF1 was identical to their full-length counterparts (unpublished data).

β-Cat cooperates with LEF1 and TCF4 to activate yeast reporter gene transcription

To determine if β-cat and TCF4 or LEF1 expressed in yeast function in regulating transcription, we generated reporter plasmids containing wild-type (WT 5X LEF) or mutated (Mut 5X LEF) LEF/TCF-dependent transcriptional elements upstream of a yeast CYC1 minimal promoter driving expression of either *Escherichia coli* LacZ or yeast HIS3 reporter genes (Fig. 2 A). WT 5X LEF and Mut 5X LEF LacZ reporters were introduced into yeast expressing full-length or NH_2-terminal–deleted forms of β-cat, TCF4, or LEF1 and cultures were assayed for β-galactosidase (β-gal) enzymatic activity (Fig. 2, B and C). Wild-type β-cat or LEF1 alone induced little β-gal activity (Fig. 2 B). Conversely, coexpression of β-cat with LEF1 increased reporter gene expression (Fig. 2 B). Similar results were obtained with TCF4 (unpublished data; Fig. 2 C; Fig. 3). No induction of β-gal activity was observed with Mut 5X LEF LacZ (Figs. 2, B and C). Furthermore, deletion of the NH_2-terminal DNA binding domain of either TCF4 or LEF1 (ΔN30 and ΔN67, respectively) resulted in significant reduction of reporter gene expression in the presence of β-cat (Fig. 2 C). The ΔN90 mutant of β-cat retained full ability to activate reporter gene expression as expected (Fig. 2 C). All of the β-cat and LEF or TCF proteins were efficiently expressed (Fig. 2 D; unpublished data).

Expression of both β-cat and TCF4 (Fig. 3 B) along with the WT 5X LEF HIS3 reporter (Fig. 2 A) enabled yeast strains harboring mutations in the HIS3 gene to grow on medium lacking histidine (Fig. 3 A, upper right quadrant). Cells containing the HIS3 reporter with mutated LEF/TCF sites (Fig. 3 A, lower quadrants), or cells grown under conditions where β-cat and TCF4 were not expressed (Fig. 3 A, left quadrants) were unable to grow. Similar results were obtained with LEF1 in conjunction with β-cat (unpublished data). Thus, β-cat and TCF/LEF are capable of using the conserved yeast nuclear import and transcriptional machinery to function in the specific activation of reporter gene expression.

E-cad and APC-25 bind to β-cat and inhibit β-cat/TCF4 transcriptional activation

β-cat expressed in our system is constitutively active as a transcriptional coactivator, due at least in part to its localization in the nucleus. To recreate the β-cat–E-cad interaction, an expression plasmid encoding the plasma membrane teth-

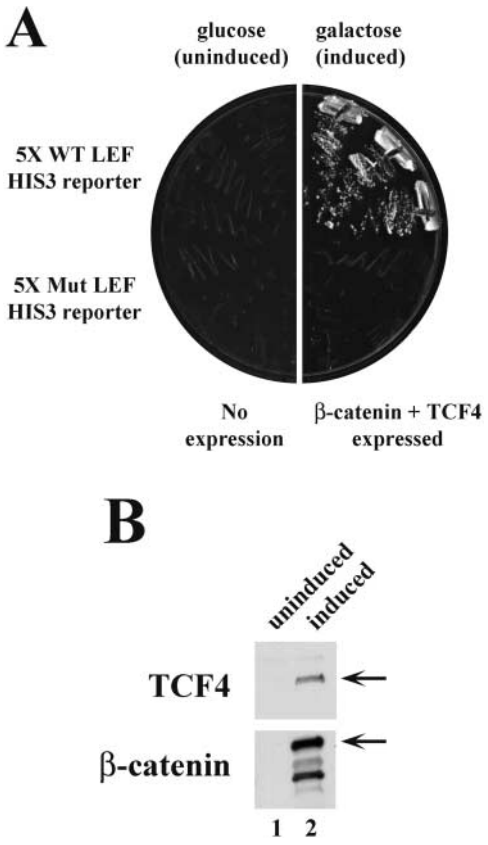


Figure 3. **β-cat– and TCF4–dependent transcription of the HIS3 reporter gene allows for yeast cell growth on medium lacking histidine.** His^- yeast containing β-cat and TCF4 plasmids with WT or Mut 5X LEF HIS3 reporter plasmids were grown on media lacking histidine with either glucose or galactose. (A) Photograph of yeast growth after 4 d. Yeast grown on glucose do not express β-cat or TCF4 and therefore cannot grow on medium lacking histidine (left). Yeast cells grown on galactose are induced to express β-cat and TCF4. Cells containing a WT 5X LEF HIS3 reporter are able to grow, whereas those containing a Mut 5X LEF HIS3 reporter cannot (right). (B) Western blot demonstrating expression of TCF4 and β-cat proteins under induced (lane 2), but not uninduced (lane 1), conditions. Blots were probed with antibodies directed against the FLAG epitope (top) and against β-cat (bottom).

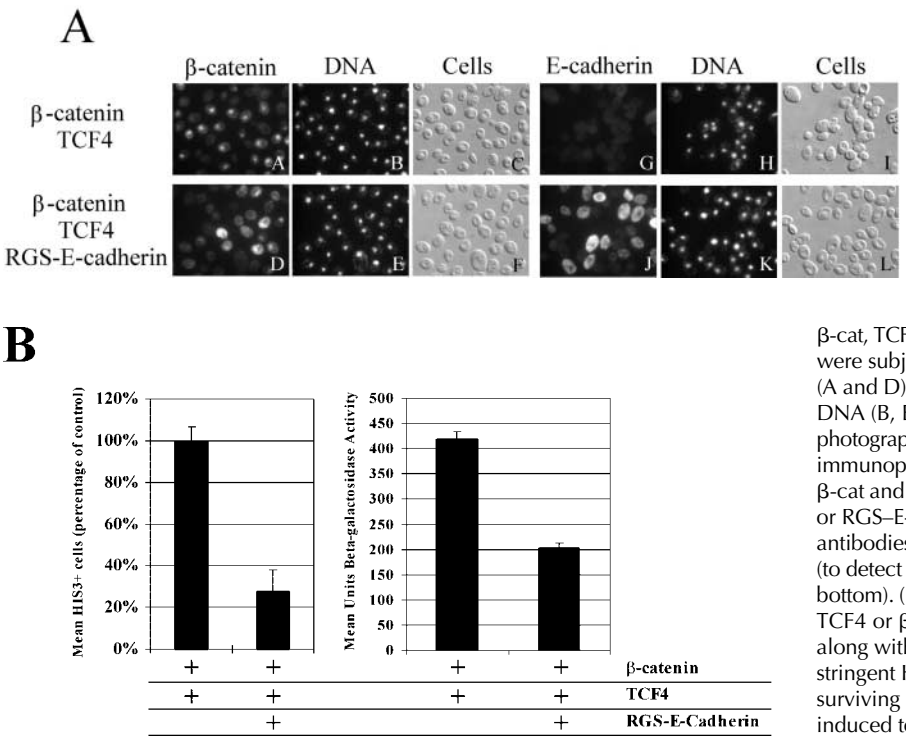
ering domain of human regulator of G protein signaling 4 (RGS4) fused to the NH_2 terminus of the cytoplasmic tail of mouse E-cad was introduced. RGS–E-cad localized to the cytoplasm and plasma membrane (Fig. 4 A, left, panel J). Nuclear β-cat redistributed to the cytoplasm in cells coexpressing RGS–E-cad (Fig. 4 A, left, panels A and D). In cells expressing only β-cat and TCF4, TCF4 coimmunoprecipitated with β-cat (Fig. 4 A, right, lane 3). However, when RGS–E-cad complexed with β-cat, the amount of TCF4 in β-cat immunoprecipitates was reduced (Fig. 4 A, right, lane 4). Furthermore, RGS–E-cad expression reduced the ability of β-cat–TCF4 to induce both HIS3 and LacZ reporter genes by 30% and 50%, respectively (Fig. 4 B). Although there was no detectable β-cat in the nucleus in the presence of RGS–E-cad, β-gal expression was only reduced by 50%, illustrating the relative sensitivity of reporter gene expression compared with IF. Thus, binding of β-cat to RGS–E-cad results in its redistribution to the cytoplasm, reduction in β-cat–TCF4 complex formation, and concomitant reduction in LEF/TCF-dependent reporter gene expression.

In mammalian cells, APC complexes with cytoplasmic β-cat, resulting in its Axin-dependent phosphorylation by GSK3β and targeting to the ubiquitin-mediated degradation pathway. The central 733 aa of APC (APC-25) are sufficient to bind β-cat and promote its degradation (Munemitsu et al., 1995). We expressed APC-25 in yeast containing stably integrated β-cat and TCF4 along with either HIS3 or LacZ reporter genes. APC-25 localized throughout the cell, mainly in the cytoplasm (Fig. 5 A, left, panel J). In the presence of APC-25, β-cat redistributed from the nucleus to the cytoplasm (Fig. 5 A, left, compare panels D and A). In the absence of APC-25, β-cat associated with TCF4 (Fig. 5 A, right, lane 2). APC-25 coimmunoprecipitated with β-cat (Fig. 5 A, right, lane 4), correlating with a loss of TCF4 in the β-cat immunoprecipitates (Fig. 5 A, right, lane 4). Furthermore, the ability of strains containing β-cat and TCF4 along with APC-25 to grow on media stringently selecting for HIS3 expression was dramatically reduced (Fig. 5 B). β-cat plus TCF4–induced LacZ reporter activity was also inhibited in the presence of APC-25 (Fig. 5 B). Thus, similar to results with RGS–E-cad, expression of APC-25 led to a dramatic reduction in β-cat/TCF4–dependent reporter gene expression accompanied by redistribution of β-cat from the nucleus to the cytoplasm. Steady-state β-cat levels, examined by Western blot, were similar with or without APC-25, despite the association of β-cat with APC-25 and its redistribution to the cytoplasm (Fig. 5 A, right).

APC-25, Axin, and GSK3β associate with β-cat

In mammalian cells, a complex containing APC, Axin, β-cat, and GSK3β forms in the cytoplasm, and negatively regulates the transcriptional activity of β-cat by targeting it to the ubiquitin-mediated degradation machinery. To systematically define the role of each of these components in regulation of β-cat signaling, various combinations of APC-25, Axin, and GSK3β were introduced into yeast containing stably integrated β-cat, TCF4, and either HIS3 or LacZ reporter plasmids, followed by evaluation of their ability to associate with β-cat and to modulate LEF/TCF-dependent transcription. As described above, TCF4 efficiently coimmunoprecipitated with β-cat (Fig. 6 A, anti-FLAG, IP, lanes 5–8), but not in the presence of APC-25 (Fig. 6 A, antiFLAG, IP, lanes 1–4). APC-25 and Axin, either alone or in combination, complexed with β-cat (Fig. 6 A, anti-HA, IP, lanes 1, 2, and 6). GSK3β was not found in β-cat immunoprecipitates either alone or in combination with APC-25 (Fig. 6 A, GSK3β, IP, lanes 3 and 7), despite being present in total cell lysates (GSK3β, T, lanes 3 and 7). GSK3β associated with β-cat only when coexpressed with Axin (Fig. 6 A, GSK3β, IP, lanes 4 and 8), consistent with reports indicating a role for Axin as a scaffolding protein in the β-cat complex (Hart et al., 1998). Of note is that none of the protein combinations described reduced steady-state levels of β-cat in total protein extracts (Fig. 6 A, β-cat, T, lanes 1–8).

Wherever APC-25 was present, β-cat localized predominantly to the cytoplasm (Fig. 6 B, panels M, P, S, and V). Ex-



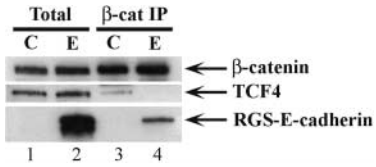
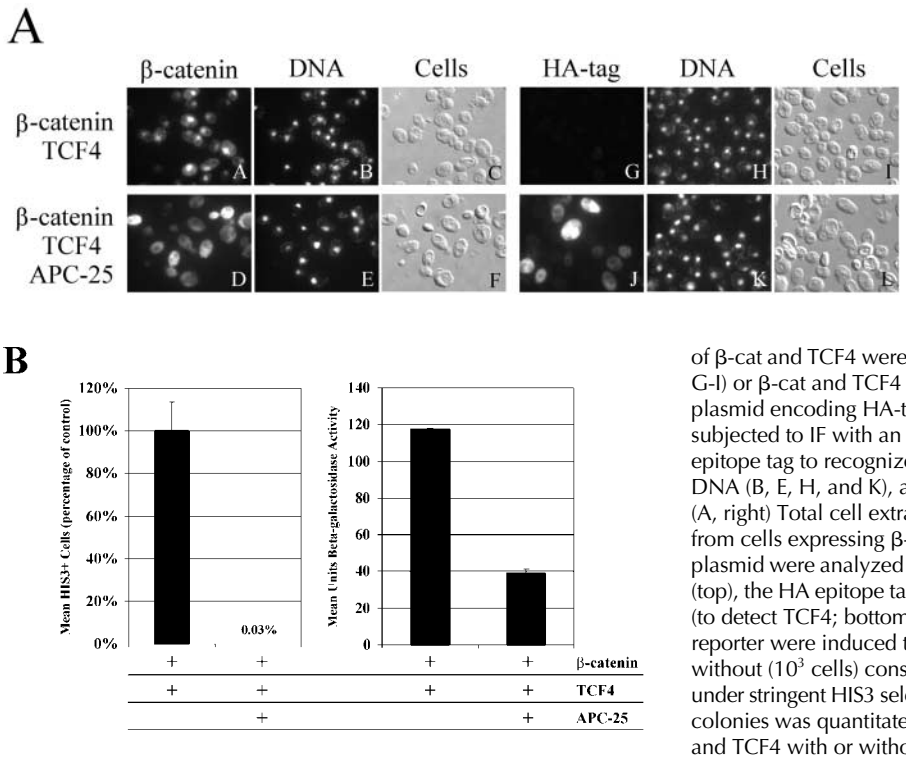


Figure 4. **The cytoplasmic domain of E-cad inhibits β-cat/TCF4 transcriptional activity in yeast by sequestration in the cytoplasm.** (A, left) Cells induced to express β-cat and TCF4 (A–C and G–I) or β-cat, TCF4, and RGS–E-cad (D–F and J–L) from plasmids were subjected to IF with an antibody against β-cat (A and D) or E-cad (G and J). Proteins (A, D, G, and J), DNA (B, E, H, and K), and cells (C, F, I, and L) were photographed. (A, right) Total cell extracts (Total) or β-cat immunoprecipitates (β-cat IP) from cells expressing β-cat and TCF4 plasmids plus either control plasmid (C) or RGS–E-cad (E) were analyzed by Western blot with antibodies against β-cat (top), the FLAG epitope tag (to detect TCF4; middle), or E-cad (to detect RGS-E-cad; bottom). (B, left) HIS$^-$ cells induced to express β-cat and TCF4 or β-cat, TCF4, and RGS–E-cad from plasmids along with the 5X LEF HIS3 reporter were plated under stringent HIS3 selection conditions, and the number of surviving colonies were quantitated. (B, right) Cells induced to express β-cat and TCF4 or β-cat, TCF4, and RGS–E-cad from plasmids along with the 5X LEF LacZ reporter were assayed for β-gal activity.

pression of Axin or GSK3β had no detectable effect on the localization of β-cat (Fig. 6 B, panels A, D, G, and J) or of APC-25 (Fig. 6 C, panels M, P, S, and V). Attempts to determine the localization of GSK3β failed due to nonspecific antibody recognition of yeast proteins (unpublished data).

Expression of APC-25 with β-cat and TCF4 consistently decreased LacZ reporter gene expression to a similar extent regardless of the presence of other regulatory proteins (Fig. 6 D). Axin and/or GSK3β expression with β-cat plus TCF4, in the absence of APC-25, had no significant effect



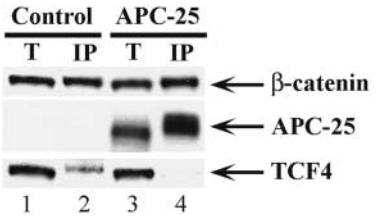


Figure 5. **APC-25 inhibits β-cat/TCF4 transcriptional activation in yeast by sequestration of β-cat in the cytoplasm.** (A, left) Cells with stably integrated copies of β-cat and TCF4 were induced to express β-cat and TCF4 (A–C and G-I) or β-cat and TCF4 in the presence of a constitutive expression plasmid encoding HA-tagged APC-25 (D–F and J–L) and were subjected to IF with an antibody against β-cat (A and D) or the HA epitope tag to recognize APC-25 (G and J). Proteins (A, D, G, and J), DNA (B, E, H, and K), and cells (C, F, I, and L) were photographed. (A, right) Total cell extracts (T) and β-cat immunoprecipitates (IP) from cells expressing β-cat and TCF4 plus either APC-25 or control plasmid were analyzed by Western blot with antibodies against β-cat (top), the HA epitope tag (to detect APC-25; middle), or the FLAG tag (to detect TCF4; bottom). (B, left) HIS$^-$ cells with the 5X LEF HIS3 reporter were induced to express β-cat and TCF4 with (10^6 cells) or without (10^3 cells) constitutively expressed APC-25, were plated under stringent HIS3 selection conditions, and the number of surviving colonies was quantitated. (B, right) Cells induced to express β-cat and TCF4 with or without constitutively expressed APC-25 along with the 5X LEF LacZ reporter were assayed for β-gal activity.

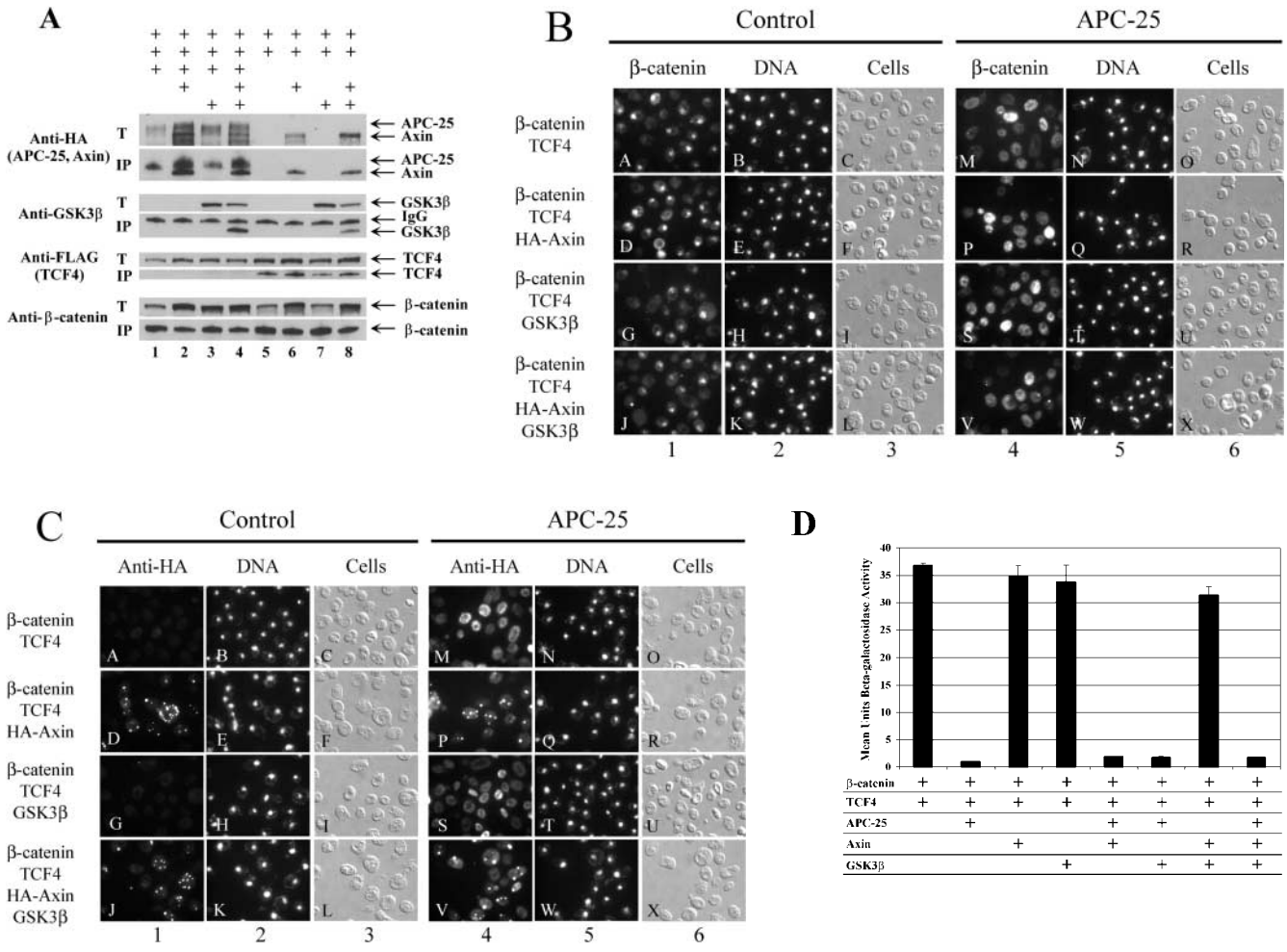


Figure 6. **Analysis of complex formation, subcellular localization, and effects on transcriptional activity using combinations of APC-25, Axin, and GSK3β with β-cat/TCF4.** (A) Expression vectors for APC-25, Axin, and GSK3β were introduced into yeast cells containing stably integrated β-cat and TCF4. β-cat was immunoprecipitated from protein extracts followed by Western blot with antisera against the HA tag (to detect APC-25 and Axin), human GSK3β, the FLAG tag (to detect TCF4), or β-cat in total extracts (T) and β-cat immunoprecipitates (IP). (B and C) Cells containing integrated β-cat and TCF4 alone (A–C and M–O) or with Axin (D–F and P–R), GSK3β (G–I and S–U), or Axin plus GSK3β (J–L and V–X) and with either a control vector (Control) or an APC-25 expression vector (APC-25) were subjected to IF with an antibody against β-cat (B) or the HA tag (to detect Axin or APC-25) (C). Proteins (columns 1and 4), DNA (columns 2 and 5), and cells (columns 3 and 6) were photographed. (D) Yeast cells with integrated β-cat and TCF4 and combinations of APC-25, Axin, and GSK3β as well as the 5X LEF LacZ reporter were assayed for β-gal activity.

on β-gal activity (Fig. 5 B; Fig. 6 D). These data indicate that of the proteins tested, only APC-25 can inhibit β-cat signaling, and this correlates with diminished complex formation between β-cat and TCF4 as well as a redistribution of β-cat from the nucleus to the cytoplasm, where it binds to APC-25.

β-cat is constitutively phosphorylated on serine/threonine

In mammalian cells, βTRCP recognizes phosphorylated β-cat and targets it to the ubiquitin degradation machinery. Consequently, we examined phosphorylation of β-cat by Western blot using antibodies specific for β-cat phosphorylated at serine/threonine residues 33, 37, 41, and 45. Phosphorylation of β-cat was detectable in yeast with or without expression of exogenous GSK3β (Fig. 7 A), indicating that it must be phosphorylated by an endogenous yeast kinase and could serve as a substrate for ubiquitin-mediated degradation. Densitometric scanning and normalization of the data indicated that coexpression of APC-25 or Axin in the presence or absence of exogenous GSK3β led to a slight increase in β-cat phosphorylation (Fig. 7 A).

βTRCP decreases both the level of β-cat and β-cat–mediated transcriptional activation

Because significant differences in the steady-state levels of β-cat were not detected, the stability of β-cat was examined by pulse-chase analysis. Because the yeast system may lack a specific F-box protein required for targeting β-cat degradation, the effect of βTRCP expression on β-cat levels was examined. Yeast containing stably integrated β-cat and TCF4 with combinations of βTRCP and APC-25 were induced to express β-cat by growth in galactose-containing medium (pulse) followed by repression of β-cat synthesis by transfer into medium containing glucose (chase). Coexpression of βTRCP, with β-cat and TCF4 in the absence of APC-25,

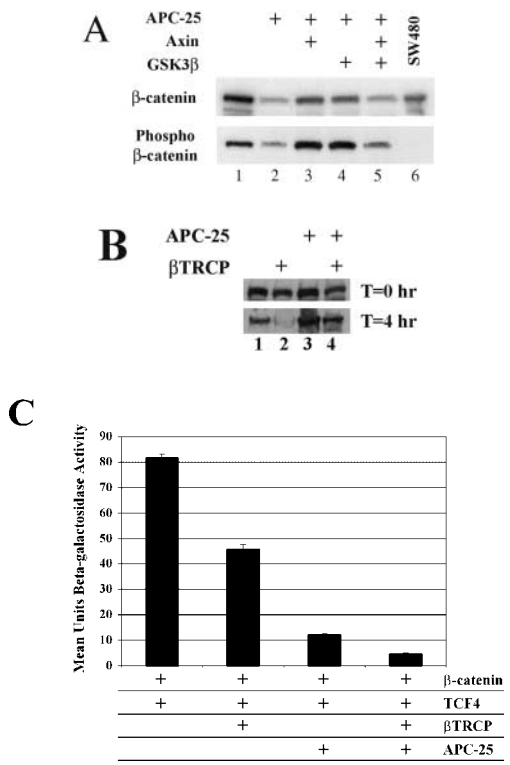


Figure 7. **β-cat stability and phosphorylation.** (A) Protein extracts from yeast expressing stably integrated β-cat and TCF4 with various combinations of APC-25, Axin, and GSK3β and SW480 cells were subjected to Western blot analysis with an antibody directed against β-cat (top) or with antisera specific for phosphorylated β-cat (bottom). (B) Cells with integrated β-cat and TCF4 along with either βTRCP (lanes 2 and 4) or control vector (lanes 1 and 3) in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of stably integrated constitutively expressed APC-25 were induced to express regulated proteins (β-cat, TCF4, and βTRCP) by growth in medium containing galactose (0 h, top). Cells were then washed into medium containing glucose to repress expression of regulated proteins for 4 h (bottom). Cell extracts prepared at 0 or 4 h were subjected to Western blot analysis for β-cat protein levels (top panel exposed 10 s and bottom panel exposed 1 min). (C) Cells with integrated β-cat and TCF4 along with various combinations of stably integrated APC-25 or plasmids encoding βTRCP and 5X LEF LacZ reporter were assayed for β-gal activity.

reduced the amount of β-cat protein (Fig. 7 B, 4-h chase, compare lanes 1 and 2). Alternatively, expression of APC-25 with β-cat and TCF4 resulted in increased β-cat protein (Fig. 7 B, 4 h, compare lanes 1 and 3). However, with the addition of βTRCP, the amount of β-cat was again diminished (Fig. 7 B, compare lanes 3 and 4). Thus, in the yeast system, β-cat is constitutively phosphorylated on serine/threonine and, in the presence of βTRCP, displays decreased levels, suggesting a reconstitution of some aspects of the ubiquitin-mediated degradation of β-cat. Various gene combinations including Axin or exogenous GSK3β did not reveal further changes in the levels of β-cat beyond those demonstrated in Fig. 7 B (unpublished data).

βTRCP reduced the ability of β-cat plus TCF4 to induce LacZ reporter expression by ~40% (Fig. 7 C). Combination of βTRCP and APC-25 decreased reporter activity beyond that seen with APC-25 alone (Fig. 7 C). These data indicate that βTRCP can reduce the capability of β-cat to activate transcription, presumably due to its facilitation of β-cat degradation; however, other mechanisms are possible.

Putative NES sequences of APC-25 are required for down-modulating β-cat signaling

APC contains NESs and is reported to enter and exit the nucleus in mammalian cells. APC-25 has six of seven 20-aa repeats (20R2–7) found in the central region of APC and several functional NES sequences have been localized to these 20-aa repeats (20R3, 4, and 7) (Fig. 8 A). Sequence alignment reveals additional potential NES sequences in repeats 2, 5, and 6 (Fig. 8 A). To explore the role of previously identified and putative NES sequences, we systematically mutated each of three hydrophobic core amino acids in the six potential NES sequences to alanines (Fig. 8 B). APC-25 with mutation of previously identified NES sequences in 20-aa repeats 3, 4, and 7 (Fig. 8 B, 20R2, 5, 6-Δ3, 4, 7) showed only a slight loss of its ability to suppress β-cat–dependent reporter gene expression (Fig. 8, C and D). Mutation of additional putative NES sequences in 20-aa repeats 2, 5, and 6, in combination with Δ3, 4, and 7 (Fig. 8 B), resulted in incremental loss of the ability of APC-25 to suppress β-cat–dependent transcription (Fig. 8, C and D). Mutation of all putative NES sequences (Fig. 8 B, 20R-Δ2, 3, 4, 5, 6, 7) resulted in complete ablation of APC-25 suppression of β-cat–dependent reporter gene activation, restoring expression to levels comparable to the absence of APC-25 (Fig. 8, C and D). These results indicate that at least one intact putative NES sequence within APC-25 is capable of conferring partial suppression of reporter activation. Interestingly, putative NES sequences in 20-aa repeat 2 are the least homologous to the consensus sequence for leucine-rich NESs (Fig. 8 A) and are alone incapable of conferring partial suppression (Fig. 8, C and D). APC, as well as the central domain of APC analogous to APC-25 (Rosin-Arbesfeld et al., 2000), rapidly shuttle between nucleus and cytoplasm in mammalian cells. In yeast, APC-25 appears to be almost exclusively cytoplasmic at steady-state (Figs. 5 and 6), raising the possibility that APC-25 enters the nucleus and is very rapidly exported. This suggests that mutation of NES sequences should result in nuclear accumulation of APC-25. Surprisingly, mutation of the known and putative NES sequences within APC-25 did not result in accumulation of APC-25 in the nucleus when examined by IF (Fig. 9 A, panels D, G, F, and I). Although mutants of APC-25 remained localized to the cytoplasm, most of them failed to retain β-cat (Fig. 9 B). β-cat was primarily cytoplasmic in yeast with wild-type APC-25, whereas mutation of the three previously defined APC NES sequences (20R2, 5, 6-Δ3, 4, 7) resulted in an increase in nuclear β-cat (Fig. 9 B, compare panels B and C). Mutation of additional putative NES sequences resulted in a further increase in nuclear β-cat, with restoration of the predominantly nuclear localization by mutants lacking all hydrophobic NES sequences (20R-Δ2, 3, 4, 5, 6, 7) or containing only the sequences in 20R2 (20R2-Δ3, 4, 5, 6, 7) (Fig. 9 B, panels O and N, respectively). Nuclear location of β-cat in cells with NES mutants of APC correlated with reporter gene activity (Fig. 8, C and D).

Mutation of previously identified as well as putative NES sequences in APC-25 resulted in decreased binding to β-cat

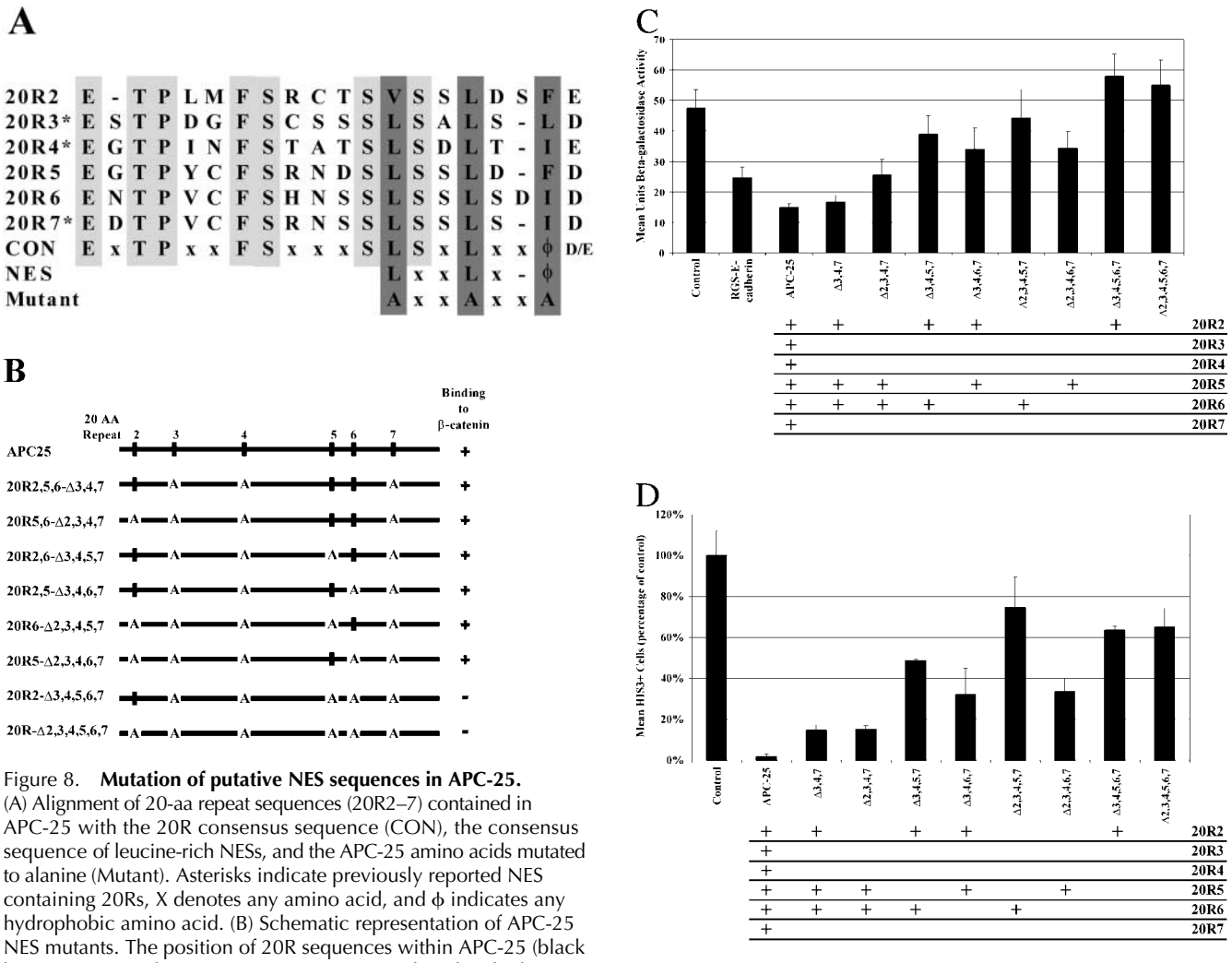


Figure 8. **Mutation of putative NES sequences in APC-25.** (A) Alignment of 20-aa repeat sequences (20R2–7) contained in APC-25 with the 20R consensus sequence (CON), the consensus sequence of leucine-rich NESs, and the APC-25 amino acids mutated to alanine (Mutant). Asterisks indicate previously reported NES containing 20Rs, X denotes any amino acid, and ϕ indicates any hydrophobic amino acid. (B) Schematic representation of APC-25 NES mutants. The position of 20R sequences within APC-25 (black boxes), repeats where NES sequences were replaced with alanines (A), and ability of APC-25 NES mutants to coimmunoprecipitate with β-cat are indicated. (C) Cells containing integrated β-cat and TCF4 alone (Control) or with plasmids expressing wild-type APC-25, APC-25 NES mutants, or RGS–E-cad as well as the 5X LEF LacZ reporter were assayed for β-gal activity. The x axis labels indicate the mutated NES sequence whereas a plus indicates the presence of a particular wild-type NES sequence in the indicated 20-aa repeat regions (20R2–7). (D) HIS$^-$ cells induced to express β-cat and TCF4 (Control) along with wild-type APC25 or APC-25 NES mutants in the presence of the 5X LEF HIS3 reporter were plated under stringent HIS3 selection conditions and surviving colonies were quantitated. The x axis labels are as in C.

and ablated binding altogether in mutants lacking NES sequences 3, 4, 5, 6, and 7 (Fig. 9 C, lanes 9 and 10). Decreased binding of NES mutants correlated with an increase in TCF4 binding to β-cat (Fig. 9 C). Of note is that putative NES sequences in 20-aa repeat 2 are alone unable to confer binding of APC-25 to β-cat (Fig. 9 C, lane 9), consistent with the inability of this APC-25 mutant to suppress reporter gene activation and to redistribute β-cat to the cytoplasm. Thus, the putative NES sequences in APC-25 also appear to be required for binding of APC-25 to β-cat, and the ability of APC-25 to modulate β-cat signaling may be defined by the ability to bind and retain β-cat in the cytoplasm.

Discussion

We introduced genes encoding key players in the mammalian β-cat signaling pathway, along with a pathway-specific transcriptional reporter gene, into the yeast *Saccharomyces cerevisiae*. Using this simplified system, it was possible to systematically define aspects of protein complex formation, subcellular localization, and signaling regulation in the β-cat pathway in the absence of the signaling network complexities that exist in mammalian cells.

Protein complex formation in yeast

Similar to other cellular systems, β-cat and TCF/LEF proteins readily formed a transcriptionally competent complex. Both APC-25 and RGS–E-cad displaced TCF/LEF upon binding to β-cat, confirming previous reports that APC, E-cad, and LEF1 compete for binding to the armadillo repeats of β-cat (Rubinfeld et al., 1993; Hulsken et al., 1994; Sadot et al., 1998; Orsulic et al., 1999). We show that mutation of putative NES sequences within 20-aa repeats (20Rs) of APC-25 results in defective binding of APC-25 to β-cat and prevents displacement of β-cat from TCF4. This is the first report where all 20Rs have been mutated in the same APC protein. Our results confirm that the 20R region is critical for β-cat binding and reveal differences in the capacity of different re-

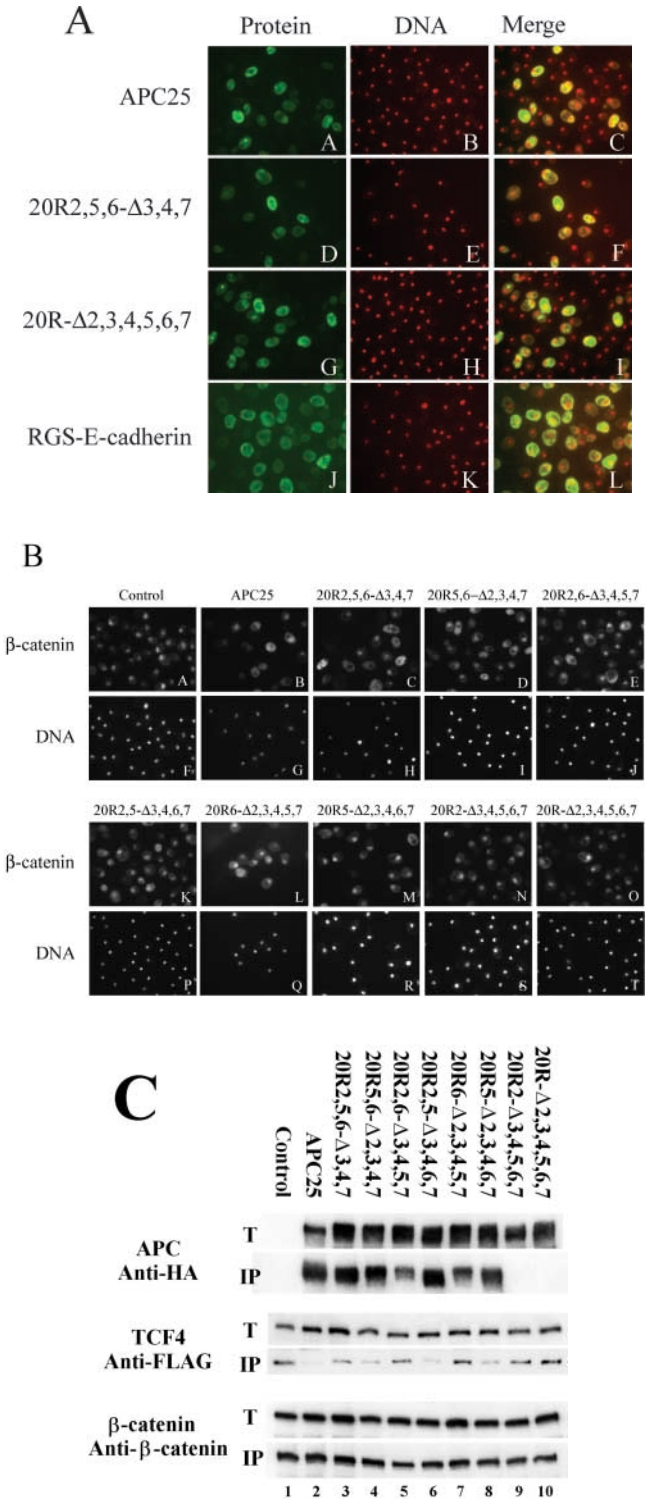


Figure 9. **Subcellular localization and complex formation with β-cat is altered in the presence of APC NES mutants.** (A) Yeast cells with integrated β-cat and TCF4 in combination with integrated wild-type APC-25 (APC-25) or APC-25 NES mutants or expressing RGS–E-cad from a plasmid were subjected to IF with an antibody against the HA epitope (APC-25 proteins) or against E-cad. Proteins (Protein, panels A, D, G, and J) and DNA (DNA, panels B, E, H, and K) were photographed. False color images were captured and merged (Merge, panels C, F, I, and L), overlapping signals of protein (green) and DNA (red) are indicated by yellow color. (B) Yeast cells with integrated β-cat and TCF4 alone plus either a control vector (Control), wild-type APC-25 expression vector (APC-25), or expression vectors for the indicated APC-25 NES mutants were subjected to IF with an antibody against β-cat. Proteins (panels A–E and K–O) and DNA (panels F–J and P–T) were photographed. (C) β-cat immunoprecipitates of protein extracts from cells expressing β-cat and TCF4 (Control) and either wild-type APC-25 or APC-25 NES mutants were analyzed by Western blot. Antisera against the HA tag (to detect APC-25 proteins), FLAG tag (to detect TCF4), or β-cat were used to identify the corresponding proteins in total cell extracts (T) as well as in complex with β-cat (IP).

peats for binding to β-cat. Residues comprising the 20R2 region alone are unable to sustain binding to β-cat, whereas those of the 20R5 and 20R6 regions are individually sufficient for interaction with β-cat. Other studies showed that Axin interacted directly with β-cat and GSK3β to bridge these two proteins (Hart et al., 1998; Ikeda et al., 1998; Itoh et al., 1998; Sakanaka et al., 1998). This result is borne out in yeast where Axin associated with β-cat, but we only observed GSK3β in complex with β-cat in the presence of Axin.

β-cat and LEF/TCF must cooperate to activate transcription

LEF/TCF transcription factors are known to require additional factors for transcriptional activation (Carlsson et al., 1993; Giese and Grosschedl, 1993). Consistent with these data, neither TCF4 nor LEF1 display transcriptional activity when expressed alone. Similarly, β-cat by itself is inactive and the combination of β-cat and either LEF1 or TCF4 is required for activation of transcription. Under these conditions, β-cat complexes with LEF1 or TCF4, confirming the necessity of cooperation between β-cat and LEF/TCF transcription factors for transcriptional activation. Furthermore, the ability of β-cat and LEF/TCF to function without additional introduced pathway components demonstrates that these proteins, in conjunction with the conserved yeast basal transcriptional machinery, are sufficient to activate pathway-specific reporter gene transcription.

Nuclear localization of LEF/TCF and β-cat

In mammalian cells, LEF/TCF proteins are found predominately in the nucleus, whereas β-cat localizes to the plasma membrane as a component of adherens junctions as well as to the cytoplasm and nucleus (Gumbiner, 1995; Eastman and Grosschedl, 1999). β-cat enters the nucleus via an unconventional mechanism involving direct interaction with the nuclear pore complex in an NLS-independent manner (Fagotto et al., 1998; Yokoya et al., 1999). Both β-cat and TCF4 independently and constitutively localized to the nucleus when introduced into yeast cells, suggesting that they utilize the basic yeast nuclear transport machinery and that no other specific β-cat pathway components are required for β-cat entry and accumulation in the nucleus. In the yeast system, β-cat and LEF/TCF localize to the nucleus under conditions where reporter gene expression is activated. If β-cat is relocalized to the cytoplasm by RGS–E-cad or APC-25 coexpression, there is a concomitant decrease in reporter gene transcription. Restoration of β-cat nuclear accumulation by mutations in APC-25 that disrupt complex formation results in restoration of reporter gene expression. Thus, consistent with other systems, our data indicate that nuclear localization of β-cat is required

for its role in transcriptional activation (Funayama et al., 1995; Behrens et al., 1996; Molenaar et al., 1996; Orsulic and Peifer, 1996; Schneider et al., 1996). Interestingly, despite binding to β-cat, Axin had no effect either alone or in combination with other regulators on β-cat localization, transcriptional activation, or complex formation with TCF4. It is possible that the interaction is too weak to sequester β-cat in the cytoplasm or that additional components are required.

Regulation of β-cat signaling and nuclear localization

In normal mammalian cells, in the absence of a Wnt signal or an activating mutation, the steady-state localization of β-cat is cytoplasmic, mostly at the plasma membrane. Surprisingly, in the yeast system, β-cat and TCF/LEF are almost exclusively nuclear, and reporter gene transcription is constitutive. This supports a model where nuclear localization of β-cat represents a default state of the cell and active inhibition of β-cat signaling is necessary. Therefore, the "resting" state in a mammalian cell would require an active pathway to counteract the nuclear localization of β-cat. Mechanisms that could downregulate the nuclear accumulation of β-cat include inhibition of nuclear import, active nuclear export, cytoplasmic sequestration, and degradation. Inactivation of this inhibitory mechanism, via stimulation by Wnt or other growth factor signals, would lead to activated β-cat–modulated transcription.

It is likely that a regulated balance exists between active export of β-cat from the nucleus and its constitutive import. Export of β-cat from *Xenopus* nuclei in vitro is saturable, indicating a need for specific interactions for export (Wiechens and Fagotto, 2001). This hypothesis is further supported by studies suggesting that β-cat is exported from the nucleus by APC in a manner dependent upon the CRM-1 export receptor (Henderson, 2000; Rosin-Arbesfeld et al., 2000). To test this mechanism in the yeast model system, we expressed a set of APC-25 proteins with mutations in previously defined and additional putative NES sequences. Because wild-type APC-25 and APC-25–β-cat complexes are cytoplasmic, and because APC is hypothesized to shuttle between the nucleus and cytoplasm, we expected to find that mutation of relevant NES sequences would lead to accumulation of APC-25 in the nucleus along with associated β-cat. Surprisingly, APC-25 mutants lacking all potential NES sequences were not detected accumulating in the yeast nucleus. Phosphorylation of a serine near the second NLS of APC-25 may be required for its efficient import into the nucleus (Zhang et al., 2001), and if this does not occur in yeast, APC-25 may inefficiently enter the nucleus and the effect of mutating NES sequences would not be evident. In this case, the observed redistribution of β-cat out of the nucleus is due to complex formation with cytoplasmic wild-type APC-25 and consequent sequestration. Alternatively, APC-25 may enter the yeast nucleus and be efficiently exported, independent of the NES sequences that were mutated. This could be due to the presence of cryptic NES sequences in APC-25, or NES-independent export.

An alternate mechanism to inhibit active nuclear localization of β-cat is sequestration via interaction with cytoplasmic proteins. This is supported by a report demonstrating that β-cat can exit the nucleus independently of CRM1 and RanGTP, suggesting passive export (Wiechens and Fagotto, 2001). In the yeast system, β-cat was redistributed from the nucleus to cytoplasm upon binding either cytoplasmic APC-25 or plasma membrane RGS–E-cad, supporting the model of regulation by cytoplasmic retention. Other studies with mammalian cells showed that β-cat nuclear localization and signaling can be inhibited by cadherin binding (Sadot et al., 1998; Orsulic et al., 1999; Gottardi et al., 2001). In tumor cells where E-cad expression is lost and β-cat signaling is activated, this may represent an important loss of regulatory function, underscoring the physiological relevance of an active inhibitory mechanism for attenuation of this signaling pathway.

Regulation of β-cat degradation

In the yeast system, complex formation between β-cat and either APC-25 or RGS–E-cad leads to cytoplasmic accumulation of β-cat and loss of transcriptional activation, however, there is no evidence for degradation of β-cat protein. In fact, complex formation with APC-25 appears to stabilize β-cat when analyzed in a pulse-chase experiment. This is similar to the stabilized β-cat–APC or β-cat–E-cad complexes observed in mammalian cells responding to Wnt-1, where β-cat degradation is inhibited (Hinck et al., 1994; Papkoff et al., 1996). In SW480 colon carcinoma cells with a truncating APC mutation, β-cat accumulates to high levels, yet much of the protein remains in the cytoplasm and associated with APC (Munemitsu et al., 1995; Kawahara et al., 2000; Barker and Clevers, 2001; Brabletz et al., 2001). These findings suggest, first, that complex formation between β-cat and proteins such as APC can be uncoupled from degradation and, second, that inhibition of degradation of β-cat is a prerequisite, but not sufficient, for nuclear accumulation.

In an effort to define the minimal requirements for β-cat degradation, we introduced additional genes into yeast containing β-cat and TCF4. APC-25, Axin, or GSK3β, alone or in all possible combinations, did not lead to measurable differences in steady-state levels of β-cat, despite detection of the expected complexes between these proteins. In contrast, βTRCP led to decreased β-cat protein levels when expressed without APC-25, Axin, or GSK3β. No further decrease in β-cat levels was observed when these proteins were included. In yeast, β-cat was constitutively phosphorylated on serine/threonine, potentially by one of the four known endogenous yeast GSK3 proteins (Andoh et al., 2000). Exogenous GSK3β does not lead to significant additional phosphorylation of β-cat, either because the protein is already maximally phosphorylated or because mammalian GSK3β is not active in our yeast system. However, a temperature-sensitive yeast mutant lacking all four GSK3 genes can be rescued by expression of mammalian GSK3β, suggesting that it is functional in yeast (Andoh et al., 2000). Taken together, these data suggest that when β-cat is appropriately phosphorylated, potentially by a yeast GSK3 homologue, βTRCP expression is sufficient for its enhanced turnover. In mammalian cells, APC and Axin would be needed to enable phosphorylation of β-cat, presumably by the complexed GSK3β, whereas in the yeast system, β-cat is phosphorylated without additional scaffolding proteins.

We have developed a novel, defined yeast system for the study of β-cat signaling. Our results support a general model where activated β-cat signaling is a default process, and in normal, unstimulated cells, this pathway must be actively in-

hibited at several levels. Regulation of β-cat–TCF/LEF transcriptional activation can be accomplished through modulation of β-cat stability, protein complex formation, subcellular compartmentalization, and nuclear import/export. This yeast system will allow further study of the complex β-cat pathway in a simplified format. Furthermore, the dual reporter system described here will enable the design of genetic strategies for the selection of additional genes that regulate β-cat signaling.

Materials and methods

Plasmids

Mammalian genes were amplified by PCR from plasmids or Marathon-ready cDNA libraries (CLONTECH Laboratories, Inc.) and subcloned into pRS shuttle vectors or vectors for high level–regulated or constitutive expression in yeast (American Type Culture Collection [ATCC] accession nos. 87538, 87669, and 87670). Full-length human β-cat (NH_2-terminal HA tag) and human GSK3β were amplified from a human mammary library, the APC-25 fragment of APC (Munemitsu et al., 1995) from a human brain library, βTRCP from a human testis library, and human Axin form I (Ikeda et al., 1998; Genbank/EMBL/DDBJ accession no. AF009674) from a human fetal brain library. The ΔN90 (NH_2-terminal HA tag) mutant was generated by PCR amplification of β-cat lacking the NH_2-terminal 90 aa. NH_2-terminal FLAG-tagged human LEF1 and ΔN67 mutant were gifts from Don Ayer (University of Utah, Salt Lake City, UT). Human TCF4 and ΔN30 mutant were PCR amplified from IMAGE clones (ATCC accession nos. 1288178 and 184930). To generate yeast LEF/TCF-dependent reporter constructs, five tandem copies of the wild-type or mutated LEF/TCF binding site (5′-CCTTTGAA-3′ or 5′-CAATTCAA-3′, respectively, provided by Dorre Gruenberg, Aventis Pharmaceuticals) were cloned into the polylinker of pNB404 (ATCC accession no. 87513) upstream of the CYC1 promoter to generate WT or Mut 5X LEF LacZ. The LacZ gene was replaced with a HIS3 gene from pRS423 to generate HIS3 reporter plasmids. Reporter constructs containing the LYS2 nutritional marker were generated by subcloning the entire reporter cassette from pNB404-based vectors into pRS317. GAL–RGS–E-cad constructs were generated by fusing PCR products encoding the plasma membrane targeting NH_2-terminal 33 aa of the human RGS4 gene (Srinivasa et al., 1998) and the COOH-terminal 150 aa of mouse E-cad, amplified from a mouse embryo cDNA library. For each APC-25 NES mutant, the three hydrophobic amino acids were substituted with alanine using a single oligonucleotide designed using the QuickChange site-directed mutagenesis kit (Stratagene).

Yeast strains

Growth and maintenance of yeast strains, introduction of plasmids, and all genetic manipulations were performed as previously described (Lee et al., 1996). Wild-type yeast strains FY833 and FY834 were used as a basis for all strains generated (ATCC accession nos. 90844 and 90845) and were crossed with strain BY4704 containing designer deletion alleles (ATCC accession no. 200868) to generate strains containing additional selectable markers to accommodate the multiple genes expressed. Different plasmids with mammalian genes were generated such that a selection of different nutritional markers was available. Yeast strains with integrated copies of mammalian genes under the control of GAL1 (β-cat and TCF4) or ADH1 (APC-25 constructs) promoters were generated by homologous recombination using standard genetic techniques (Abelson and Simon, 1991).

Assay for β-gal in liquid cultures

β-gal assays and calculation of activity units were performed exactly as previously described (Ausubel et al., 1994). Triplicate yeast cultures of 50–100 cells were induced for protein expression in medium containing 2% galactose, and an OD_{600} was determined for each sample.

Histidine reporter plating assay

Yeast strains containing the desired plasmid(s) were grown on appropriate selective medium. Aliquots of 10^6 or 10^3 cells were plated in triplicate on selective medium lacking histidine with 10 mM 3-amino-triazol. Aliquots were also plated on selective medium containing histidine to determine the number of cells plated that were capable of growth. After 4 d of growth, colonies were counted and the percentage of viable cells surviving was calculated.

Immunofluorescence microscopy

IF was performed as previously described (Lee et al., 1996). Mouse monoclonal anti–β-cat (Transduction Laboratories), anti–E-cad (Transduction Laboratories), and anti-FLAG M5 (Sigma-Aldrich) antibodies were used at a 1:500 dilution. Mouse monoclonal anti-HA (BabCo) antiserum was used at a 1:200 dilution. Proteins were visualized with a 1:500 dilution of FITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Nuclei were stained with 10 μg/ml of DAPI. Cells were photographed using differential interference microscopy (DIC) optics (100×) with a SPOT camera (Diagnostic Instruments) using SPOT and Adobe Photoshop® software.

Western blot analysis and immunoprecipitation

Protein extracts from yeast cells were prepared as previously described (Lee et al., 1996). Yeast proteins (40 μg total yeast protein) were separated on 10% SDS-PAGE (Bio-Rad Laboratories), transferred to nitrocellulose, and probed with mouse monoclonal anti-HA, polyclonal anti-GSK3β (Transduction Labs), and anti–β-cat antibodies at a 1:2,000 dilution, anti-FLAG M5 at a 1:1,000 dilution, or a mixture of two antibodies recognizing phosphorylated β-cat (Cell Signaling Technology) at 1:1,000 each. HRP-conjugated secondary antibodies were used at a 1:5,000 dilution (Santa Cruz Biotechnology, Inc.), and blots were developed with ECL detection reagents (Amersham Biosciences). For immunoprecipitations, 0.5–1 mg total yeast protein extract was incubated with 50 μl of a 50% slurry of protein A–sepharose beads and 1 μg of β-cat antibody per sample for 2 h at 4°C. Immune complexes were washed three times with lysis buffer and solubilized in SDS-PAGE sample buffer before gel electrophoresis. 12–25-fold more total protein was used for immunoprecipitation than was analyzed in the comparable total protein lane of gels. Digital images were generated using Adobe Photoshop® software.

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