

# Chibby cooperates with 14-3-3 to regulate $\beta$ -catenin subcellular distribution and signaling activity

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$\beta$ -Catenin functions in both cell–cell adhesion and as a transcriptional coactivator in the canonical Wnt pathway. Nuclear accumulation of  $\beta$ -catenin is the hallmark of active Wnt signaling and is frequently observed in human cancers. Although  $\beta$ -catenin shuttles in and out of the nucleus, the molecular mechanisms underlying its translocation remain poorly understood. Chibby (Cby) is an evolutionarily conserved molecule that inhibits  $\beta$ -catenin–mediated transcriptional activation. Here, we identified 14-3-3 $\epsilon$  and 14-3-3 $\zeta$  as Cby-binding partners using affinity purification/mass spectrometry.

14-3-3 proteins specifically recognize serine 20 within the 14-3-3-binding motif of Cby when phosphorylated by Akt kinase. Notably, 14-3-3 binding results in sequestration of Cby into the cytoplasm. Moreover, Cby and 14-3-3 form a stable tripartite complex with  $\beta$ -catenin, causing  $\beta$ -catenin to partition into the cytoplasm. Our results therefore suggest a novel paradigm through which Cby acts in concert with 14-3-3 proteins to facilitate nuclear export of  $\beta$ -catenin, thereby antagonizing  $\beta$ -catenin signaling.

## Introduction

$\beta$ -Catenin (armadillo in *Drosophila melanogaster*) is a multifunctional protein that participates in several cellular processes, including cell–cell adhesion and the canonical Wnt signaling (Takemaru, 2006). At the plasma membrane,  $\beta$ -catenin binds to type I cadherins to mediate actin filament assembly via  $\alpha$ -catenin (Nelson and Nusse, 2004; Gates and Peifer, 2005). The remaining cytoplasmic  $\beta$ -catenin is continuously degraded by the action of the so-called “destruction complex” (Kimelman and Xu, 2006). In this complex, the tumor suppressors Axin and adenomatous polyposis coli (APC) function as scaffold proteins that facilitate sequential phosphorylation of  $\beta$ -catenin at the N terminus by casein kinase I $\alpha$  and glycogen synthase kinase 3 (GSK3; Liu et al., 2002). Phosphorylated  $\beta$ -catenin is then recognized by the E3 ubiquitin ligase receptor  $\beta$ TrCP and targeted for ubiquitin-mediated proteasomal degradation. Binding of a Wnt ligand to cell surface receptors, Frizzled, and low-density lipoprotein receptor-related proteins 5 and 6 ultimately leads to inactivation of the  $\beta$ -catenin destruction complex (Cadigan and

Liu, 2006). As a consequence,  $\beta$ -catenin accumulates in the cytoplasm, translocates into the nucleus, and interacts with members of the DNA-binding T cell factor/lymphoid enhancer factor (Tcf/Lef) family to activate Wnt target genes (Stadeli et al., 2006; Willert and Jones, 2006). Wnt/ $\beta$ -catenin signaling plays key roles during embryonic development and in maintenance of organs and tissues in adults (Cadigan and Nusse, 1997; Pinto and Clevers, 2005). Moreover, perturbations of this signaling cascade have been linked to a range of human diseases, especially cancer (Moon et al., 2004; Clevers, 2006). For instance,  $\beta$ -catenin signaling is aberrantly activated in >70% of colorectal cancers and to a lesser extent in other tumor types, promoting cancer cell proliferation, survival, and migration (Polakis, 2000; Lustig and Behrens, 2003).

Although it is well established that  $\beta$ -catenin can enter and exit the nucleus, the precise molecular mechanisms still remain largely unclear. It contains neither NLS nor nuclear export signal (NES) sequences.  $\beta$ -Catenin appears to be imported into the nucleus in an importin/karyopherin-independent fashion potentially by directly interacting with nuclear pore components (Fagotto et al., 1998; Yokoya et al., 1999). Conversely, APC has been shown to harbor functional NES sequences and facilitate nuclear export of  $\beta$ -catenin by the classical chromosome region maintenance protein 1 (CRM-1)–dependent pathway (Henderson, 2000; Rosin-Arbesfeld et al., 2000). However, subsequent studies challenged this view by demonstrating that  $\beta$ -catenin can

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Abbreviations used in this paper: APC, adenomatous polyposis coli; Cby, Chibby; CRM-1, chromosome region maintenance protein 1; DN-14-3-3, dominant-negative 14-3-3; GSK3, glycogen synthase kinase 3; hLuc, synthetic Renilla luciferase; KD, kinase-dead; Lef, lymphoid enhancer factor; MBP, maltose-binding protein; NES, nuclear export signal; OA, okadaic acid; rPP2A, recombinant protein phosphatase 2A; Tcf, T cell factor; WT, wild-type.

The online version of this paper contains supplemental material.

exit from the nucleus independent of APC and CRM-1 (Eleftheriou et al., 2001; Wiechens and Fagotto, 2001). More recently, Axin was identified as a nucleo-cytoplasmic shuttling factor that enhances cytoplasmic localization of  $\beta$ -catenin (Cong and Varmus, 2004; Wiechens et al., 2004). Clearly, these findings suggest the existence of multiple mechanisms that fine-tune nuclear  $\beta$ -catenin levels. Intracellular distribution of  $\beta$ -catenin may also be regulated by cytoplasmic and nuclear retention through interaction with its binding partners (Tolwinski and Wieschaus, 2001; Townsley et al., 2004; Krieghoff et al., 2006).

We previously reported a  $\beta$ -catenin antagonist Cbyby (Cby; Takemaru et al., 2003). Cby is a 14.5-kD protein evolutionarily conserved from fly to human. We demonstrated that Cby physically interacts with  $\beta$ -catenin in a manner that competes with Tcf/Lef factors, and thus represses  $\beta$ -catenin-mediated gene activation. Reduction of Cby levels in *D. melanogaster* embryos by RNAi results in hyperactivation of this pathway (Takemaru et al., 2003; Tolwinski and Wieschaus, 2004), which highlights the biological importance of Cby's function. Recently, we found that Cby promotes adipocyte and cardiomyocyte differentiation of pluripotent stem cells through inhibition of  $\beta$ -catenin signaling (Li et al., 2007; Singh et al., 2007).

To further elucidate the molecular and cellular functions of Cby, we set out to isolate Cby-binding partners. Here, we detail the isolation of 14-3-3 proteins as novel Cby interactors. We show that 14-3-3 proteins interact with Cby upon phosphorylation of serine 20 by Akt kinase. Notably, direct docking of 14-3-3 induces cytoplasmic sequestration of Cby protein. An alanine substitution for serine 20 in Cby eliminates binding to 14-3-3 and partially compromises Cby's ability to inhibit  $\beta$ -catenin signaling. We also demonstrate that Cby and 14-3-3 form a trimolecular complex with  $\beta$ -catenin in tissue culture cells. Intriguingly, Cby and 14-3-3 collaborate to promote cytoplasmic localization of  $\beta$ -catenin. In support of this model, nuclear-targeted Akt inhibits  $\beta$ -catenin-dependent transcriptional activation. Our findings, therefore, unravel a novel mechanism controlling the dynamic nucleo-cytoplasmic trafficking of  $\beta$ -catenin.

## Results

### Identification of the $\epsilon$ and $\zeta$ isoforms of 14-3-3 proteins as Cby-binding partners

To extend our understanding of Cby's function, we searched for Cby-interacting proteins using a maltose-binding protein (MBP) pull-down approach. Recombinant MBP or MBP-Cby fusion proteins were incubated with cell lysates prepared from human embryonic kidney (HEK) 293T cells. Cby-associated proteins were pulled down using amylose beads and resolved by SDS-PAGE, yielding several proteins that coprecipitated specifically with MBP-Cby in comparison to the MBP control (Fig. 1 A). The protein bands were excised, digested with trypsin, and processed for mass spectrometry. One of them, with an apparent molecular mass of 28 kD, was identified as 14-3-3 $\zeta$ . Although mass spectrometry was not able to identify the copurifying 30-kD protein, prior studies have found copurification of 14-3-3 $\zeta$  and 14-3-3 $\epsilon$  (Chen et al., 2003; Ory et al., 2003). These findings prompted us to examine whether this protein was 14-3-3 $\epsilon$ .

As shown in Fig. 1 B, an anti-pan-14-3-3 antibody that recognizes all isoforms of 14-3-3 proteins reacted equally with both bands (lane 1), but an anti-14-3-3 $\epsilon$ -specific antibody detected the upper band more intensely (lane 2), which suggests that both 14-3-3 $\epsilon$  and  $\zeta$  were copurified with Cby.

### 14-3-3 interacts with Cby

14-3-3 proteins are a family of highly conserved dimeric proteins, comprised of seven isoforms in mammals ( $\beta$ ,  $\gamma$ ,  $\epsilon$ ,  $\sigma$ ,  $\zeta$ ,  $\tau$ , and  $\eta$ ; Fu et al., 2000; Aitken, 2006). The family members are ubiquitously expressed and modulate diverse biological processes, in particular signal transduction pathways and transcription, through direct protein-protein interactions.

To validate the interaction between Cby and 14-3-3 proteins, we performed coimmunoprecipitation experiments using cell lysates from HEK293T cells cotransfected with Flag-Cby and individual HA-tagged 14-3-3 isoforms ( $\epsilon$ ,  $\zeta$ , and  $\eta$ ; Fig. 1 C). The 14-3-3 proteins were immunoprecipitated with anti-HA antibodies and analyzed by Western blot analysis using anti-Flag antibodies to detect Cby (Fig. 1 C, left) or vice versa (right). In a result consistent with the initial finding, the 14-3-3 $\zeta$  and  $\epsilon$  isoforms bound to Cby to a similar extent. In addition, Cby also coimmunoprecipitated with 14-3-3 $\eta$ , which indicates that Cby interacts with multiple 14-3-3 isoforms.

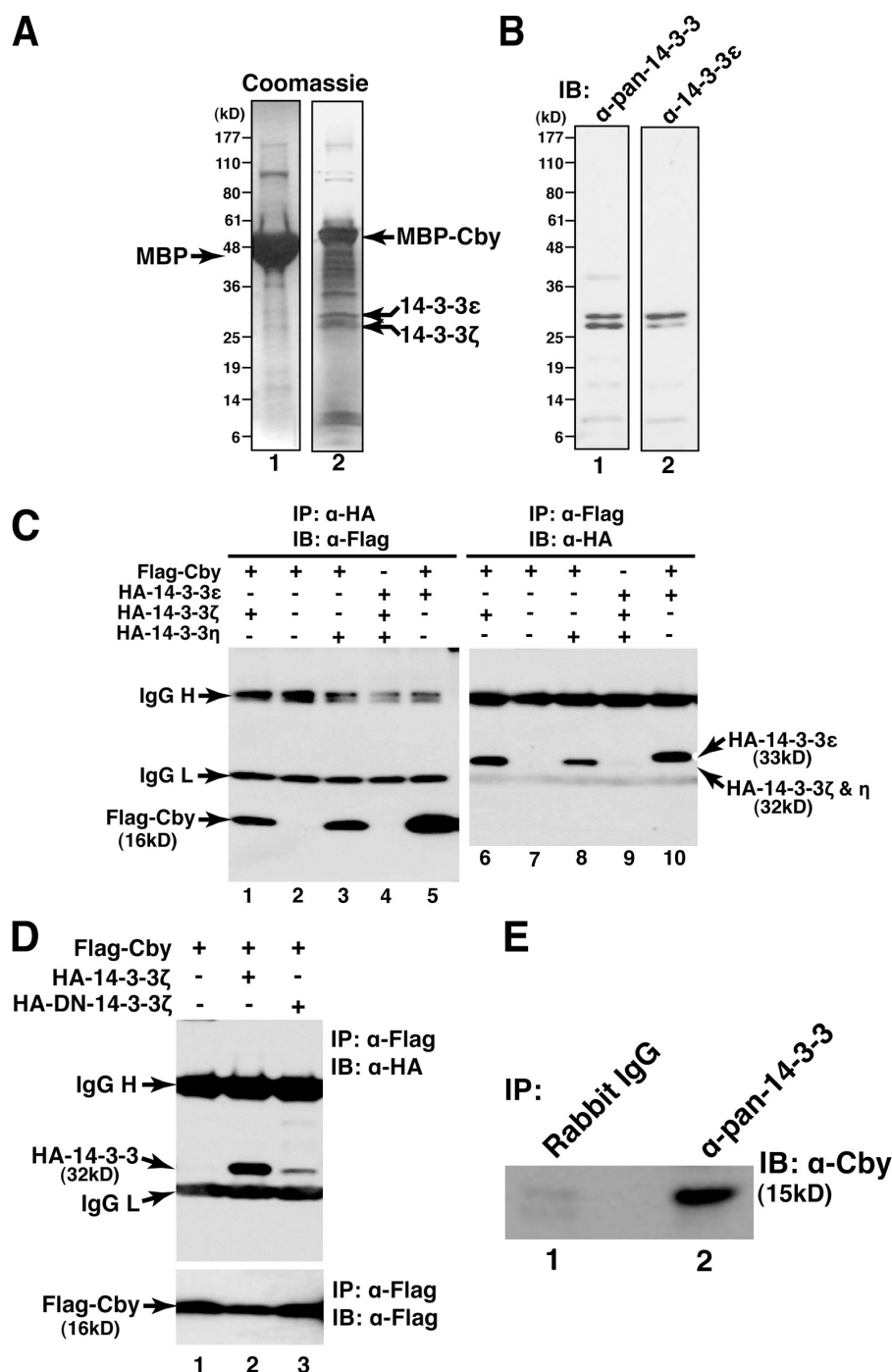
Next, we took advantage of a dominant-negative form of 14-3-3 $\zeta$  (DN-14-3-3 $\zeta$ ; R56A/R60A), which is unable to bind to its substrates yet retains the ability to form homo- or heterodimers with other 14-3-3 members (Xing et al., 2000). As determined by coimmunoprecipitation assays (Fig. 1 D), DN-14-3-3 $\zeta$  exhibited significantly reduced affinity to Cby compared with the wild-type (WT) protein, which confirms that the interaction between Cby and 14-3-3 is specific.

To further evaluate the physiological relevance of the Cby-14-3-3 interaction, the association of endogenous proteins was examined (Fig. 1 E). Immunoprecipitates with rabbit anti-pan-14-3-3 antibodies were prepared from HEK293T cells in which both Cby and 14-3-3 are relatively abundantly expressed. Cby was readily detected in the 14-3-3 immunoprecipitates (Fig. 1 E, lane 2) but not those performed using control rabbit IgG (lane 1). Collectively, these results establish clearly that 14-3-3 proteins specifically interact with Cby.

### The 14-3-3-binding motif in the N-terminal region of Cby is essential for its interaction with 14-3-3

14-3-3 proteins typically bind to their target proteins through one of the two consensus sequences, RSXpSXP (mode I) and RXXXpSXP (mode II), where pS represents phosphoserine (Muslin et al., 1996; Yaffe et al., 1997). Inspection of the human Cby amino acid sequence revealed a potential motif in its N-terminal region, <sup>16</sup>RKSASLS<sup>22</sup>, that closely matched the canonical mode II motif (Fig. 2 A). Serine 20 was predicted to be a crucial phosphorylation site. Worthy of note, this motif is highly conserved among vertebrate Cby homologues (unpublished data).

To study whether the putative 14-3-3-binding site is functional, we performed site-directed mutagenesis to introduce a series of

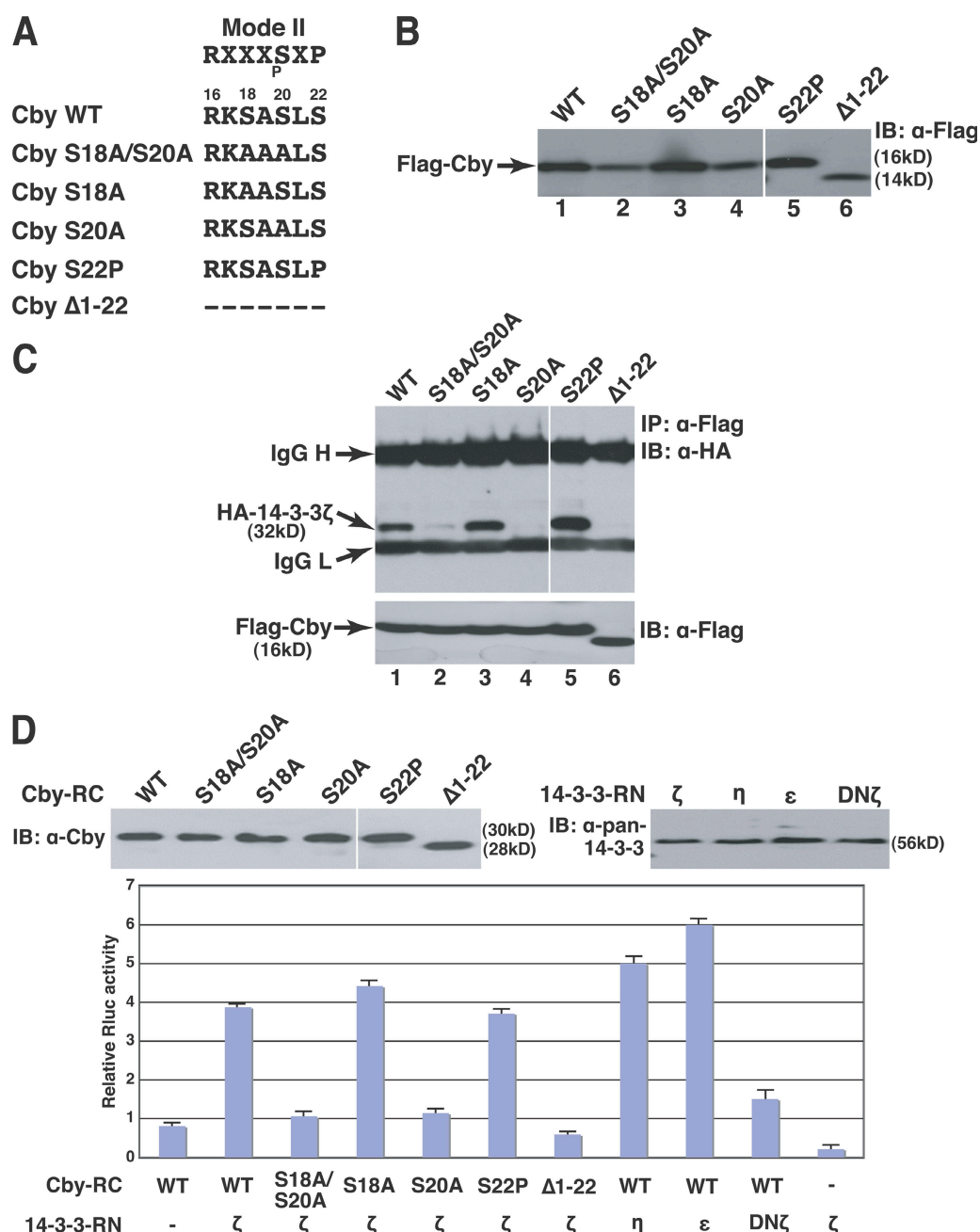


**Figure 1. Physical interaction between Cby and 14-3-3 proteins.** (A) Purification of 14-3-3 proteins as Cby-binding partners by an MBP pull-down approach. Lysates from HEK293T cells were incubated with bacterially expressed and purified MBP (lane 1) or MBP-Cby fusion protein (lane 2). Associated proteins were precipitated, separated by a 4–20% gradient SDS-PAGE, and visualized by Coomassie staining. (B) Immunoblotting with an anti-pan-14-3-3 antibody confirmed the copurification of 14-3-3 proteins with MBP-Cby (lane 1). An anti-14-3-3ε-specific antibody detected the upper band more intensely (lane 2). (C) HEK293T cells were transiently transfected with the indicated combinations of expression plasmids for Flag-Cby and HA-14-3-3 (ε, ζ, and η). Total cell lysates were immunoprecipitated with anti-HA antibodies and detected with anti-Flag antibodies (left) or vice versa (right). All three isoforms of 14-3-3 were expressed at comparable levels (not depicted). IgG H, IgG heavy chain; IgG L, light chain. (D) DN-14-3-3 exhibits significantly reduced affinity for Cby. Lysates from HEK293T cells transfected with Flag-Cby alone or together with HA-14-3-3ζ or HA-DN-14-3-3ζ were immunoprecipitated with anti-Flag antibodies and analyzed by Western blotting with anti-HA antibodies. Precipitated Flag-Cby levels were similar in all lanes, as shown in the bottom panel. (E) Coimmunoprecipitation of endogenous proteins. Cell lysates from nontransfected HEK293T cells were immunoprecipitated with control IgG (lane 1) or anti-pan-14-3-3 antibodies (lane 2) and immunoblotted for Cby.

mutations within this motif (Fig. 2 A). Upon overexpression of N-terminally Flag-tagged proteins in HEK293T cells, CbyS18A and CbyS22P were found to be present at levels similar to WT Cby (CbyWT), whereas the other Cby mutants (S18A/S20A, S20A, and Δ1-22) were expressed at slightly lower levels (Fig. 2 B). The Cby mutants were then individually coexpressed with HA-tagged 14-3-3ζ in HEK293T cells, immunoprecipitated using anti-Flag antibodies, and immunoblotted for 14-3-3ζ with anti-HA antibodies. As shown in Fig. 2 C, CbyS18A or CbyS22P, which harbors a motif that matches perfectly the mode II consensus sequence, interacted with 14-3-3ζ as efficiently as CbyWT. However, Cby mutants bearing a serine-to-alanine substitution

or deletion of serine 20 (S18A/S20A, S20A, and Δ1-22) almost completely lost their ability to bind to 14-3-3ζ.

To confirm the importance of serine 20 in 14-3-3 binding, we used the split synthetic Renilla luciferase (hRluc) assays for detecting protein–protein interactions (Paulmurugan and Gambhir, 2003). Cby was fused in-frame to the C-terminal portion of hRluc (Fig. 2 D, Cby-RC), whereas 14-3-3 was linked to the N-terminal portion of hRluc (14-3-3-RN). Interactions between these fusion proteins would bring the N- and C-terminal portions of hRluc into close proximity and restore its activity. The constructs were transfected into HEK293T cells in various combinations, and hRluc activities were measured. As demonstrated

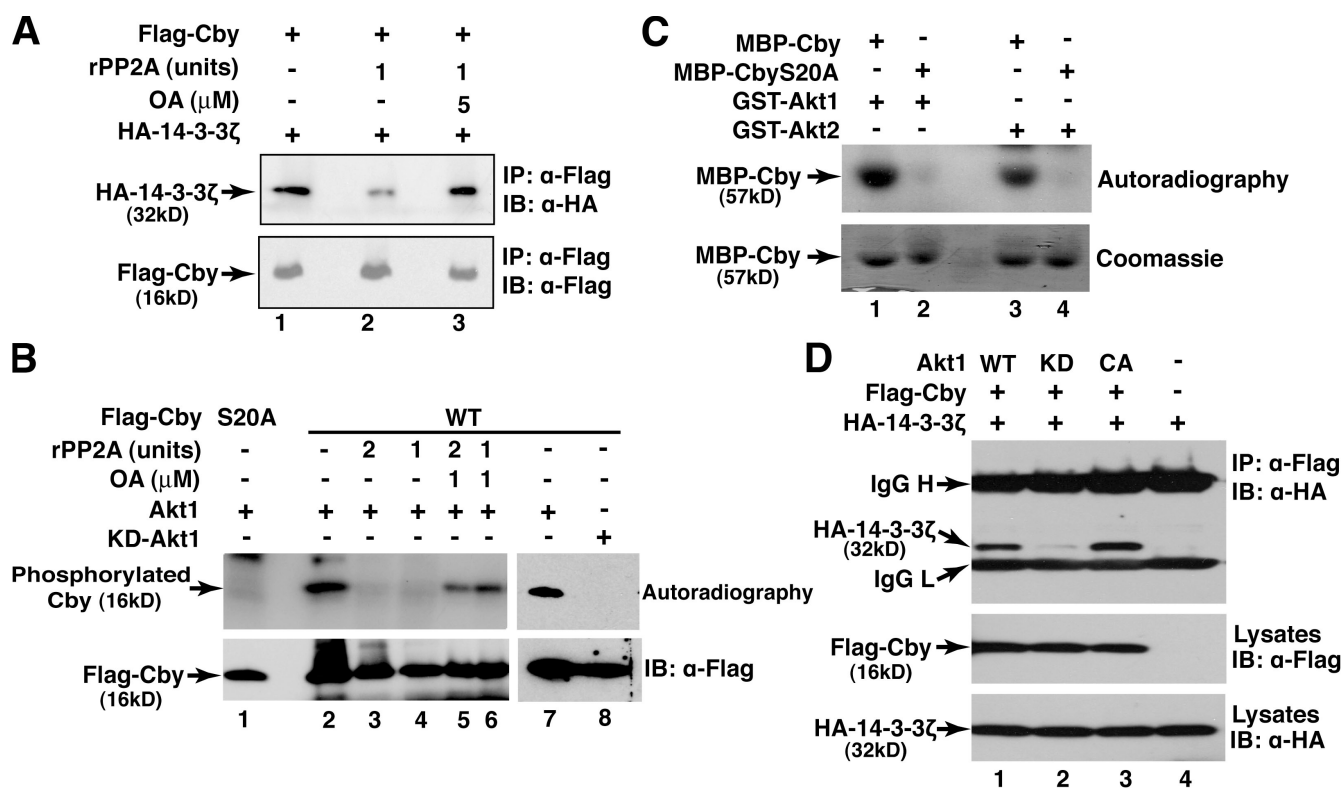


**Figure 2. Binding of 14-3-3 to Cby requires serine 20 within the N-terminal 14-3-3-binding motif of Cby.** (A) Cby contains a mode II 14-3-3-binding consensus sequence RXXXpSXP, where pS represents phosphoserine. Shown are the Cby point and deletion mutants used in this study. CbyΔ1-22 lacks the N-terminal 22 amino acids. (B) Expression levels of Cby mutants. Lysates from HEK293T cells transfected with an equal amount of an expression plasmid for Flag-tagged WT or mutant Cby were subjected to Western blotting with an anti-Flag antibody. (C) Cby serine 20 is crucial for binding to 14-3-3. HEK293T cells were transfected with Flag-tagged WT or mutant Cby and HA-14-3-3ζ, followed by coimmunoprecipitation with anti-Flag antibodies and immunoblotting with anti-HA antibodies. Note that to compensate for protein levels, the amounts of DNA for CbyS18A/S20A, CbyS20A, and CbyΔ1-22 were appropriately increased for transfection, and hence, roughly similar expression levels were observed for all the Cby mutants (bottom). IgG H, IgG heavy chain; IgG L, light chain. (D) Confirmation of Cby-14-3-3 interactions by split hLuc protein fragment-assisted complementation. WT or mutant Cby was fused in-frame to the C-terminal portion of hLuc (Cby-RC), whereas 14-3-3 was fused to its N-terminal part (14-3-3-RN). These expression constructs (200 ng each) were transfected into HEK293T cells as indicated, and Renilla luciferase activities were measured 24 h after transfection. A firefly luciferase plasmid was cotransfected to normalize transfection efficiency. Immunoblotting with anti-Cby antibodies (top left) or anti-pan-14-3-3 antibodies (top right) showed that these fusion proteins were stably expressed. Transfections were carried out in triplicate and the means ± SD are shown. White lines indicate that intervening lanes have been spliced out.

in Fig. 2 D, cotransfection of CbyWT, S18A, or S22P and 14-3-3ζ hLuc vectors resulted in high levels of hLuc activity. In contrast, coexpression of CbyS18A/20A, S20A, or Δ1-22 and 14-3-3ζ hLuc fusion proteins produced only basal levels of hLuc activity.

In addition, CbyWT efficiently bound to 14-3-3η and ε but not to DN-14-3-3ζ. Collectively, these data demonstrate that the Cby serine 20 residue within the mode II 14-3-3-binding motif mediates the interaction with 14-3-3 proteins.





**Figure 3. Cby serine 20 is phosphorylated by Akt, facilitating its interaction with 14-3-3.** (A) Interaction between Cby and 14-3-3 depends on phosphorylation. HEK293T cells were transfected with a Flag-Cby expression vector, and cell lysates were immunoprecipitated using anti-Flag affinity beads. The precipitated materials were preincubated in the presence or absence of rPP2A and its inhibitor OA. These samples were then mixed with an equal amount of total cell lysates from HEK293T cells expressing HA-14-3-3ζ, and the beads were precipitated, washed, and analyzed by SDS-PAGE and Western blotting with anti-HA antibodies. (B) Akt phosphorylates Cby at serine 20. HEK293T cells were transfected with an expression plasmid for Flag-tagged CbyWT or S20A, and Flag-Cby was immunoprecipitated using anti-Flag affinity beads. The bead-bound materials were incubated with [ $\gamma$ - $^{32}$ P]ATP and affinity-purified HA-tagged Akt1 or KD-Akt1 from transfected HEK293T cells in the presence or absence of rPP2A and OA as indicated. The reaction mixtures were then resolved by SDS-PAGE and visualized by autoradiography. (C) In vitro Akt kinase assay. Recombinant MBP-Cby or MBP-CbyS20A was incubated with active GST-Akt1 or Akt2 in the presence of [ $\gamma$ - $^{32}$ P]ATP. Proteins were separated by SDS-PAGE and detected by autoradiography. Roughly equal amounts of MBP-Cby fusion proteins were observed in all lanes upon Coomassie blue staining (bottom). (D) Coexpression of KD-Akt1 abolishes the interaction between Cby and 14-3-3. HEK293T cells were cotransfected with Flag-Cby, HA-14-3-3ζ, and either WT, KD, or myristoylated, constitutively active (CA) Akt1; and cell lysates were immunoprecipitated with anti-Flag antibodies followed by Western blot analysis with anti-HA antibodies. IgG H, IgG heavy chain; IgG L, light chain.

### Binding of 14-3-3 to Cby is regulated by phosphorylation

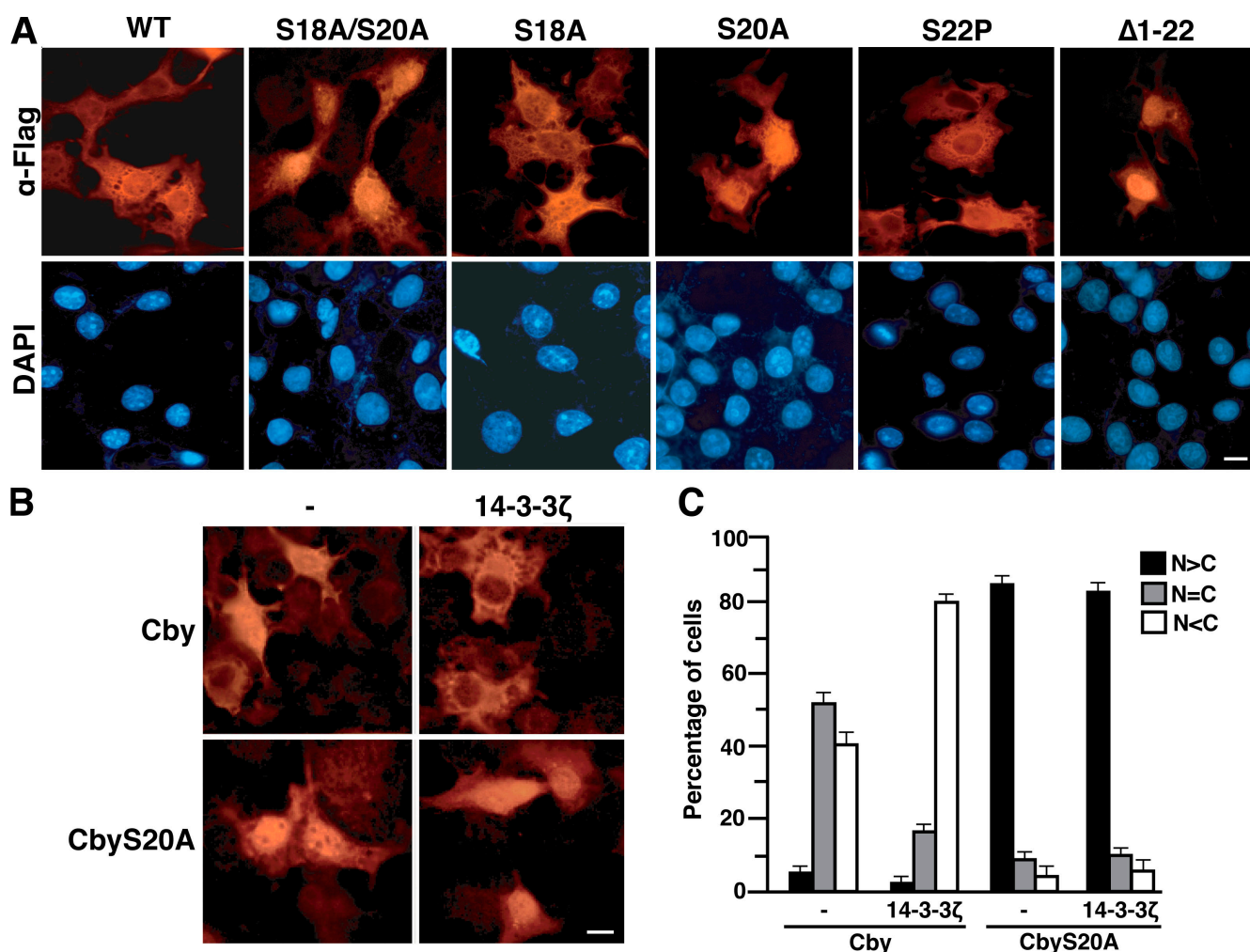
In the majority of cases, 14-3-3 proteins recognize serine-phosphorylated substrates (Muslin et al., 1996; Yaffe et al., 1997). Having established that Cby serine 20 is key in its binding to 14-3-3, we investigated if phosphorylation regulates the interaction. To this end, Flag-Cby was transiently expressed in HEK293T cells and immunoprecipitated using anti-Flag affinity beads, followed by treatment with recombinant protein phosphatase 2A (rPP2A) in the presence or absence of its potent inhibitor okadaic acid (OA). The samples were then mixed with an equal amount of lysates from HEK293T cells expressing HA-14-3-3ζ, the beads were precipitated and washed, and the proteins were then separated by SDS-PAGE and immunoblotted for 14-3-3 using anti-HA antibodies. As shown in Fig. 3 A (lane 2), phosphatase treatment significantly diminished Cby-14-3-3 interactions, and the inhibition was prevented in the presence of OA (lane 3). These results indicate that 14-3-3 binding to Cby depends upon the phosphorylation state of Cby. Consistent with this finding, we did not detect interactions between Cby and

14-3-3 by in vitro pull-down assays using bacterially produced Cby (unpublished data).

### Cby is a substrate for Akt kinase

We hypothesized that Cby is phosphorylated at serine 20 by a kinase in tissue culture cells to enable specific recognition by 14-3-3. Analysis of the human Cby sequence for potential phosphorylation sites using the Scansite program (<http://scansite.mit.edu>; Yaffe et al., 2001) identified serine 20 as a putative phosphorylation site for the serine/threonine kinase Akt (also known as protein kinase B).

To determine whether Cby is a substrate for Akt kinase, we performed an in vitro phosphorylation assay. Affinity-purified Flag-CbyWT or S20A recovered from transiently transfected HEK293T cells was incubated with affinity-purified HA-tagged Akt1 or kinase-dead (KD) Akt1 (K179A) in the presence of [ $\gamma$ - $^{32}$ P]ATP with or without rPP2A and OA. These samples were then analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3 B, Cby was indeed phosphorylated by Akt1 (lanes 2 and 7) but not by KD-Akt1 (lane 8). Incubation with rPP2A resulted in



**Figure 4. 14-3-3 proteins sequester Cby in the cytoplasm.** (A) The 14-3-3-binding motif in Cby is critical for the regulation of its subcellular localization. COS7 cells were transiently transfected with an expression vector encoding Flag-tagged WT or mutant Cby. Cells were fixed 24 h after transfection and immunostained with an anti-Flag antibody. Nuclei were stained with DAPI. (B) 14-3-3 facilitates the cytoplasmic relocation of Cby but not CbyS20A. COS7 cells were cotransfected with an expression construct for Flag-tagged Cby or CbyS20A and an empty control or HA-14-3-3ζ plasmid, followed by immunostaining with an anti-Flag antibody. Bars, 10 μm. (C) Quantitative analysis of the results in B. The subcellular localization of Flag-Cby was scored as follows: N > C, predominantly nuclear; N = C, evenly distributed between the nucleus and cytoplasm; N < C, predominantly cytoplasmic. Error bars represent the means ± SD of three independent experiments.

dephosphorylation of Cby (Fig. 3 B; lanes 3 and 4), whereas OA treatment prevented Cby dephosphorylation (lanes 5 and 6). Finally, Akt1 could not phosphorylate CbyS20A (Fig. 3 B; lane 1). To confirm and extend these findings, we performed an *in vitro* kinase assay using bacterially expressed and purified proteins. MBP-Cby or MBP-CbyS20A were incubated with GST-Akt1 or GST-Akt2 in the presence of [ $\gamma$ - $^{32}$ P]ATP, and the proteins were resolved by SDS-PAGE and visualized by autoradiography. In agreement with the results shown in Fig. 3 B, both Akt1 and Akt2 phosphorylated Cby to a similar extent but were not able to phosphorylate CbyS20A (Fig. 3 C). These observations argue that Cby is a bona fide Akt substrate and that Akt kinase phosphorylates Cby at the serine 20 residue.

Next, we sought to investigate if phosphorylation of Cby by Akt regulates binding to 14-3-3. For this purpose, Flag-Cby and HA-14-3-3ζ were coexpressed in HEK293T cells with WT, KD, or constitutively active Akt1, followed by immunoprecipitation of Cby with an anti-Flag antibody and immunoblotting for 14-3-3ζ

with an anti-HA antibody (Fig. 3 D). Constitutively active Akt1 slightly, but reproducibly, augmented the interaction of Cby with 14-3-3ζ in comparison with WT-Akt1 (Fig. 3 D, compare lanes 1 and 3). More importantly, KD-Akt1 almost completely abrogated their interaction (Fig. 3 D; lane 2). Coexpression of various forms of Akt1 did not affect Cby protein levels (Fig. 3 D; middle panel). These results further support the notion that phosphorylation of Cby serine 20 by Akt facilitates the formation of Cby-14-3-3 complexes.

#### Cytoplasmic sequestration of Cby by 14-3-3

To gain insights into the functional significance of the Cby-14-3-3 interaction, we examined subcellular distribution of the Cby mutants. When overexpressed in COS7 cells, Cby localized to the cytoplasm or to both the nucleus and cytoplasm, especially in cells with high levels of Cby expression (Fig. 4, A and C). Notably, Cby mutants defective in 14-3-3 binding (S18A/S20A, S20A, and Δ1-22) preferentially localized to the nucleus (Fig. 4 A).

In contrast, the other Cby mutants that retained the ability to bind to 14-3-3 exhibited a localization pattern similar to that of WT protein. These results reveal a strong correlation between Cby–14-3-3 complex formation and the cytoplasmic localization of Cby.

One of the well-established functions of 14-3-3 proteins is to control the intracellular distribution of their ligands (Muslin and Xing, 2000; Eckardt, 2001). Our data also support the idea that 14-3-3 modulates the intracellular localization of Cby. To test this assumption directly, we investigated whether ectopic expression of 14-3-3 influences Cby localization. As shown in Fig. 4 (B and C), overexpression of 14-3-3 $\zeta$  sequestered Cby into the cytoplasm. In marked contrast, 14-3-3 $\zeta$  had essentially no major effect on the predominant nuclear localization of CbyS20A. Consistent with a prior finding (van Hemert et al., 2004), the majority of 14-3-3 $\zeta$  was seen in the cytoplasmic compartment where it colocalized with Cby (see Fig. 7 B; not depicted). Although 14-3-3 proteins are, in some cases, known to affect the stability of their substrates (Chen et al., 2003; LeBron et al., 2006), Cby protein levels remained unaltered upon coexpression with 14-3-3 $\zeta$  or DN-14-3-3 $\zeta$  (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200709091/DC1>). Collectively, we conclude that 14-3-3 binding induces relocation of Cby into the cytoplasm.

#### 14-3-3 binding influences the antagonistic activity of Cby on $\beta$ -catenin signaling

In the nucleus, Cby physically interacts with  $\beta$ -catenin and represses  $\beta$ -catenin–mediated transcriptional activation by competing with Tcf/Lef transcription factors (Takemaru et al., 2003). Our data suggest that 14-3-3 proteins bind to Cby and sequester it in the cytoplasm. This raises the interesting possibility that Cby–14-3-3 interactions regulate  $\beta$ -catenin signaling. To address this question, we performed Tcf/Lef luciferase reporter (TopFlash) assays (Korinek et al., 1997) in HEK293T cells using the three different 14-3-3 isoforms and Cby. Transfection of stabilized  $\beta$ -catenin stimulated TopFlash activity  $\sim$ 25-fold (Fig. 5 A). Previous studies have shown that 14-3-3 $\zeta$  mildly enhances  $\beta$ -catenin–dependent activation of TopFlash (Tian et al., 2004; Fang et al., 2007). Consistent with their finding, expression of 14-3-3 $\zeta$  stimulated TopFlash activation by  $\beta$ -catenin, whereas that of either the 14-3-3 $\eta$  or  $\epsilon$  isoform resulted in a small but consistent decrease in TopFlash activity. In all cases, coexpression of Cby showed repressive effects on TopFlash activity. Given the fact that all three 14-3-3 isoforms bind to Cby (Figs. 1 C and 2 D) and sequester it into the cytoplasmic compartment (Fig. 4 B and not depicted), we concluded that overexpression of 14-3-3 proteins is most likely to exert pleiotropic effects on the TopFlash reporter, potentially by interacting with multiple cellular target proteins.

To clarify whether Cby–14-3-3 interactions influence  $\beta$ -catenin signaling, we tested 14-3-3 binding–deficient Cby mutants in TopFlash assays (Fig. 5 B). As expected, cotransfection of Cby mutants capable of 14-3-3 binding (S18A and S22P) substantially repressed TopFlash activation by  $\beta$ -catenin similar to that observed for CbyWT, with no significant effect on the mutant reporter TopFlash. However, Cby mutants unable to bind 14-3-3 (S18A/S20A, 20A, and  $\Delta$ 1-22) displayed a reduced

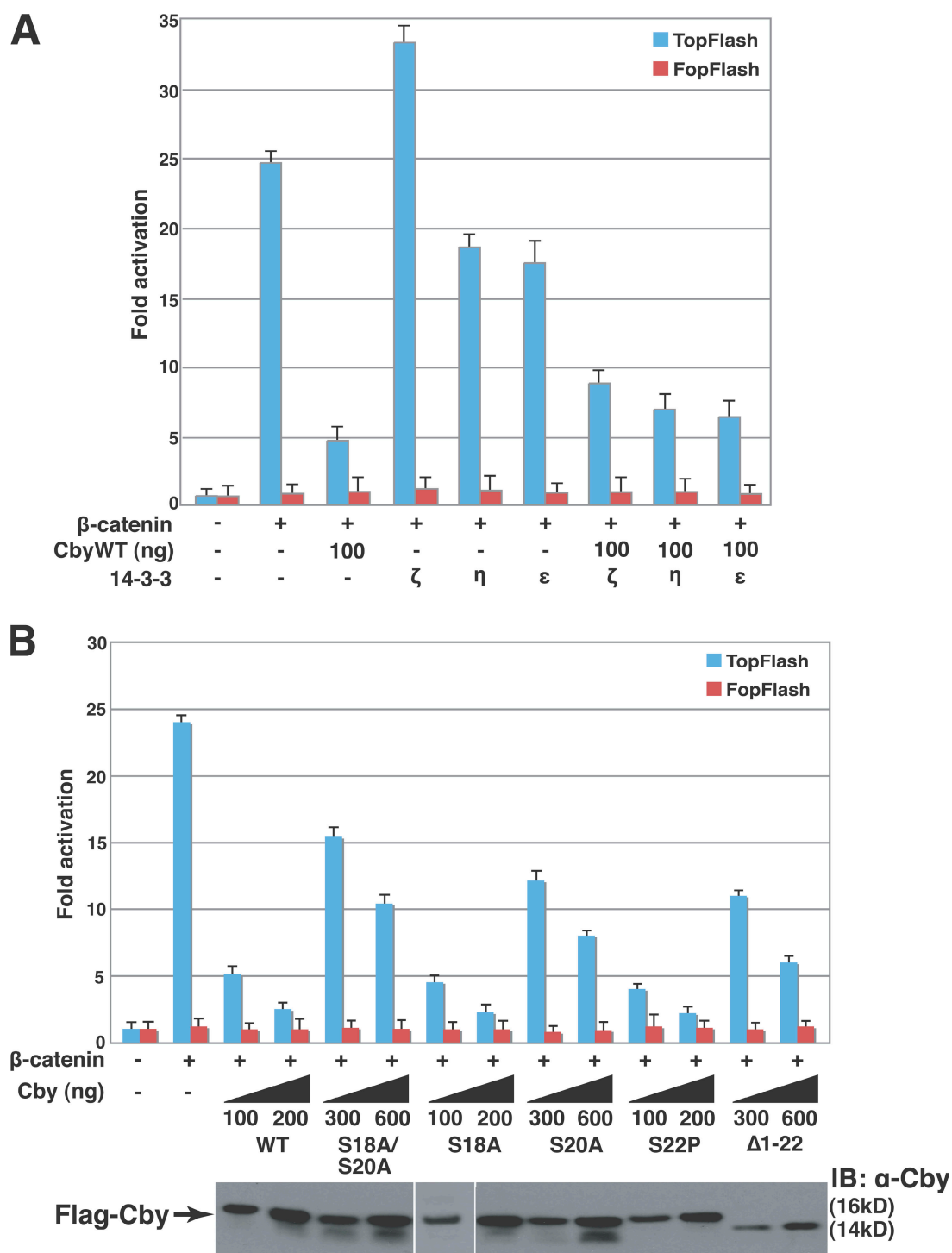
ability to repress  $\beta$ -catenin signaling. These results were unexpected, as these mutants are predominantly localized in the nucleus (Fig. 4 A) and were therefore expected to antagonize  $\beta$ -catenin signaling more efficiently.

#### $\beta$ -Catenin is present in a complex with Cby and 14-3-3

Our finding raised the possibility that inhibition of  $\beta$ -catenin activity by Cby might involve additional mechanisms besides competing with Tcf/Lef factors for  $\beta$ -catenin binding. To explore potential mechanisms, we first assessed whether  $\beta$ -catenin forms a complex with Cby and 14-3-3 by coimmunoprecipitation experiments. As shown in Fig. 6 A, both  $\beta$ -catenin and 14-3-3 coimmunoprecipitated with Cby (lane 4). In good agreement with the formation of a Cby–14-3-3– $\beta$ -catenin complex, we also found that increased expression of 14-3-3 or  $\beta$ -catenin does not interfere with Cby– $\beta$ -catenin or Cby–14-3-3 complex formation (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200709091/DC1>). Recent studies have shown that  $\beta$ -catenin associates with 14-3-3 $\zeta$  (Tian et al., 2004; Fang et al., 2007). Thus, we further tested if the Cby–14-3-3 interaction directly contributes to the formation of trimolecular complexes. HEK293T cells were cotransfected with  $\beta$ -catenin–Myc, HA–14-3-3 $\zeta$ , and Flag-tagged CbyWT or S20A for coimmunoprecipitation assays. Coexpression of the 14-3-3 binding–defective CbyS20A mutant largely decreased the amount of 14-3-3 $\zeta$  coimmunoprecipitated with  $\beta$ -catenin (Fig. 6 B, top, compare lanes 1 and 2). As anticipated, CbyS20A bound to  $\beta$ -catenin indistinguishably from CbyWT (Fig. 6 B, third panel from the top, compare lanes 1 and 2) because the  $\beta$ -catenin–binding domain resides in the C-terminal half of Cby (Takemaru et al., 2003). These results suggest that  $\beta$ -catenin, Cby, and 14-3-3 form a complex, and that the interaction between Cby and 14-3-3 is crucial for stable ternary complex formation.

#### Cby acts in concert with 14-3-3 to sequester $\beta$ -catenin into the cytoplasm

From the results presented so far, we reasoned that Cby might collaborate with 14-3-3 to relocate  $\beta$ -catenin into the cytoplasm, leading to inhibition of target gene activation. To test this, we analyzed the intracellular localization of a  $\beta$ -catenin C-terminal truncation mutant missing amino acids 665–781 that fails to bind to Cby (Takemaru et al., 2003). Upon overexpression in COS7 cells, a stabilized form of full-length  $\beta$ -catenin ( $\beta$ -cateninS33Y) was distributed throughout the cytoplasm and nucleus, as revealed by immunofluorescence staining (Fig. 7, A and C). In sharp contrast, the C-terminally truncated mutant ( $\beta$ -cateninS33Y $\Delta$ C) was detected primarily in the nucleus, which implies that Cby binding ultimately controls  $\beta$ -catenin distribution. Intriguingly, coexpression with Cby and 14-3-3 $\zeta$  resulted in a dramatic cytoplasmic shift of  $\beta$ -cateninS33Y, whereas no major changes were observed in the predominant nuclear localization of  $\beta$ -cateninS33Y $\Delta$ C (Fig. 7, B and C). However, we noted that ectopic expression of either Cby or 14-3-3 $\zeta$  alone was sufficient to relocate  $\beta$ -catenin to the cytoplasm (Fig. 7 C), presumably because of the existence of endogenous proteins, as both Cby and 14-3-3 are relatively abundant in COS7 cells.

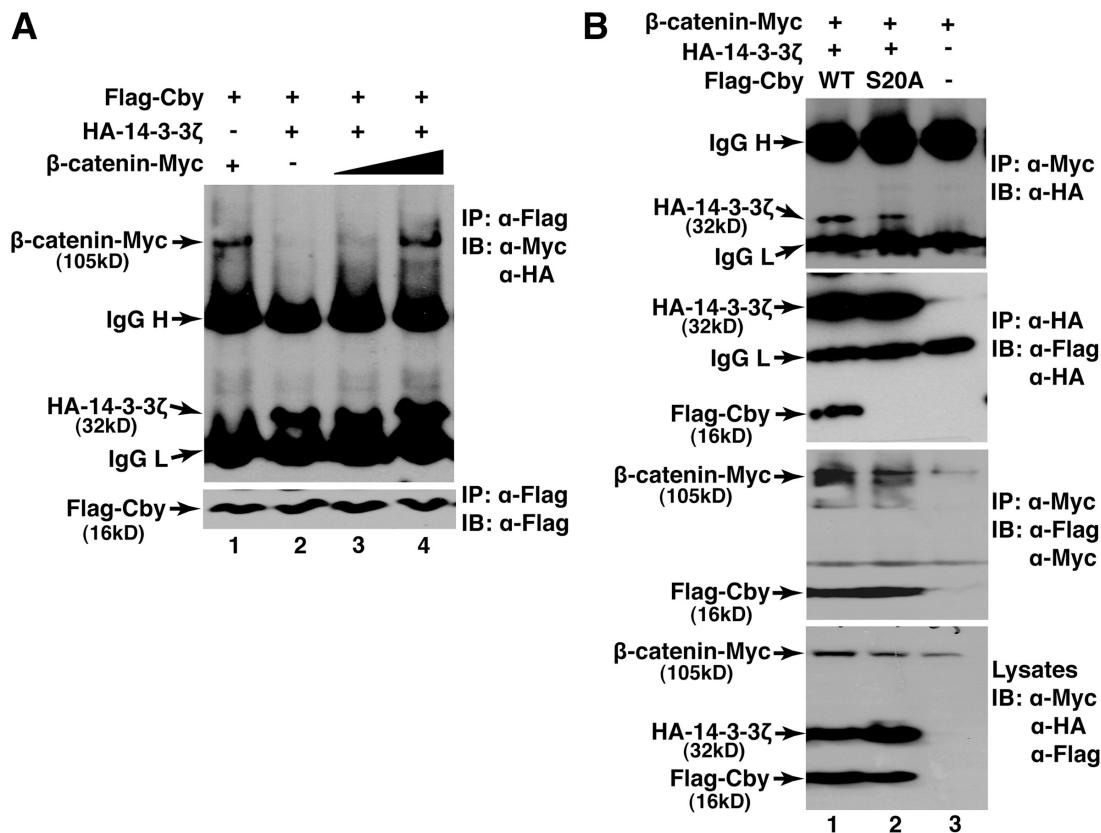


**Figure 5. Association of Cby with 14-3-3 influences  $\beta$ -catenin signaling.** (A) Effects of different 14-3-3 isoforms on  $\beta$ -catenin-mediated transcriptional activation were evaluated by TopFlash assays. (B) The ability of Cby mutants to repress  $\beta$ -catenin signaling was tested by TopFlash assays. HEK293T cells were transfected with 10 ng of TopFlash or mutant FopFlash luciferase reporter with or without 10 ng of an expression vector for stabilized  $\beta$ -catenin ( $\beta$ -catenin-Myc), 200 ng of a HA-tagged 14-3-3 plasmid, and the indicated amounts of a Flag-tagged Cby expression vector. Luciferase activity was measured 24 h after transfection and normalized to Renilla luciferase activity used as an internal control. Transfections were performed in triplicate and the means  $\pm$  SD are shown. Western blot analysis with anti-Cby antibodies showed that Cby proteins were expressed at similar levels. Note that, to compensate protein levels, higher amounts of DNA for CbyS18A/20A, CbyS20A, and Cby $\Delta$ 1-22 were used for transfection. White lines indicate that intervening lanes have been spliced out.

In support of this notion, ectopic expression of 14-3-3 binding-defective CbyS20A substantially retained  $\beta$ -catenin in the nucleus (Fig. 7 C), most likely by competing with endogenous Cby.

Furthermore, overexpression of 14-3-3 $\zeta$  created no significant effect on CbyS20A-dependent nuclear localization of  $\beta$ -catenin (Fig. 7 C). These data are, therefore, consistent with a model in





**Figure 6. Cby, 14-3-3, and β-catenin form a complex.** (A) Coimmunoprecipitation of β-catenin and 14-3-3 with Cby. Total cell lysates from HEK293T cells expressing Flag-Cby, HA-14-3-3ζ, and stabilized β-catenin-Myc in the indicated combinations were immunoprecipitated with anti-Flag antibodies and subjected to Western blotting with a mixture of anti-Flag and anti-HA antibodies. (B) Cby-14-3-3 interaction is crucial for stable tripartite complex formation. HEK293T cells were cotransfected with expression constructs for β-catenin-Myc, HA-14-3-3ζ, and Flag-tagged CbyWT or S20A as shown. Cell lysates were immunoprecipitated with either anti-Myc or anti-HA antibodies and immunoblotted with the indicated antibodies. IgG H, IgG heavy chain; IgG L, light chain.

which Cby cooperates with 14-3-3 to sequester β-catenin into the cytoplasm, thereby attenuating β-catenin signaling.

### Nuclear Akt suppresses β-catenin-mediated transcriptional activation

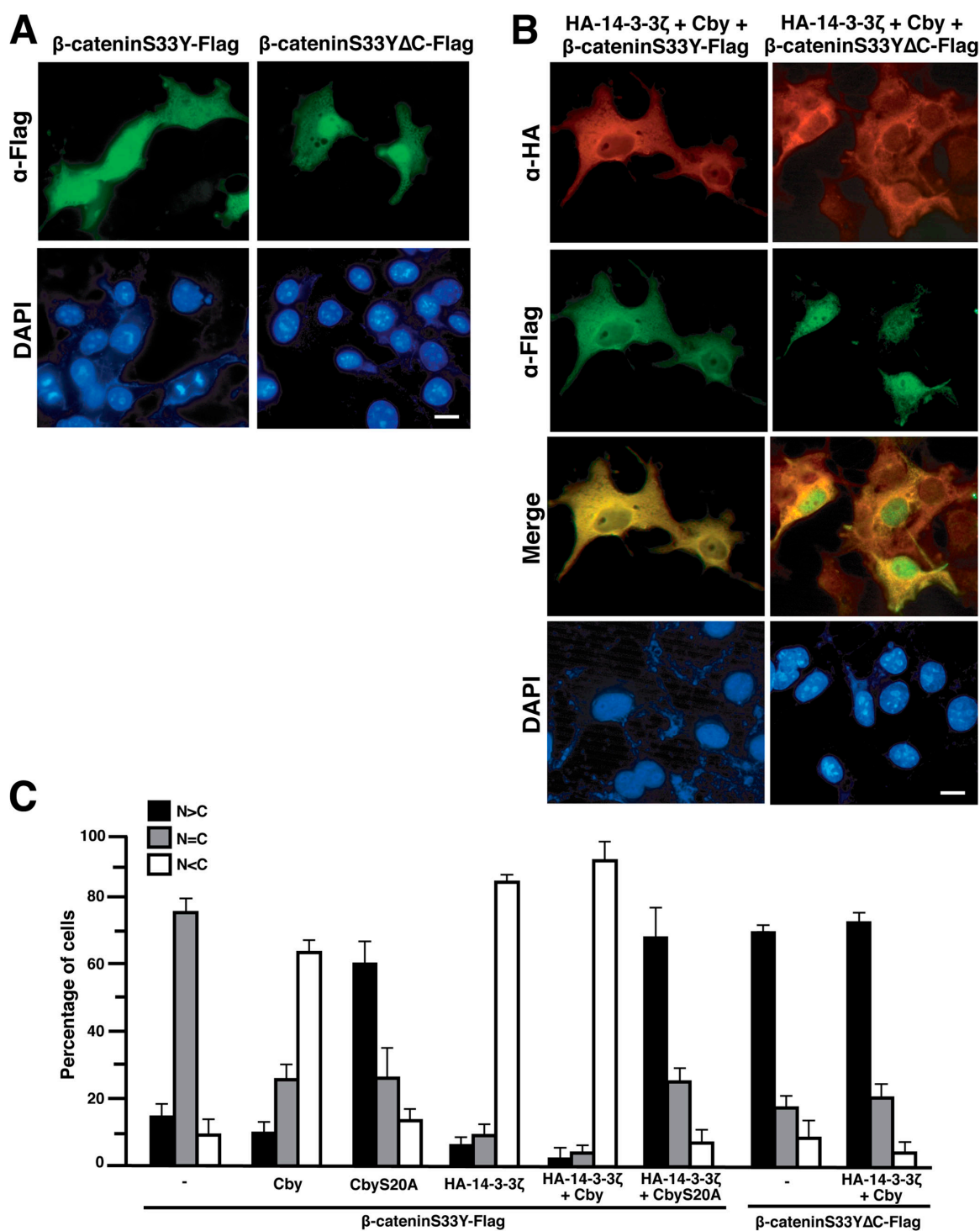
Previous studies point toward an activating role of Akt in the Wnt-β-catenin pathway (Tian et al., 2004; Fang et al., 2007). In contrast, our findings suggest that Cby phosphorylation by Akt facilitates 14-3-3 binding followed by nuclear export of β-catenin, leading to inhibition of β-catenin signaling. To resolve the seemingly contradicting data, we hypothesized that different pools of Akt could have distinct functions in controlling β-catenin activity. To test this, we assessed the effects of various forms of Akt on β-catenin-mediated transcriptional activation using TopFlash assays (Fig. 8). As expected from previous studies (Tian et al., 2004; Fang et al., 2007), a myristoylated, membrane-targeted (Myr) Akt1 moderately enhanced β-catenin-dependent activation of TopFlash. However, Akt1, Akt2, or constitutively active Akt2 (Akt2DD; T309D/S474D) decreased TopFlash activity. Notably, a nuclear-targeted form of Akt1 (NLS-Akt1), Akt2 (NLS-Akt2), or Akt2DD (NLS-Akt2DD) more potently inhibited TopFlash activation by β-catenin. Moreover, cotransfection of Cby with nuclear Akt further reduced β-catenin transcriptional activity compared

with NLS-Akt alone. These results further support our conclusion that the Cby-14-3-3-β-catenin complex assembly occurs upon phosphorylation by Akt in the nucleus and highlight the complex nature of the cross-talk between the canonical Wnt and Akt signaling pathways.

## Discussion

The Wnt-β-catenin pathway is pivotal for numerous important cellular events during embryonic development, tissue homeostasis, and tumorigenesis. The key component of this pathway, β-catenin, exerts its signaling activity only in the nucleus. Therefore, nuclear import/export of β-catenin represents a crucial step in regulating signaling competent β-catenin levels and serves as an attractive target for anti-cancer therapies, but the underlying molecular mechanisms are poorly defined.

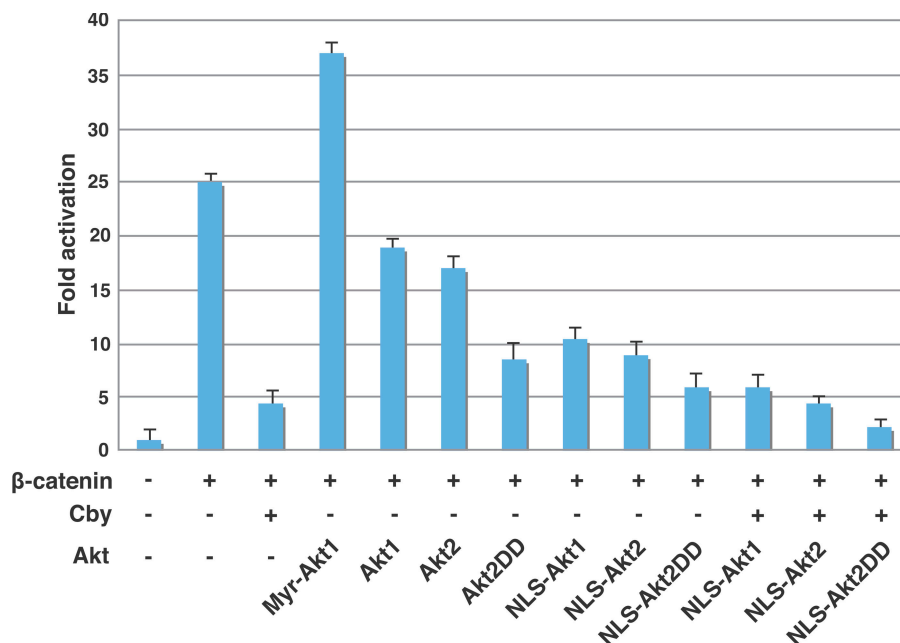
In this study, we have identified 14-3-3 proteins as novel Cby-binding partners. We demonstrated that 14-3-3 proteins specifically bind to serine 20 within the 14-3-3-binding consensus motif of Cby. Their interaction entirely depends upon phosphorylation of this critical serine residue by Akt kinase. Furthermore, we found that association with 14-3-3 triggers cytoplasmic relocation of Cby protein. Intriguingly, Cby and 14-3-3 form a tripartite complex with β-catenin and act cooperatively



**Figure 7. Cby and 14-3-3 relocate  $\beta$ -catenin to the cytoplasm.** (A) A  $\beta$ -catenin mutant incapable of binding Cby preferentially localizes to the nucleus. COS7 cells were transfected with either stabilized  $\beta$ -cateninS33Y-Flag or a C-terminal truncation mutant ( $\beta$ -cateninS33Y $\Delta$ C-Flag) defective in Cby binding and then immunostained with anti-Flag antibodies. (B)  $\beta$ -CateninS33Y-Flag or  $\beta$ -catenin $\Delta$ C-Flag were coexpressed with HA-14-3-3 $\zeta$  and untagged Cby in COS7 cells. After 24 h, cells were double stained with anti-Flag (green) and anti-HA (red) antibodies. (C) Quantification of  $\beta$ -catenin localization. The subcellular distribution of Flag-tagged  $\beta$ -catenin was scored as in Fig. 4 C. Error bars represent the means  $\pm$  SD of three independent experiments.  $N > C$ , predominantly nuclear;  $N = C$ , evenly distributed between the nucleus and cytoplasm;  $N < C$ , predominantly cytoplasmic. Bars, 10  $\mu$ m.

to sequester  $\beta$ -catenin into the cytoplasmic compartment. In support of our model, Cby collaborates with nuclear Akt to repress  $\beta$ -catenin-dependent transcriptional activation. Our results, therefore, suggest that inhibition of  $\beta$ -catenin signaling

by Cby involves at least two distinct molecular mechanisms (Fig. 9); i.e., competing with Tcf/Lef transcription factors for binding to  $\beta$ -catenin in the nucleus (Takemaru et al., 2003) and facilitating nuclear export of  $\beta$ -catenin via interaction with

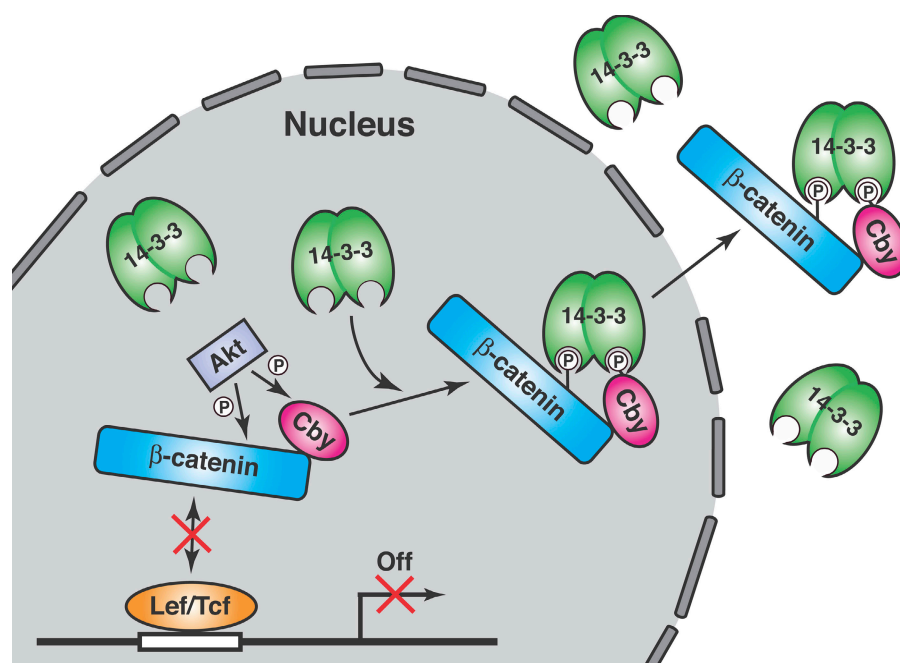


**Figure 8. Nuclear Akt enhances inhibition of  $\beta$ -catenin signaling by Cby.** HEK293T cells were transiently transfected with 10 ng of TopFlash luciferase reporter together with the indicated combinations of expression constructs for stabilized  $\beta$ -catenin ( $\beta$ -catenin-Myc; 10 ng), Cby (100 ng), and different forms of Akt (200 ng). Luciferase activity was measured as described in the legend for Fig. 5. Myr-Akt1 predominantly localized to the plasma membrane, whereas NLS-Akt was exclusively nuclear. The other Akt forms were primarily distributed throughout the cytoplasm (not depicted). Transfections were carried out in triplicate and the means  $\pm$  SD are shown.

14-3-3 upon its phosphorylation by Akt. Consistent with this, Cby mutants defective in 14-3-3 binding (S18A/S20A, 20A, and  $\Delta$ 1-22) unexpectedly exhibit significantly reduced ability to repress TopFlash activation by  $\beta$ -catenin (Fig. 5 B) even though they accumulate in the nucleus (Fig. 4 A). Hence, the nuclear export and repressor activities of Cby may equally contribute to the rapid down-regulation of nuclear  $\beta$ -catenin activity. However, it is also plausible that one mechanism predominates over the other, depending on the cellular context.

14-3-3 $\zeta$  has been found to associate with  $\beta$ -catenin (Tian et al., 2004). Later, it was found that Akt phosphorylates  $\beta$ -catenin at serine 552, which appears to enhance its interaction with 14-3-3 $\zeta$  (Fang et al., 2007). In both cases, ectopic ex-

pression of 14-3-3 $\zeta$  resulted in a moderate activation (two- to fourfold) of  $\beta$ -catenin-dependent transcription in TopFlash assays. We found that 14-3-3 $\zeta$  enhances, whereas 14-3-3 $\eta$  and  $\epsilon$  isoforms repress,  $\beta$ -catenin activation of the TopFlash reporter (Fig. 5 A). One possible explanation for this observation is that 14-3-3 overexpression exerts complex biological effects, which makes our interpretation of the TopFlash results difficult. In fact, 14-3-3 proteins have been shown to interact with a plethora of target proteins ranging from transcription factors to various signaling molecules (Dougherty and Morrison, 2004; Pozuelo Rubio et al., 2004). However, it is interesting to note that, consistent with our results (Fig. 7 C), ectopic expression of 14-3-3 $\zeta$  was found to cause the cytoplasmic enrichment of  $\beta$ -catenin



**Figure 9. Dual mechanism model for inhibition of  $\beta$ -catenin signaling activity by Cby.** In the nucleus, Cby binds to  $\beta$ -catenin and competes with Tcf/Lef transcription factors, leading to repression of target gene expression. In addition, phosphorylation of Cby and  $\beta$ -catenin by Akt facilitates 14-3-3 binding, resulting in nuclear export of  $\beta$ -catenin to the cytoplasm. These two distinct mechanisms might be required to achieve full repression of  $\beta$ -catenin transcriptional activity. For details, see Discussion.

(Tian et al., 2004), potentially by interacting with endogenous Cby. Nonetheless, our findings indicate that the Cby–14-3-3 interaction significantly contributes to their stable complex formation with  $\beta$ -catenin (Fig. 6 B). More importantly, 14-3-3 itself is not sufficient to sequester  $\beta$ -catenin into the cytoplasm, as 14-3-3 binding-defective CbyS20A retains  $\beta$ -catenin in the nucleus even in the presence of excess 14-3-3 (Fig. 7 C). Therefore, we speculate that phosphorylation of  $\beta$ -catenin and Cby by Akt provokes 14-3-3 binding to form a stable ternary complex followed by  $\beta$ -catenin nuclear export and termination of its signaling (Fig. 9). Similarly, it has been shown that Akt phosphorylates FoxO Forkhead transcription factors, and this promotes their interaction with 14-3-3, leading to their nuclear exit and cytoplasmic retention (Brunet et al., 1999; Van Der Heide et al., 2004). It is also noteworthy that, in *Caenorhabditis elegans*, 14-3-3/PAR-5 binds to and mediates nuclear export of TCF/POP-1 (Lo et al., 2004).

The key negative regulators of the Wnt– $\beta$ -catenin pathway, APC and Axin, have been shown to affect the nuclear cytoplasmic shuttling of  $\beta$ -catenin (Henderson, 2000; Rosin-Arbesfeld et al., 2000; Cong and Varmus, 2004; Wiechens et al., 2004). Both proteins carry functional NLS and NES sequences and export  $\beta$ -catenin from the nucleus to the cytoplasm in a CRM-1-dependent manner, most likely for degradation. The Cby–14-3-3 pathway represents yet another mechanism that controls  $\beta$ -catenin subcellular distribution. In addition to two putative NLS sequences (Takemaru et al., 2003), Cby harbors a potential NES, and its localization is sensitive to leptomycin B (unpublished data), which is indicative of a CRM-1-dependent process. At present, the relationship between APC–, Axin–, and Cby–14-3-3-dependent  $\beta$ -catenin nuclear export pathways is unclear. However, Cby does not form a complex with APC or Axin (unpublished data), which implies that the Cby–14-3-3 route operates independently of that of APC and Axin.

Akt is a major effector of the phosphatidylinositol-3-kinase signaling, which is activated by a diverse array of extracellular stimuli, and is known to promote cell growth, survival, and tumor formation (Hennessey et al., 2005). Several lines of evidence indicate that Akt plays a positive role in Wnt– $\beta$ -catenin signaling, most likely through phosphorylation and inactivation of GSK3, although its precise mechanism of action remains largely debatable (Brazil et al., 2002). Here, we provide evidence that nuclear-targeted Akt inhibits, whereas membrane-tethered Akt stimulates,  $\beta$ -catenin-mediated transcriptional activation (Fig. 8). It is possible that membrane/cytoplasmic activated Akt favorably phosphorylates and thus inactivates GSK3, whereas nuclear Akt phosphorylates  $\beta$ -catenin and Cby, which in turn facilitates 14-3-3 binding and subsequent nuclear exclusion of the ternary complex. The opposing roles of Akt in the Wnt– $\beta$ -catenin pathway may be temporally and spatially controlled in an in vivo scenario. Once activated at the plasma membrane in response to diverse stimuli, Akt translocates into the nucleus, where it phosphorylates and modulates the activity of nuclear factors including Forkhead transcription factors and p300/CBP coactivators (Andjelkovic et al., 1997; Brunet et al., 1999; Borgatti et al., 2003; Huang and Chen, 2005). However, little is known about the physiological functions of nuclear Akt.

Our results reveal that the subcellular compartmentalization of Akt differentially influences  $\beta$ -catenin signaling. This is reminiscent of GSK3, as it both positively and negatively affects Wnt– $\beta$ -catenin signaling depending on its intracellular location (Zeng et al., 2005). It is also conceivable that phosphorylation of Cby at serine 20 is catalyzed by additional kinases because 14-3-3-binding motifs have been shown to be substrates for various protein kinases (Dougherty and Morrison, 2004; Aitken, 2006). Clearly, further work will be required to define the cross-talk between the Wnt– $\beta$ -catenin and Akt signaling pathways.

In conclusion, our study reveals a new molecular mechanism for the regulation of  $\beta$ -catenin subcellular distribution by the combinatorial action of Cby and 14-3-3 proteins. As deregulation of 14-3-3 has been implicated in the development of several types of human cancer (Hermeking, 2003; Wilker and Yaffe, 2004), it would be of great interest to determine if  $\beta$ -catenin signaling is aberrantly activated in these tumor cells.

## Materials and methods

### Plasmids and protein expression

Expression vectors for human Cby (Takemaru et al., 2003),  $\beta$ -catenin–Myc (Takemaru et al., 2003), and  $\beta$ -cateninS33Y-Flag (Li et al., 2007) have been described previously. Akt expression constructs were provided by J.E. Pessin (State University of New York at Stony Brook, Stony Brook, NY). Human 14-3-3 $\epsilon$ ,  $\zeta$ , and  $\eta$  cDNAs were PCR amplified from HEK293T cDNA using a forward primer containing the HA sequence at the 5' end, digested with EcoRI and XbaI, and subcloned into the pCS2+ vector. Similarly, PCR fragments for Flag-Cby $\Delta$ 1-22 and  $\beta$ -cateninS33Y $\Delta$ C (amino acids 1–664)-Flag were inserted into the pCS2+ and pQCXIP (Clontech Laboratories, Inc.), respectively. Cby single or double point mutants and DN-14-3-3 $\zeta$  (R56A/R60A) were created using the QuickChange site-directed mutagenesis kit (Stratagene). For hRluc protein fragment-assisted complementation assays (Paulmurugan and Gambhir, 2003), 14-3-3 or Cby cDNAs were amplified by PCR using plasmid templates and ligated in-frame with the N-terminal portion (amino acids 1–239) or the C-terminal portion (amino acids 240–321) of hRluc into the pJCH510 or pJCH511 vector (gifts from J.-C. Hsieh, State University of New York at Stony Brook). We verified all constructs by DNA sequencing. MBP and MBP-Cby were expressed in *E. coli* BL21 cells and purified by amylose resin (New England Biolabs, Inc.) as described previously (Takemaru et al., 2003).

### Cell culture and transfection

HEK293T and COS7 cells were purchased from American Type Culture Collection, and maintained in DME with 10% FBS and 100 U/ml penicillin-streptomycin. For transient transfection, cells were seeded onto 6- or 12-well tissue culture dishes, cultured overnight, and then transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Empty vector was added to adjust the total amount of DNA to be the same in every transfection.

### Purification of Cby-associated proteins

HEK293T cell lysates were prepared in lysis buffer containing 20 mM Tris-HCl, pH 8.0, 135 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1% Triton X-100, 10% glycerol, and complete protease inhibitor cocktail (Roche); cleared by centrifugation at 12,000 rpm for 30 min; and incubated with MBP or MBP-Cby for 1 h at 4°C. Subsequently, amylose beads (New England Biolabs, Inc.) were added and continuously rotated for 2 h at 4°C. The beads were then collected, extensively washed, and boiled in SDS sample buffer, followed by the separation of bound proteins on a 4–20% SDS-PAGE. Each band pulled down specifically with MBP-Cby was cut out from the gel stained with SimplyBlue SafeStain (Invitrogen), digested with trypsin, and analyzed by mass spectrometry (QSTAR and matrix-assisted laser desorption ionization–time of flight) in the Proteomics Center at State University of New York at Stony Brook.

### Coimmunoprecipitation and Western blotting

Transfected or nontransfected HEK293T cells were lysed, and coimmunoprecipitation and immunoblotting were performed essentially as described



previously (Takamaru and Moon, 2000; Li et al., 2007). For phosphatase treatment, Flag-Cby was expressed in HEK293T cells, immunoprecipitated using anti-Flag M2 affinity beads (Sigma-Aldrich), and incubated in phosphatase reaction buffer (50 mM imidazole, pH 7.2, 0.2 mM EGTA, 0.02%  $\beta$ -mercaptoethanol, and 0.1 mg/ml BSA) for 45 min at 30°C with or without rPP2A (Millipore) and its inhibitor OA (Sigma-Aldrich) as indicated. Subsequently, these samples were mixed with an equal amount of cell lysates from HEK293T cells expressing 14-3-3 $\zeta$  followed by coimmunoprecipitation and Western blot analysis. The primary antibodies used were as follows: rabbit anti-Cby (Takamaru et al., 2003); mouse anti-Flag M2 (Sigma-Aldrich); rat anti-HA (Roche); mouse anti-Myc (Invitrogen); and rabbit anti-pan-14-3-3, anti-14-3-3 $\epsilon$ , and rabbit IgG (Santa Cruz Biotechnology, Inc.).

#### TopFlash and hLuc protein fragment-assisted complementation assays

HEK293T cells were seeded onto 12-well plates and transfected with the appropriate combinations of plasmids in triplicate. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and a luminometer (Berthold Technologies) as described previously (Takamaru et al., 2003; Li et al., 2007). An expression plasmid (10 ng) for Renilla luciferase (pRL-TK) or firefly luciferase (pCMV-Luc) was cotransfected to normalize transfection frequency.

#### In vitro kinase assays

Flag-CbyWT or S20A and HA-Akt1 or KD-Akt1 were individually expressed in HEK293T cells, and purified using anti-Flag M2 affinity beads (Sigma-Aldrich) and anti-HA affinity beads (Roche), respectively. The immunoprecipitated Flag-CbyWT or S20A was incubated with purified HA-Akt1 or KD-Akt1 in 50  $\mu$ l of kinase assay buffer containing 20 mM Pipes, pH 7.0, 15 mM MnCl<sub>2</sub>, 7 mM  $\beta$ -mercaptoethanol, 0.25 mM  $\beta$ -glycerophosphate, and 0.4 mM spermine on ice for 5 min in the absence or presence of rPP2A and OA. After incubation, 10  $\mu$ l of the kinase assay buffer containing 10  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP was added to each reaction and further incubated for 30 min at 37°C. The kinase reaction was terminated by the addition of SDS sample buffer and boiling, and then resolved by SDS-PAGE and visualized by autoradiography. For in vitro kinase assays using bacterially expressed and purified proteins, 10  $\mu$ g of MBP-Cby or MBP-CbyS20A was mixed with GST-Akt1 or GST-Akt2 (Cell Signaling Technology) in 25  $\mu$ l of the kinase assay buffer containing 10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP, incubated for 30 min at 37°C, followed by SDS-PAGE and autoradiography.

#### Immunofluorescence microscopy

Transfected COS7 cells were grown on glass coverslips, fixed with methanol/acetone (1:1, vol/vol), permeabilized with 0.2% Triton X-100, and blocked with 1% BSA in PBS. Flag-tagged Cby and  $\beta$ -catenin were detected using mouse anti-Flag M2 antibodies (Sigma-Aldrich) followed by TRITC-labeled goat anti-mouse IgG and FITC-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), respectively. HA-14-3-3 $\zeta$  was immunostained using a rat anti-HA antibody (Roche) followed by TRITC-labeled goat anti-rat IgG (Jackson ImmunoResearch Laboratories). Nuclei were counterstained with DAPI (Sigma-Aldrich), and stained cells were analyzed by a fluorescent microscope (DM5000; Leica) with an HCX PL Fluotar 100 $\times$  1.30 NA oil objective lens (Leica). All images were acquired at room temperature with a camera (DFC300 FX; Leica) using Application Suite software version 2.8.1 (Leica). To quantify subcellular localization, independent transfections were performed at least three times and a minimum of 100 cells were counted for each transfection. In cotransfections, only cells expressing both proteins were scored.

#### Online supplemental material

Fig. S1 shows that overexpression of 14-3-3 $\zeta$  or DN-14-3-3 $\zeta$  does not affect Cby protein levels. Fig. S2 demonstrates that increased expression of 14-3-3 $\zeta$  or  $\beta$ -catenin does not interfere with Cby- $\beta$ -catenin or Cby-14-3-3 complex formation. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200709091/DC1>.

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## References

- Aitken, A. 2006. 14-3-3 proteins: a historic overview. *Semin. Cancer Biol.* 16:162–172.
- Andjelkovic, M., D.R. Alessi, R. Meier, A. Fernandez, N.J.C. Lamb, M. Frech, P. Cron, P. Cohen, J.M. Lucocq, and B.A. Hemmings. 1997. *J. Biol. Chem.* 272:31515–31524.
- Borgatti, P., A.M. Martelli, G. Tabellini, A. Bellacosa, S. Capitani, and L.M. Neri. 2003. Threonine 308 phosphorylated form of Akt translocates to the nucleus of PC12 cells under nerve growth factor stimulation and associate with the nuclear matrix protein nucleolin. *J. Cell. Physiol.* 196:79–88.
- Brazil, D.P., J. Park, and B.A. Hemmings. 2002. PKB binding proteins. Getting in on the Akt. *Cell.* 111:293–303.
- Brunet, A., A. Bonni, M.J. Zigmond, M.Z. Lin, P. Juo, L.S. Hu, M.J. Anderson, K.C. Arden, J. Blenis, and M.E. Greenberg. 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell.* 96:857–868.
- Cadigan, K.M., and Y.I. Liu. 2006. Wnt signaling: complexity at the surface. *J. Cell Sci.* 119:395–402.
- Cadigan, K.M., and R. Nusse. 1997. Wnt signaling: a common theme in animal development. *Genes Dev.* 11:3286–3305.
- Chen, H.K., P. Fernandez-Funez, S.F. Acevedo, Y.C. Lam, M.D. Kaytor, M.H. Fernandez, A. Aitken, E.M. Skoulakis, H.T. Orr, J. Botas, and H.Y. Zoghbi. 2003. Interaction of Akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. *Cell.* 113:457–468.
- Clevers, H. 2006. Wnt/beta-catenin signaling in development and disease. *Cell.* 127:469–480.
- Cong, F., and H. Varmus. 2004. Nuclear-cytoplasmic shuttling of Axin regulates subcellular localization of beta-catenin. *Proc. Natl. Acad. Sci. USA.* 101:2882–2887.
- Dougherty, M.K., and D.K. Morrison. 2004. Unlocking the code of 14-3-3. *J. Cell Sci.* 117:1875–1884.
- Eckardt, N.A. 2001. Transcription factors dial 14-3-3 for nuclear shuttle. *Plant Cell.* 13:2385–2389.
- Eleftheriou, A., M. Yoshida, and B.R. Henderson. 2001. Nuclear export of human beta-catenin can occur independent of CRM1 and the adenomatous polyposis coli tumor suppressor. *J. Biol. Chem.* 276:25883–25888.
- Fagotto, F., U. Gluck, and B.M. Gumbiner. 1998. Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin. *Curr. Biol.* 8:181–190.
- Fang, D., D. Hawke, Y. Zheng, Y. Xia, J. Meisenhelder, H. Nika, G.B. Mills, R. Kobayashi, T. Hunter, and Z. Lu. 2007. Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. *J. Biol. Chem.* 282:11221–11229.
- Fu, H., R.R. Subramanian, and S.C. Masters. 2000. 14-3-3 proteins: structure, function, and regulation. *Annu. Rev. Pharmacol. Toxicol.* 40:617–647.
- Gates, J., and M. Peifer. 2005. Can 1000 reviews be wrong? Actin, alpha-catenin, and adherens junctions. *Cell.* 123:769–772.
- Henderson, B.R. 2000. Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nat. Cell Biol.* 2:653–660.
- Hennessy, B.T., D.L. Smith, P.T. Ram, Y. Lu, and G.B. Mills. 2005. Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat. Rev. Drug Discov.* 4:988–1004.
- Hermeking, H. 2003. The 14-3-3 cancer connection. *Nat. Rev. Cancer.* 3:931–943.
- Huang, W.C., and C.C. Chen. 2005. Akt phosphorylation of p300 at Ser-1834 is essential for its histone acetyltransferase and transcriptional activity. *Mol. Cell. Biol.* 25:6592–6602.
- Kimelman, D., and W. Xu. 2006. beta-catenin destruction complex: insights and questions from a structural perspective. *Oncogene.* 25:7482–7491.
- Korinek, V., N. Barker, P.J. Morin, D. van Wichen, R. de Weger, K.W. Kinzler, B. Vogelstein, and H. Clevers. 1997. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC<sup>−/−</sup> colon carcinoma. *Science.* 275:1784–1787.
- Krieghoff, E., J. Behrens, and B. Mayr. 2006. Nucleo-cytoplasmic distribution of beta-catenin is regulated by retention. *J. Cell Sci.* 119:1453–1463.
- LeBron, C., L. Chen, D.M. Gilkes, and J. Chen. 2006. Regulation of MDMX nuclear import and degradation by Chk2 and 14-3-3. *EMBO J.* 25:1196–1206.
- Li, F.Q., A.M. Singh, A. Mofunanya, D. Love, N. Terada, R.T. Moon, and K.I. Takamaru. 2007. Chibby promotes adipocyte differentiation through inhibition of beta-catenin signaling. *Mol. Cell. Biol.* 27:4347–4354.
- Liu, C., Y. Li, M. Semenov, C. Han, G.H. Baeg, Y. Tan, Z. Zhang, X. Lin, and X. He. 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell.* 108:837–847.

- Lo, M.C., F. Gay, R. Odom, Y. Shi, and R. Lin. 2004. Phosphorylation by the beta-catenin/MAPK complex promotes 14-3-3-mediated nuclear export of TCF/POP-1 in signal-responsive cells in *C. elegans*. *Cell*. 117:95–106.
- Lustig, B., and J. Behrens. 2003. The Wnt signaling pathway and its role in tumor development. *J. Cancer Res. Clin. Oncol.* 129:199–221.
- Moon, R.T., A.D. Kohn, G.V. De Ferrari, and A. Kaykas. 2004. WNT and beta-catenin signalling: diseases and therapies. *Nat. Rev. Genet.* 5:691–701.
- Muslin, A.J., and H. Xing. 2000. 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell. Signal.* 12:703–709.
- Muslin, A.J., J.W. Tanner, P.M. Allen, and A.S. Shaw. 1996. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*. 84:889–897.
- Nelson, W.J., and R. Nusse. 2004. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science*. 303:1483–1487.
- Ory, S., M. Zhou, T.P. Conrads, T.D. Veenstra, and D.K. Morrison. 2003. Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. *Curr. Biol.* 13:1356–1364.
- Paulmurugan, R., and S.S. Gambhir. 2003. Monitoring protein-protein interactions using split synthetic renilla luciferase protein-fragment-assisted complementation. *Anal. Chem.* 75:1584–1589.
- Pinto, D., and H. Clevers. 2005. Wnt, stem cells and cancer in the intestine. *Biol. Cell*. 97:185–196.
- Polakis, P. 2000. Wnt signaling and cancer. *Genes Dev.* 14:1837–1851.
- Pozuelo Rubio, M., K.M. Geraghty, B.H. Wong, N.T. Wood, D.G. Campbell, N. Morrice, and C. Mackintosh. 2004. 14-3-3-affinity purification of over 200 human phosphoproteins reveals new links to regulation of cellular metabolism, proliferation and trafficking. *Biochem. J.* 379:395–408.
- Rosin-Arbesfeld, R., F. Townsley, and M. Bienz. 2000. The APC tumour suppressor has a nuclear export function. *Nature*. 406:1009–1012.
- Singh, A.M., F.Q. Li, T. Hamazaki, H. Kasahara, K. Takemaru, and N. Terada. 2007. Chibby, an antagonist of the Wnt/beta-catenin pathway, facilitates cardiomyocyte differentiation of murine embryonic stem cells. *Circulation*. 115:617–626.
- Stadeli, R., R. Hoffmans, and K. Basler. 2006. Transcription under the control of nuclear Arm/beta-catenin. *Curr. Biol.* 16:R378–R385.
- Takemaru, K., S. Yamaguchi, Y.S. Lee, Y. Zhang, R.W. Carthew, and R.T. Moon. 2003. Chibby, a nuclear beta-catenin-associated antagonist of the Wnt/Wingless pathway. *Nature*. 422:905–909.
- Takemaru, K.-I. 2006. Catenin, beta. *UCSD-Nature Molecule Pages*. (doi:10.1038/mp.a000506.01; accessed September 3, 2007).
- Takemaru, K.I., and R.T. Moon. 2000. The transcriptional coactivator CBP interacts with  $\beta$ -catenin to activate gene expression. *J. Cell Biol.* 149:249–254.
- Tian, Q., M.C. Feetham, W.A. Tao, X.C. He, L. Li, R. Aebersold, and L. Hood. 2004. Proteomic analysis identifies that 14-3-3zeta interacts with beta-catenin and facilitates its activation by Akt. *Proc. Natl. Acad. Sci. USA*. 101:15370–15375.
- Tolwinski, N.S., and E. Wieschaus. 2001. Armadillo nuclear import is regulated by cytoplasmic anchor Axin and nuclear anchor dTCF/Pan. *Development*. 128:2107–2117.
- Tolwinski, N.S., and E. Wieschaus. 2004. A nuclear function for armadillo/beta-catenin. *PLoS Biol.* 2:E95.
- Townsley, F.M., A. Cliffe, and M. Bienz. 2004. Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function. *Nat. Cell Biol.* 6:626–633.
- Van Der Heide, L.P., M.F. Hoekman, and M.P. Smidt. 2004. The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. *Biochem. J.* 380:297–309.
- van Hemert, M.J., M. Niemantsverdriet, T. Schmidt, C. Backendorf, and H.P. Spaink. 2004. Isoform-specific differences in rapid nucleocytoplasmic shuttling cause distinct subcellular distributions of 14-3-3 sigma and 14-3-3 zeta. *J. Cell Sci.* 117:1411–1420.
- Wiechens, N., and F. Fagotto. 2001. CRM1- and Ran-independent nuclear export of beta-catenin. *Curr. Biol.* 11:18–27.
- Wiechens, N., K. Heinle, L. Englmeier, A. Schohl, and F. Fagotto. 2004. Nucleocytoplasmic shuttling of Axin, a negative regulator of the Wnt-beta-catenin pathway. *J. Biol. Chem.* 279:5263–5267.
- Wilker, E., and M.B. Yaffe. 2004. 14-3-3 proteins—a focus on cancer and human disease. *J. Mol. Cell. Cardiol.* 37:633–642.
- Willert, K., and K.A. Jones. 2006. Wnt signaling: is the party in the nucleus? *Genes Dev.* 20:1394–1404.
- Xing, H., S. Zhang, C. Weinheimer, A. Kovacs, and A.J. Muslin. 2000. 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades. *EMBO J.* 19:349–358.
- Yaffe, M.B., K. Rittinger, S. Volinia, P.R. Caron, A. Aitken, H. Leffers, S.J. Gamblin, S.J. Smerdon, and L.C. Cantley. 1997. The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell*. 91:961–971.
- Yaffe, M.B., G.G. Leparc, J. Lai, T. Obata, S. Volinia, and L.C. Cantley. 2001. A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nat. Biotechnol.* 19:348–353.
- Yokoya, F., N. Imamoto, T. Tachibana, and Y. Yoneda. 1999. beta-catenin can be transported into the nucleus in a Ran-unassisted manner. *Mol. Biol. Cell*. 10:1119–1131.
- Zeng, X., K. Tamai, B. Doble, S. Li, H. Huang, R. Habas, H. Okamura, J. Woodgett, and X. He. 2005. A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature*. 438:873–877.