Stony Brook University

Chibby forms a homodimer through a heptad repeat of leucine residues in its C-terminal coiled-coil motif

A Dissertation Presented

by

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to

The Graduate School

in partial fulfillment of the requirements

for the Degree of

Doctor of Philosophy

in

Genetics

Stony Brook University

December 2009

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The Graduate School
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Abstract of the Dissertation

Chibby forms a homodimer through a heptad repeat of leucine residues in its C-terminal coiled-coil motif

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The Wnt/β-catenin signaling pathway plays crucial roles in embryonic development, in maintenance of organs and tissues in adults and in many other processes. Chibby (Cby) is an evolutionarily conserved molecule that physically interacts with the key downstream coactivator β-catenin and represses its transcriptional activation potential. Although Cby harbors a predicted coiled-coil motif in the C-terminal region, its molecular nature and functional importance remain largely unexplored.

In this thesis/dissertation, I report that Cby forms a stable complex with itself. Alanine substitutions of two or more of four critical leucine residues within the C-terminal heptad repeat completely eliminate the Cby-Cby interaction. The Cby oligomer predominantly exists as a homodimer. Cby dimerization-deficient

mutants still retain the ability to bind to β-catenin and to repress β-catenin-dependent gene activation. More importantly, Cby homodimerization is required for its efficient interaction with the nuclear import receptor importin-α and subsequent nuclear translocation.

This comprehensive mutational analysis of the Cby coiled-coil domain reveals that the four heptad leucine residues play an essential role in mediating Cby homodimerization. Although monomeric Cby is sufficient to bind to β-catenin and block β-catenin-mediated transcriptional activation, homodimer formation of Cby is indispensable for its efficient nuclear import.

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List of Abbreviations

APC	Adenomatous polyposis coli
BRG1	Brahma-related gene 1
Cby	Chibby
CK1	Casein kinase 1
CtBP	C-terminal binding protein
DKK	Dickkopf
DMEM	Dulbecco's modified Minimal Essential Medium
DMS	Dimethyl suberimidate
Dvl	Dishevelled
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FAP	Familial adenomatous polyposis
FGF	Fibroblast growth factor
Fz	Frizzled
GST	Glutathione-S-transferase
GSK3	Glycogen synthase kinase 3
HDAC	histone deacetylase
hRluc	Renilla luciferase (synthetic)
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
Lef	Lymphoid enhancer factor
LMB	Leptomycin B

MBP	Maltose binding protein
LRP	Low-density lipoprotein receptor-related protein
NES	Nuclear export signal
NLS	Nuclear localization signal
NPC	Nuclear pore complex
OPPG	Osteoporosis pseudoglioma syndrome
PC1	Polycystin 1
PC2	Polycystin 2
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
RNAi	RNA interference
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SFRP	Soluble frizzled-related protein
TC1	Thyroid cancer 1
Tcf	T-cell factor
WIF	Wnt-inhibitor protein
Wls	Wntless
WTX	Wilms Tumor gene on X chromosome
β-Trcp	β-transducin repeat-containing protein

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Acknowledgements

My heartfelt appreciation to my advisor, Dr. Ken-Ichi Takemaru for his guidance and direction in making this dissertation possible; for his support, patience, sincere criticisms and for pushing me to bring out the best in me. He made my time in Stony Brook a very pleasant one. Also to my Supervisor, Dr. Feng-Qian Li, who helped me with ideas, discussion and analyzed my results to make sense out of them. Thanks to her, I have grown as a scientist from the first day I joined the lab. She taught me so much!

I would like to thank my thesis committee members, Dr. Dan Bogenhagen, Dr. Gerald Thomsen, Dr. Peter Gergen and Dr. Hsien-Yu Wang for their time, effort and support, in particular to Dr. Dan Bogenhagen for the use of his gel filtration column and reagents and for taking his time to teach me how to use it. Thanks to Dr. Matthew Bobiak, Dr. Miguel Gracia-Diaz and Dr. Elena Yakubovskaya for advice.

Thanks to all who provided a supportive environment in Stony Brook. Thanks to Damon Love, for all his help in my technical issues, to Vici Fisher for all the laughs, and to all present and former members of the Takemaru Lab. Thanks to friends outside the lab: especially Banke Fagbemi and Tracy Callendar. Thanks to Genetics Program Coordinator, Kate Bell for all her help, for the candies and small talks!

Special thanks to my parents for believing in me and supporting me in my decision to leave Nigeria to pursue my dreams in America. I am eternally grateful

for all your sacrifices and support! Thanks to my brother, Chike and my sisters Tobe and Onyeka, to Pastor Reuben Chizor for keeping me focused, to my Aunt, Akunna Aneke, to Caroline Thomas for words of encouragement and to a host of relatives and friends here and in Nigeria. You all made this possible. Most importantly, thanks to my Lord and Savior, Jesus Christ

Chapter 1: General Introduction

The Wnt/β-catenin pathway

Wnt proteins constitute a signaling family of conserved, secreted cysteine-rich glycoproteins, which are involved in a variety of biological processes such as cell proliferation, differentiation, cell fate specification, migration and polarity [1-4]. Wnt proteins undergo extensive protein modification before being transported and released into the extracellular matrix. Wnts are glycosylated and palmitoylated in the endoplasmic reticulum (ER) [5, 6]. *Drosophila* melanogaster porcupine and its homologue in worms (in mom-1) have been shown to be integral membrane acyltransferases responsible for palmitoylation of Wnt proteins in the ER, which is necessary for Wnt export from the ER [7]. After post-translational modification, Wnts require another integral protein Wntless (Wls)/evenness interrupted [8, 9]. Wls encodes a seven-pass transmembrane protein that is highly conserved across species from worms to men. Wls directs Wnt from the Golgi to the cell surface, where it can associate with extracellular molecules such as glypicans and lipoproteins that act to limit diffusion and modulate signaling ability [10]. Inactivation of this gene in Wnt-producing cells leads to the retention of the Wnt protein in the cell [11]. Retromer complex, involved in intracellular trafficking, acts to direct Wnt proteins from the Golgi apparatus into specialized intracellular components dedicated to long-range Wnt secretion. Retromer complex also recycles Wls back to the Golgi, or else, Wls is

degraded [12]. A number of secreted proteins such as Dickkopf (Dkk), Wnt-inhibitor protein (WIF), soluble Frizzled-related proteins (SFRP) can bind Wnts and antagonize signaling [10].

Binding of Wnt ligands to specific receptor complexes on target cells can activate one of three different pathways: the canonical Wnt/β-catenin pathway, the planar cell polarity pathway (PCP) and the Wnt/Ca^{2+} pathway. Each of these pathways delivers a different set of instructions to the recipient cell by activating specific sets of target genes. The most characterized is the Wnt/β-catenin pathway, in which β-catenin acts as its major player [13, 14].

β-Catenin is a multifunctional adaptor protein, which was originally identified as a component of the cadherin-mediated cell-cell adhesion system, where it links cadherins to α-catenin [15]. It also functions as a transcriptional co-activator in the canonical Wnt pathway [16-19]. In the absence of a Wnt ligand (Figure 1.1), β-catenin is recruited to a destruction complex containing the tumor suppressors Axin and Adenomatous polyposis coli (APC) and protein phosphatase 2A (PP2A) [11, 20]. Casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3) sequentially phosphorylate β-catenin at a series of highly conserved serine/threonine residues near its N-terminal region. β-catenin is first phosphorylated at serine 45 by CK1α and then at threonine 41, serine 37 and serine 33 by GSK3 [21]. Subsequently, the phosphorylated form of β-catenin is recognized by the F box/WD-repeat β-transducin repeat-containing protein (β-TrCP), a component of the E3 ubiquitin ligase complex that polyubiquitinates β-catenin for degradation via the proteasome pathway [21-23]. Therefore, in the

non-activated state, cytoplasmic β-catenin levels are kept low. In the nucleus, T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factors interact with transcriptional repressors such as Groucho, C-terminal binding protein (CtBP) and histone deacetylases (HDAC) to repress Wnt-specific target genes [11].

More recently, the tumor suppressor Wilms Tumor gene on the X chromosome (WTX) was identified as a new component of the β-catenin destruction complex [24]. WTX is mutated in a significant number of Wilms' tumor cases, a pediatric kidney syndrome [25]. WTX is found to complex with Axin, APC, β-catenin and β-Trcp through its direct interaction with β-catenin and β-Trcp and promotes ubiquitylation and degradation of β-catenin, thereby antagonizing the Wnt/β-catenin signaling [24]. The precise mechanism by which WTX facilitates β-catenin degradation is unknown. In another report, WTX was independently identified as an APC-interacting protein, which recruits APC to the plasma membrane away from microtubules. Depletion of WTX by siRNA reduced APC protein levels in the cell, while promoting APC distribution to microtubules ends [26]. Hence WTX regulation of APC localization may underlie the mechanism in which WTX promotes β-catenin degradation [20]. The role of WTX in suppressing Wnt/β-catenin signaling might be vertebrate-specific since Drosophila does not seem to encode an identifiable WTX homolog [20]. Another Axin-binding protein, Diversin has been shown to facilitate β-catenin degradation by recruiting CK1ε to phosphorylate β-catenin [27]

Activation of the pathway occurs when Wnt proteins interact with specific cell surface receptor complexes consisting of the Frizzled (Fz) family

transmembrane proteins, which are members of the larger family of seven-membrane spanning G-protein-coupled receptors and two members of the low-density lipoprotein receptor-related protein family (LRP) LRP5 and LRP6 [11]. This triggers the phosphorylation of a cytoplasmic protein, Dishevelled (Dvl) by CK1, CK2 and Par1 kinases [17], promoting its interaction with the Fz proteins, which in turn inhibits GSK3 activity [10, 28]. The resulting Dvl/receptor complexes are thought to stimulate the formation of LRP5/6 aggregates at the plasma membrane. This facilitates the phosphorylation of the cytoplasmic tail of LRP5/6 on several serines and threonines by GSK3 and CK1-γ, which provides docking sites for Axin [11]. Recruitment of Axin to the plasma membrane to the phosphorylated tails of LRP5/6 compromises the activity of the destruction complex via Axin degradation. The ubiquitous protein phosphatase 1 (PP1) has recently been identified as a conserved positive regulator of this pathway. PP1 is a major serine/threonine phosphatase that regulates a variety of cellular processes and signaling pathways. PP1 interacts with and dephosphorylates Axin at several CK1-phosphorylated serine residues; Axin dephosphorylation in turn attenuates binding of Axin to GSK3 resulting in inhibition of β-catenin degradation [29]. Axin residues that are phosphorylated by CK1 and dephosphorylated by PP1 are outside the GSK3-binding domain of Axin, suggesting that Axin undergoes a phosphorylation-dependent conformational change that regulates the GSK3-Axin interaction [29]. Together, these act synergistically to inhibit β-catenin phosphorylation and degradation [13, 20, 30].

Consistent with Fzs being members of the G-protein coupled receptors superfamily, Gαo and Gαq subunits are required for Wnt/β-catenin signaling as locking these proteins in the GTP-bound state promotes activation of the pathway [31]. It has been demonstrated that Wnt-activated G-proteins participate in the disruption of the Axin-GSK3 complex [32, 33]. Depletion of Gα-proteins inhibited Wnt-induced disruption of Axin-GSK3 complex and diminished Wnt stabilization of β-catenin and activation of G proteins in vivo in the absence of exogenous Wnt disrupted Axin-GSK3 complex and stabilized β-catenin [33]

Upon Wnt activation, stabilized β-catenin accumulates in the cytoplasm and then translocates into the nucleus, where it binds Tcf/Lef factors to exert its effect on gene transcription by functioning as a transcriptional co-activator. The primary structure of the β-catenin protein is comprised of 12 armadillo (arm) repeats flanked by N and C terminal regions [34]**.** The N- and C-terminal domains have been shown to have transcriptional activation activity, although the C-terminal domain of β-catenin shows the more transactivation potential [35, 36]. In the nucleus, β-catenin directly displaces Groucho from Tcf/Lef and converts the complex into a potent transcriptional activator, relieving repression of Wnt target genes [37]. In addition, β-catenin serves as a landing platform for the recruitment for a variety of transcriptional co-activators such as histone acetyltransferase CREB-binding protein (CBP), a component of the SWI/SWF chromatin remodeling complex Brahma-related gene 1 (BRG1) and a member of the Polymerase-Associated Factor 1 (PAF1) complex Hydrax/Parafibromin which interacts directly with the C-terminal region of β-catenin [38, 39]. The β-catenin

coactivator Pygopus indirectly interacts with the N-terminus of β-catenin through an adaptor protein Bcl9. The precise role of β-catenin/Bcl9/Pygopus complex is rather controversial. One line of evidence suggests that it facilitates nuclear import of β-catenin [40], while another study supports a direct role for this complex in enhancing stability of β-catenin to activate Wnt target genes [41]. Direct transcriptional targets of β-catenin include genes that function in cell differentiation (siamois and brachyury), signaling (vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) FGF4 and FGF18), proliferation (cyclin-D1 and c-myc) and adhesion (E-cadherin and neuronal cell-adhesion molecule (NRCAM)) [42].

Wnt/β-catenin signaling and human diseases

Dysregulation of the Wnt//β-catenin pathway has been implicated in a variety of human diseases, most importantly cancer [4]. Constitutive activation of Wnt/β-catenin signaling through loss-of-function mutations in the tumor suppressor genes APC and Axin, or through gain-of-function mutations in β-catenin is linked to diverse human cancers, including colorectal cancers and melanomas [43, 44]. The respective mutations of APC or Axin compromise their function within the β-catenin destruction complex whereas mutations of the conserved phosphorylation sites of β-catenin blocks its targeted degradation by the ubiquitin proteasome pathway [45].

This pathway was first linked to cancer when it was found to be permanently activated in both inherited familial adenomatous polyposis (FAP) and spontaneous forms of colon cancer [2]. FAP is an autosomal, dominantly

inherited cancer syndrome in which individuals who have inherited one defective APC allele suffer the spontaneous inactivation of the second APC allele at low frequency in their intestinal epithelial cells[46]. The resulting chronic activation of the Wnt pathway in these cells drives their expansion into polyps, which frequently progress to invasive colon carcinoma [45]. The Wnt signaling pathway is aberrantly activated in about 90% of sporadic colon cancers, usually as the result of mutations in APC but also less frequently because of mutations in β-catenin and Axin. These mutations promote proliferation, survival and migration of cancer cells [23, 47]. A range of other human cancers also show signs of aberrant Wnt signaling activity although with a lower frequency than colon cancer and in contrast to colon cancer, mutations are rarely due to loss of APC [45]. Instead, mutations in β-catenin seem to be the preferred route to chronic Wnt signaling dysfunction in cancers such as liver cancer, endometrial ovarian cancer, prostate cancer, melanoma and Wilms tumor [43, 45, 48]. In other cancers and diseases that show active Wnt signaling, despite the absence of APC, Axin or β-catenin mutations, reports indicate that this can occur through epigenetic silencing of genes encoding natural Wnt pathway inhibitors such as SRFP and WIF or increased expression of pathway components including Wnt ligands, Fzd receptors and Dvl family members [49-52]. Accordingly, elucidating the function of core components of the pathway may prove therapeutic for cancer treatment [3, 45, 53].

The Wnt/β-catenin pathway has been implicated in regulating bone formation [54]. This was triggered by the discovery of LRP5 loss-of-function

mutations in patients with osteoporosis pseudoglioma syndrome (OPPG), a recessive disorder characterized by low bone mass and abnormal eye vasculature [55]. Conversely, patients with autosomal dominant high bone mass diseases harbor LRP5 gain-of-function mutation [56]. Thus LRP5 activity likely correlates with bone mass via regulation of osteoblast (bone-forming cell) proliferation [57]. Given that reduction of SFRP and DKK also results in increased bone mass, secreted Wnt antagonists have become important targets for treatment of osteoporosis [57].

Chibby, a β-catenin-associated antagonist of the Wnt pathway

A yeast-based RAS recruitment screen was performed using the C-terminal transactivation domain of β-catenin as bait, to isolate proteins that potentially modulate its transducing activity. This resulted in identification of a novel protein Chibby (Cby) [58].

Cby is a 14.5-kDa protein that is evolutionarily conserved from fly to human [58]. It harbors a functional nuclear export signal (NES) in the N-terminal region and a functional nuclear localization signal and a conserved α-helical coiled-coil motif in the C-terminal region (Figure 1.2). Cby physically interacts with the C-terminal activation domain of β-catenin [58]. Crystal structural studies for full-length β-catenin suggest that Cby binds to the Helix C located at the C-terminal end of the central Arm repeat region of β-catenin [59]. The C-terminal half of Cby is required for association with β-catenin [58]. Cby represses β-catenin signaling activity by two distinct molecular mechanisms (Figure 1.3) [60]. Cby binds to β-catenin, preventing its interaction with Tcf/Lef factors in the

nucleus, thereby inhibiting activation of Wnt target genes. In addition, upon phosphorylation of Cby serine 20 by Akt kinase, Cby and 14-3-3 chaperone proteins form a stable trimolecular complex with β-catenin, and cooperate to promote cytoplasmic localization of β-catenin, leading to down-regulation of β-catenin–mediated transcriptional activation. These two mechanisms might be required to achieve full repression of β-catenin transcriptional activity [61]. In *Drosophila melanogaster* embryos, depletion of Cby by RNA interference (RNAi) results in hyperactivation of the Wnt/β-catenin pathway [58, 62, 63], highlighting the biological importance of Cby's function. Previous studies have also demonstrated that Cby facilitates adipocyte and cardiomyocyte differentiation of pluripotent stem cells by inhibiting β-catenin signaling using *in vitro* cell culture models [60, 64]. This is consistent with previous reports that Wnt/β-catenin signaling inhibits differentiation into these cell lineages [65, 66].

Since chronic activation of β-catenin oncoprotein is a critical step in the pathogenesis of many human cancers [43], Cby is a tumor suppressor candidate [58, 62]. In accordance with this notion, Cby expression is down regulated in thyroid and uterine tumors [58, 62] in which elevation of β-catenin signaling has been reported [67, 68]. Cby was isolated as a candidate gene that is critical in the pathogenesis of ependymomas, which are the third most common primary brain tumors in children [69]. The study showed that genomic abnormalities that resulted in transcriptional inactivation of Cby are present in up to 60% of ependymomas, further highlighting the probable role of Cby as a tumor suppressor candidate [69]. Thus, Cby expression might be a feasible therapeutic

strategy for the treatment of human cancers by inhibiting oncogenic β-catenin. Activation of β-catenin/Tcf target genes induced by mutations in the Wnt pathway component constitutes the primary transforming event in colorectal cancer, inducing increased expression of c-myc, which accelerates proliferation [70]. Following disruption of β-catenin/Tcf-4 activity in colorectal cancer cells, there is decreased expression of c-myc with induction of p21, which in turn mediates G1 arrest and differentiation [70]. Thus β-catenin/Tcf-4 complex can act as the switch that controls proliferation and differentiation in healthy and malignant intestinal epithelial cells [70]. Since a common feature of all Wnt-driven cancers is the constant association of Tcf-β-catenin in the nucleus, which leads to chronic activation of a genetic program that promotes cancer formation by stimulating cell growth, blocking apoptosis and altering cell movement [45], one potential approach would be to use Cby or its domain as a small peptide inhibitor to prevent continuous association of Tcf-β-catenin in the nucleus.

Cby-interacting proteins

β-Catenin, 14-3-3 proteins, thyroid cancer protein 1 (TC1) and polycystin 2 (PC2) are proteins that have been shown to interact with Cby. 14-3-3 proteins are a family of highly conserved, acidic, dimeric proteins, which are ubiquitously expressed in all eukaryotic cells [71-73]. They are comprised of 7 isoforms in mammals (β, γ, ε, σ, ζ, τ and η) [71]. They bind to a wide array of cellular proteins and can modify the function of their targets by altering their catalytic activity, cellular localization, incorporation into protein complexes or their susceptibility to proteases and phosphatases [71, 72, 74]. In an attempt to

identify Cby-binding partners using affinity purification and mass spectrometry, two isoforms of 14-3-3, ζ and ε were isolated. 14-3-3 binds typically to phosphorylated serines/threonines in their target [71]. 14-3-3 proteins specifically recognize serine 20 in Cby upon phosphorylation by Akt kinase. A substitution of alanine for serine 20 completely abolishes the interaction between Cby and 14-3-3 [61]. It has been demonstrated that 14-3-3 binding induces relocation of Cby into the cytoplasm [61].

TC1 is another novel Cby-interacting protein. It is present only in vertebrates and encodes a protein of about 106 amino acids [75]. TC1 expression has been shown to be upregulated in thyroid and high-grade gastric cancer [75, 76], suggesting that it might be implicated in poor differentiation and/or aggressive behavior of cancers. However TC1 is not tumorigenic by itself. This led to the search for TC1-binding partners. A yeast two-hybrid screen was performed using full-length TC1 as bait, resulting in isolation of Cby. TC1 contains a putative coiled-coil motif and single NES and NLS sequences in its C-terminal [77]. TC1 acts to relieve the antagonistic activity of Cby, thereby positively regulating the Wnt/β-catenin pathway [77]. Mechanistically, the C-terminal half of TC1 physically interacts with the C-terminal region of Cby, hence competing with β-catenin for binding to Cby [77]. TC1 was shown to upregulate the expression of β-catenin target genes, implicated in cancer invasion and proliferation such as matrix metallopeptidase (MMP) 7 and 14, c-myc and cyclin D1 [77]. TC1 localizes mainly in the nucleolus but upon cotransfection with Cby,

intracellular distribution of TC1 coincided with localization of Cby at nuclear speckles, suggesting that Cby modulates the intracellular localization of TC1 [77].

PC2, one of the proteins mutated in patients with polycystic kidney disease (PKD), is another Cby-interacting protein. PC2 is a 968 amino acid protein with a molecular mass of about 110 kDa with six putative transmembrane domains and intracellular N- and C-termini [78]. A member of the transient receptor potential (TRP) superfamily of channels, PC2 is a non-selective cation channel permeable to Ca^{2+}, K^{+} and Na^{+} [79]. The C-terminus of PC2 contains a calcium-binding EF-hand and a coiled-coil domain that mediates its self interaction and interactions with polycystin 1 (PC1) [80]. PC1 is the second protein that is also mutated in patients with PKD [80-82]. PC2 localizes at the plasma membrane and endoplasmic reticulum and is expressed in the cilia of renal epithelia [83, 84]. Cby was isolated as a binding partner of PC2 using the C-terminal region of PC2 as bait in a yeast two-hybrid screen [85]. The coiled-coil domain of Cby was required for interaction with the C-terminal region of PC2, though the coiled-coil domain of PC2 was not necessary for interaction with Cby [85]. Cby was shown to play a role in regulating the subcellular localization of PC2 [85]. In the absence of Cby, PC2 is localized in the cytoplasm. When coexpressed with Cby in mammalian cultured cells, Cby and PC2 proteins redistribute, colocalizing to the trans-Golgi network, though by itself, Cby localizes predominantly to the cytoplasm [85].

Cby seems to regulate intracellular localization of most of its interacting proteins, perhaps through its interaction with 14-3-3 proteins. Thus further

identification and characterization of Cby-interacting partners would help gain more insights into unrecognized biological roles of Cby.

Cby as a nucleocytoplasmic shuttling protein

A distinguishing characteristic of eukaryotic cells is the presence of a nuclear envelope, which separates the nucleus and the cytoplasm. The presence of the physical barrier makes it necessary to develop mechanisms in which molecules and proteins can be imported into the nucleus or exported into the cytoplasm. Transport between the nucleus and cytoplasm occurs through large proteinaceous structures that perforate the nuclear envelope called nuclear pore complexes (NPCs) [86]. NPCs allow passive diffusion of relatively small molecules. However most cargo proteins undergo active transport to access or exit the nucleus. Active transport of cargo molecules between the nucleus and cytoplasm is facilitated by specific soluble carrier proteins, karyopherins with those involved in import and export, termed importins and exportins respectively [87]. For a molecule to be able to enter or exit the nucleus, it generally harbors a nuclear localization signal (NLS), which will interact with importins or a nuclear export signal (NES), which binds to exportins.

The best understood pathway of nucleocytoplasmic transport is the classical nuclear import pathway (Figure 1.4) [88]. For import into the nucleus, the adaptor protein importin-α recognizes and binds its cargo through NLS in the cytoplasm. The N-terminal importin-β binding domain of importin-α interacts with importin-β, which mediates the docking of an NLS-containing-cargo-importin-α/β trimeric complex to the cytoplasmic side of the nuclear pore and the complex

translocates into the nucleus [89]. However, it has been shown that importin-β, in some cases, is able to recognize cargo proteins directly and functions independently of importin-α [90]. In the nucleus, binding of RanGTP to importin-β causes a conformational change, resulting in the release of the importin α-cargo complex [86, 87]. For export into the cytoplasm, NES-containing cargoes are recognized in the nucleus by the export receptor, chromosome region maintenance 1 (CRM1) complexed with Ran-GTP in the nucleus and transported through the NPC to the cytoplasm. Hydrolysis of GTP to GDP in the cytoplasm triggers the release of the cargo in the cytoplasm [88, 91].

Several lines of evidence indicate that Cby is constitutively shuttling between the nucleus and cytoplasm. 1) Cby carries both NLS and NES motifs (F.-Q. Li *et al*., manuscript under revision); 2) Cby is evenly present in both the cytoplasm and the nucleus in cell culture models [61]; 3) When co-expressed with 14-3-3 proteins; Cby is sequestered in the cytoplasm [61]. Taken together, these circumstantial data suggest that Cby is a nucleocytoplasmic protein.

Our recent study has shown that Cby indeed shuttles between the nucleus and the cytoplasm (F.-Q. Li *et al*., manuscript under revision). Cby contains a functional NLS in its C-terminal region that interacts with various isoforms of importin-α family members (1,3,5 and 6). Mutations within the NLS caused a shift in Cby localization towards the cytoplasm. Additionally, Cby has a functional NES that physically interacts with the nuclear export receptor CRM1 and inhibition of CRM1 by leptomycin B (LMB), triggers nuclear localization of Cby. These results demonstrate that Cby enters and exits the nucleus through the

classical nuclear import/export pathway. The effect of Cby shuttling on β-catenin signaling was directly assessed using Tcf/Lef luciferase reporter (TOPFLASH) assays [92]. Cby NLS mutants defective in nuclear entry repressed β-catenin signaling more potently than wild-type Cby indicating that these mutants inhibit β-catenin signaling by trapping β-catenin in the cytoplasm. On the other hand Cby NES mutant that localize exclusively in the nucleus displayed a reduced ability to inhibit β-catenin signaling. Furthermore, cytoplasmic Cby NLS mutant increased cytoplasmic localization of β-catenin while nuclear Cby NES mutant caused significant accumulation of β-catenin in the nucleus in mammalian cultured cells. The results suggest that nucleocytoplasmic shuttling of Cby directly controls β-catenin signaling and localization is the predominant mode of modulating β-catenin (F.-Q. Li *et al*., manuscript under revision).

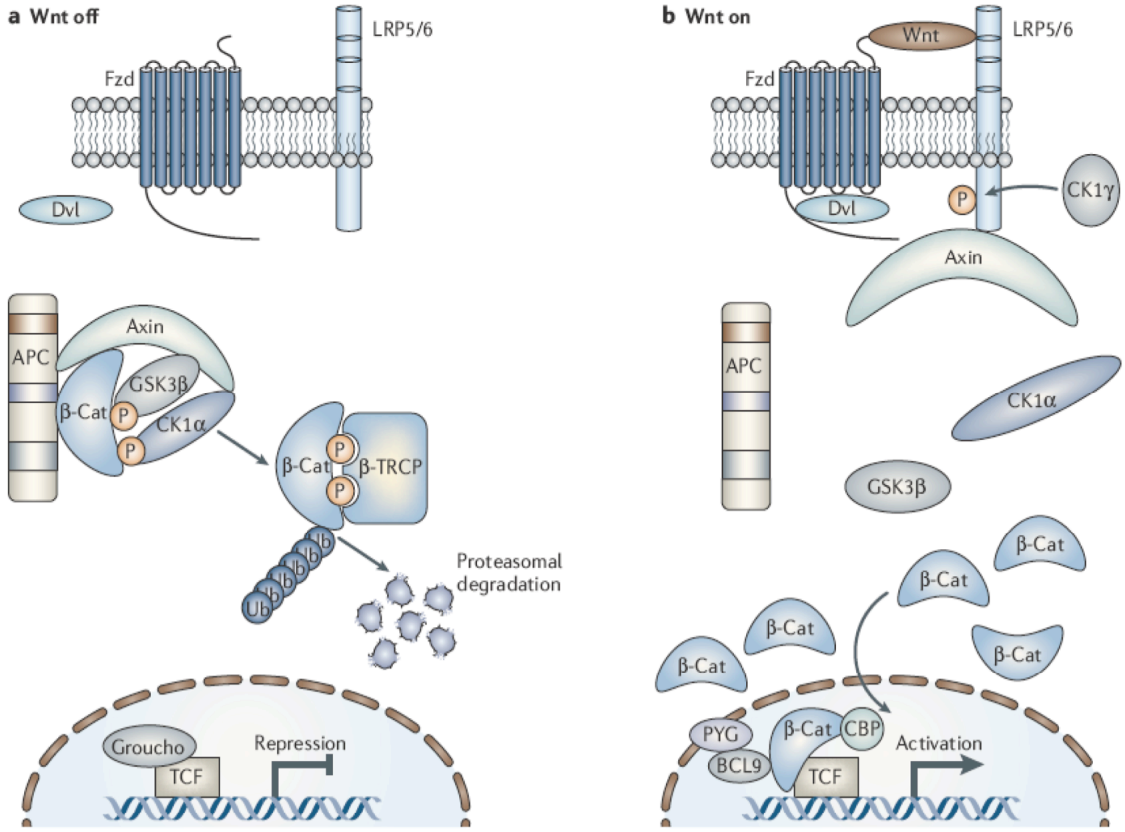
Cby as a coiled-coil protein

The coiled-coil motif is one of the principal subunits for oligomerization in proteins. Coiled-coil proteins can self-oligomerize and/or interact with other coiled-coil proteins [93]. Coiled-coils consist of amphipathic α-helices that twist around each other into a left-handed helix to form a supercoil [93, 94]. The coiled-coil domain can be represented as a helical wheel in which the seven amino acids of each heptad repeat are designated positions a to g. Hydrophobic residues usually occupy positions a and d, creating a hydrophobic dimerization interface. The other positions b, c, e, f and g contain hydrophilic residues that are surface-exposed [95].

The leucine zipper normally functions as a dimerization motif due to the presence of a leucine every 7 amino acid residues, although in some cases the canonical leucines are replaced by either isoleucine or valine [96]. The leucine zipper mediates homo- and heterodimerization of proteins and the conserved leucine residues are usually at position d in the coiled-coil domain [95]. Coiled-coil domains were initially characterized in basic leucine zipper (bZIP) transcription factors such as c-Jun and c-Fos [97]. Dimerization of bZIP proteins is a prerequisite for their binding to cognate DNA enhancer elements [97]. Point mutations generated in coiled-coil domains prevent dimerization and subsequent DNA binding [98]. Coiled-coil domains have also been shown to mediate dimerization of a wide array of proteins that do not interact with DNA [99-101].

Comparative analysis of Cby protein sequences across species showed the presence of a conserved putative coiled-coil motif in its C-terminal region [58]. A yeast-two hybrid screen using full length human Cby as bait, pulled out Cby itself as a binding partner [85]. Subsequent deletion analysis indicated that the C-terminal region of human Cby encompassing the coiled-coil domain (amino acids 60-112) was required for its self-association [85]. The region is also required for Cby's interaction with PC2 [85]. The C-terminal region of Cby has also been shown to be required for interaction with TC1 [77]. However the importance of the conserved leucines in mediating Cby interactions with itself and the type of complex Cby forms and the functional significance of Cby-Cby interactions remains largely unknown.

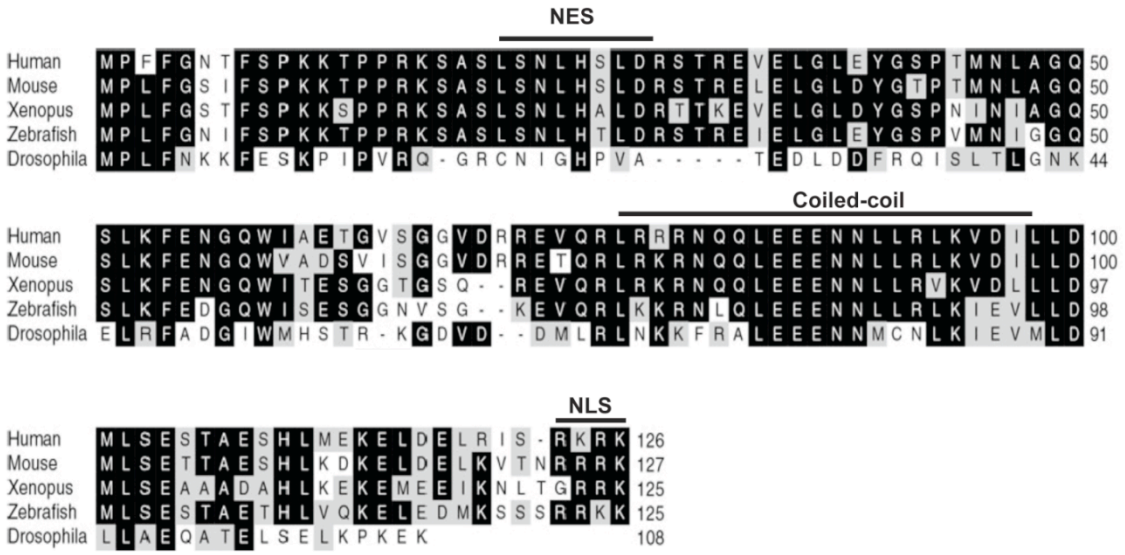
Figure 1.1



Barker N., 2008

The Wnt/β-catenin signaling pathway. (A) In the absence of a Wnt signal, β-catenin is captured by APC and Axin within the destruction complex, facilitating its phosphorylation by the kinases, CK1α and GSK3β. CK1α and GSK3β then sequentially phosphorylate a conserved set of serine and threonine residues at the N-terminal end of β-catenin. This facilitates the binding of β-Trcp, which mediates ubiqiuitinylation and efficient proteasomal degradation of β-catenin. Low levels of β-catenin in the nucleus ensures that Tcf/Lef family of transcription factors actively repress target genes by recruiting transcriptional co-repressors (Groucho) to their promoters and or enhancers. **(B)** Interaction of a Wnt ligand with its specific receptor complex containing a Frizzled family member and LRP5 or LRP6 triggers the formation of Dishevlled-Frizzled complex and phosphorylation of LRP by CK1γ, facilitating relocation of Axin to the membrane and inactivation of the destruction complex. This allows β-catenin to accumulate and enter the nucleus, where it interacts with members of the Tcf/Lef family. In the nucleus, β-catenin converts the Tcf proteins into a potent transcriptional activator by displacing Groucho proteins and recruiting an array of coactivator proteins including CREB-binding protein, Bcl9/Pygopus. This ensures activation of Wnt target genes [11].

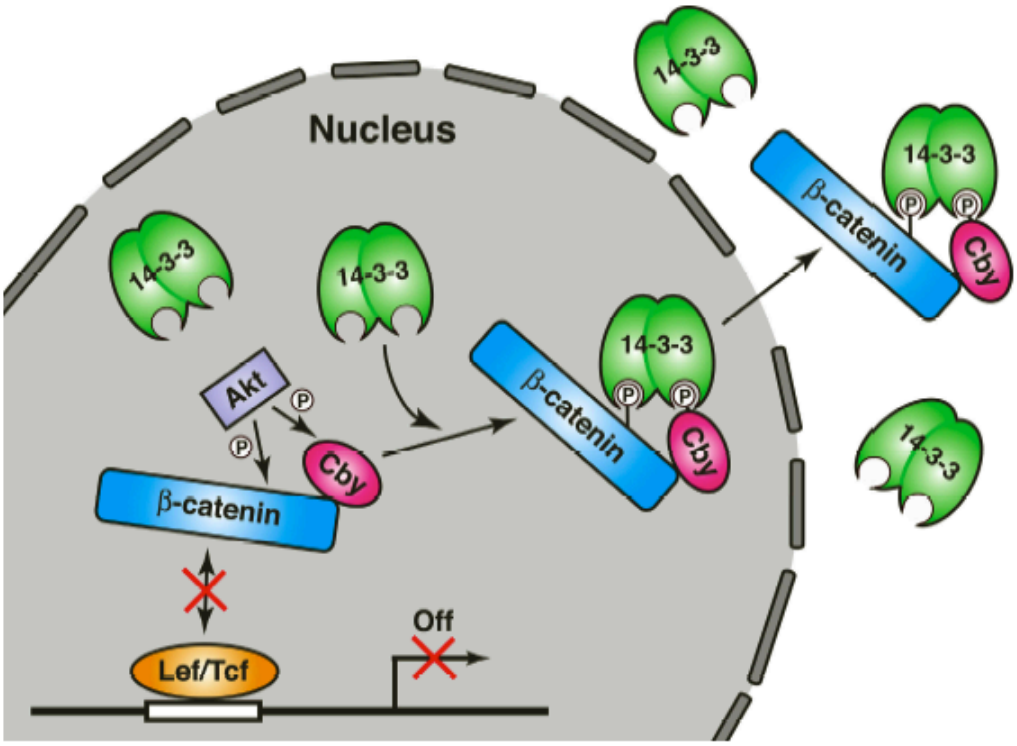
Figure 1.2.



Takemaru KI., 2003

Schematic presentation of domains in human Cby. Sequence alignment of Cby across species. Identical and similar residues are highlighted in black and gray, respectively. Human Cby contains a functional N-terminal nuclear export signal (NES) in its N-terminal region and a α-helical coiled-coil domain and a functional nuclear localization signal (NLS) in its C-terminal region. Only the coiled-coil motif and not the NLS or NES is conserved in its *Drosophila* ortholog.

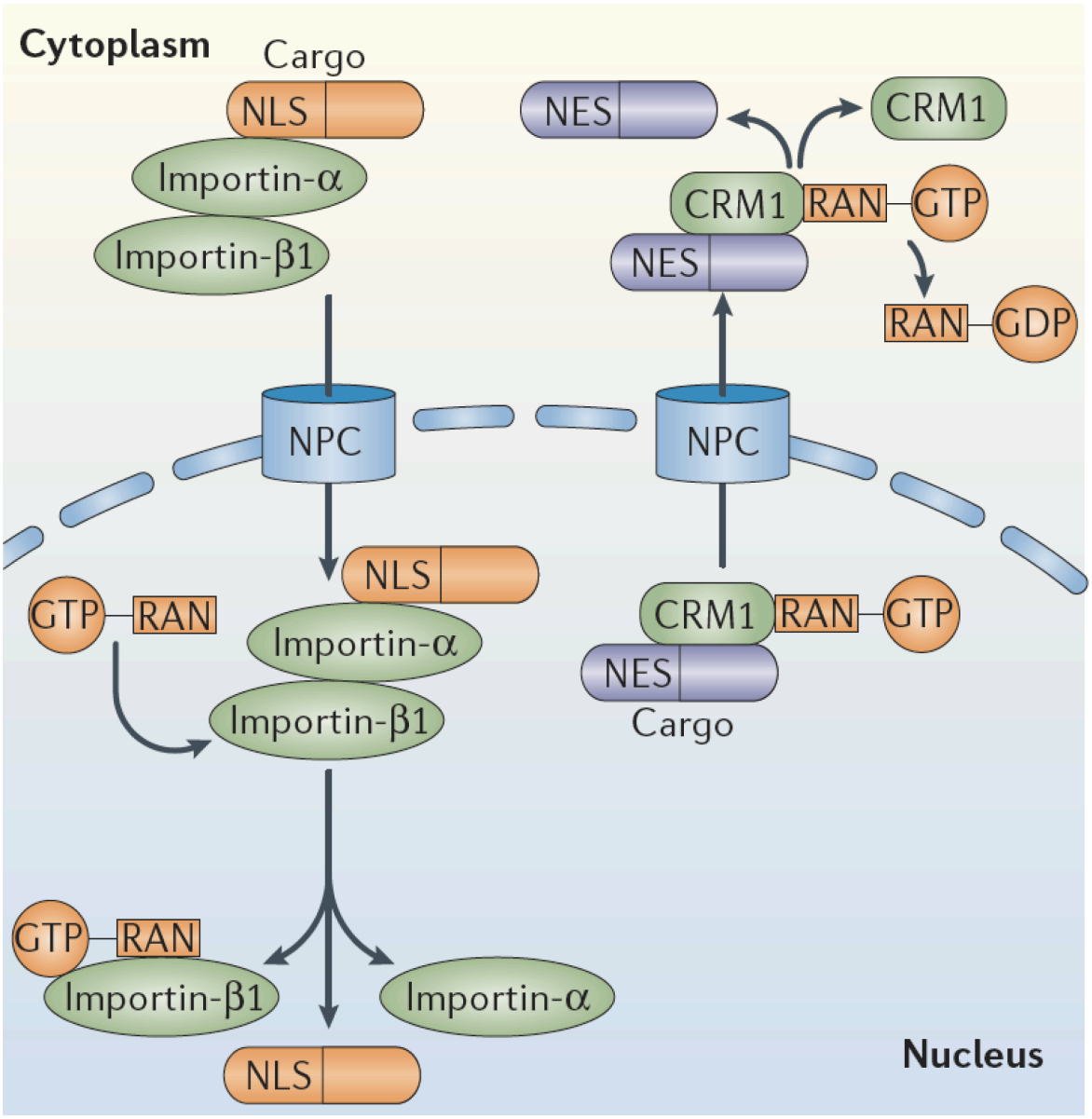
Figure 1.3



Li FQ., 2008

Dual mechanism model for the inhibition of β-catenin signaling activity by Cby. In the nucleus, Cby binds to β-catenin and competes with Tcf/Lef transcription factors, leading to repression of Wnt target genes. Additionally, phosphorylation of Cby and β-catenin by Akt facilitates 14-3-3 binding, resulting in relocation of β-catenin to the cytoplasm. These two distinct mechanisms might be required to achieve full repression of β-catenin transcriptional activity [61]

Figure 1.4



Reich NC, 2006

Schematic representation of the classical nucleocytoplasmic shuttling pathway. Nuclear-localization signal (NLS)-containing cargo is recognized in the cytoplasm by adaptor protein importin-α. Importin-α interacts with importin- β1, which mediates translocation of the complex through the nuclear pore complexes (NPC) into the nucleus. In the nucleus, binding of Ran-GTP to importin-β1 triggers the release of the cargo. Nuclear export signal (NES)-containing cargo is recognized in the nucleus by chromosome region maintenance 1 (CRM1) bound to Ran-GTP and is transported through the NPC into the cytoplasm. Hydrolysis of GTP to GDP in the cytoplasm triggers the release of the cargo [88].

Chapter 2: Materials and Methods

Expression Constructs

Expression vectors for Flag-CbyWT, MBP-CbyWT, β-catenin-Myc and His-tagged β-catenin Arm repeat 10–C-terminus (His-βcat R10-C) have been previously described [58, 102]. The GST-importin-α3 construct was a gift from Dr. Nancy Reich, Stony Brook University [103]. To generate Myc- and HA-tagged Cby expression constructs, human Cby cDNA was excised from the Flag-CbyWT vector with Eco RI and Xho I, and subcloned into pCS2+Myc and pCS2+HA, respectively. A C-terminally Flag-tagged CbyWT (CbyWT-Flag) plasmid was generated by PCR amplification of human Cby cDNA using a 3' primer containing a Flag sequence, digested with Eco RI and Xho I and subcloned into pCS2+. Both N- and C- terminally Flag-tagged Cby point mutants were created with the QuickChange site-directed mutagenesis kit (Stratagene) using the Flag-tagged CbyWT vectors as templates and primers containing the desired mutations. To obtain His-CbyWT mammalian expression plasmid, Cby cDNA was PCR-amplified, digested with Eco RI and Xho I, and subcloned into pcDNA4/HisMax A (Invitrogen). To generate His-CbyL77A/L91A and His-Cby4A constructs, the His-CbyWT construct was digested with Bam HI and Xho I, and its insert was replaced with the corresponding Cby mutant fragment from Flag-CbyL77A/L91A and Flag-Cby4A, respectively. To construct MBP-Cby mutants, the cDNA inserts were PCR-amplified, digested with Bgl II and Xho I, and ligated into pMAL-c2 (New England Biolabs). For synthetic Renilla luciferase (hRluc) protein-fragment-assisted complementation assays [104], cDNAs encoding Cby, GFP, Jun or Fos

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. All constructs were verified by DNA sequencing.

Cell culture and transfection

HEK293T and COS7 cell lines were purchased from ATCC, and maintained in DMEM with 10% FBS and 100 units/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) or SuperFect (Qiagen) according to the manufacturer's instructions. Empty vector was added to adjust the total amount of DNA to be the same in every transfection. To generate stable HEK293T cells, the His-CbyWT expression plasmid was transfected into HEK293T cells. The transfectants were expanded into medium supplemented with 500 μg/ml Zeocin (Invitrogen) 48 hours after transfection. Cells were then maintained in this medium until resistant colonies of cells were formed. The cells were then routinely maintained in culture medium supplemented with Zeocin. For LMB treatment, 17 hours after transfection, cells were treated with 40 mM LMB (Sigma) or an equal volume of methanol as negative control for 5 hours and processed for immunofluorescence microscopy.

Coimmunoprecipitation and Western blotting

HEK293T cell lysates were prepared in lysis buffer containing 20 mM Tris-HCl, pH 8.0, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol and complete protease inhibitor cocktail (Roche), and cleared by

centrifugation at 12,000 rpm for 30 min at 4°C. Coimmunoprecipitation and immunoblotting were performed as previously described [60, 61]. Cell lysates were incubated with 2 μg of anti-Myc or anti-HA antibodies overnight at 4°C followed by 2-hour incubation with protein A and protein G Sepharose beads (Roche) at 4°C. The beads were collected and washed with lysis buffer three times. For Figure 3.5, immunoprecipitates were exposed to varying concentrations of NaCl and urea in wash buffer, as indicated. Immunoprecipitates were subjected to 15% SDS-PAGE and then immunoblotting. The primary antibodies used were: mouse anti-Flag M2, 1:500 dilution (Sigma); mouse anti-Myc 9E10, 1:5,000 dilution (Invitrogen); rat anti-HA, 1:300 dilution (Roche); rabbit anti-Cby, 1:1,000 dilution[58], mouse anti-GST, 1:5,000 dilution (Novagen); rabbit anti-MBP, 1:5,000 dilution (New England BioLabs) and rabbit anti-β-catenin, 1:1,000 dilution (Sigma).

Synthetic Renilla luciferase (hRluc) protein-fragment-assisted complementation and TOPFLASH assays

HEK293T cells were seeded onto 12-well plates and transfected with appropriate combinations of plasmids. Cells were collected 17 hours later, washed with phosphate-buffered saline (PBS) and lysed with 100 μl of 1X passive lysis buffer (Promega). After clarification by centrifugation, luciferase activities in cell lysates (10 μl) were measured using the Dual Luciferase Reporter Assay System (Promega) and a Berthold luminometer according to manufacturer's instructions. An expression plasmid (10 ng) for Renilla luciferase (pRL-TK) or (5 ng) of firefly luciferase (pCMV-Luc) was co-transfected to normalize transfection efficiency. For the split hRluc assays using ViviRen live

cell substrate in Figure 3.1-4, cells were seeded onto 96-well plates and transfected with appropriate combinations of hRluc fusion constructs. The next day, ViviRen (Promega) was directly added to the tissue culture media and Renilla luciferase luminescence was measured according to the manufacturer's instructions.

Cross-linking experiments and gel filtration chromatography

His-tagged CbyWT, L77A/L91A and 4A were transiently expressed in HEK293T cells, and purified using Ni-NTA His-Bind Resin (Novagen) according to the manufacturer's instructions. The purified proteins were dialyzed against dialysis buffer containing 20 mM Hepes, pH 7.9, 100 mM NaCl, 1 mM EDTA, 0.1% NP-40 and 10% glycerol. An aliquot of the protein samples was incubated in the absence or presence of freshly prepared glutaraldehyde (0.2% final concentration) (Sigma) at 37°C for 1, 5 or 20 min or dimethyl suberimidate (DMS; 2 mg/ml final concentration) (Sigma) at room temperature for 30 min in 40 μl of the dialysis buffer. The glutaraldehyde cross-linking reaction was stopped by addition of 10 μl of 1 M Tris-HCl, pH 8.0. The samples were then mixed with SDS sample buffer, boiled and resolved by 18% SDS-PAGE, followed by immunoblotting with anti-Cby antibody.

For gel filtration experiments, His-CbyWT was purified from ten 15-cm dishes of stable HEK293T cells and dialyzed with dialysis buffer as described above. The protein sample was then loaded onto a pre-calibrated Superdex 75 gel filtration column (Amersham Biosciences), and run via fast protein liquid chromatography (FPLC) with gel filtration column buffer containing 20 mM Tris-

HCl, pH 8.0, 0.5 or 1.0 M NaCl, I mM EDTA, 2 mM DTT, 2 mM betaine-HCl, 0.02% Triton X-100 and 5% glycerol at a flow rate of 0.3 ml/min. Fractions of approximately 0.5 ml were collected and analyzed by Western blotting with anti-Cby antibody. The column was calibrated using protein standards (Amersham Biosciences): ferritin, 450 kDa; aldolase, 158 kDa; bovine serum albumin, 67 kDa; ovalbumin, 45 kDa; cytochrome C, 12 kDa.

Protein expression in bacteria and *in vitro* pull-down assays

E. coli BL21 cells were transformed with expression constructs for MBP and MBP-Cby fusion proteins, GST and GST-importin α3 fusion proteins, while *E*. coli BL21 DE3 cells were transformed with expression construct for His-βcatR10-C. Bacteria were grown at 37°C for 3 hours to middle logarithmic phase (optical density at 600 nm of 0.6 to 1.0). For MBP proteins, bacterial cultures were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Sigma) for 3 hours at 30°C. GST fusion culture was induced with 1 mM IPTG for 3 hours at 37°C, while His fusion culture was induced with 0.5 mM IPTG for 3 hours at 37°C. MBP fusion proteins were purified using amylose resin (New England BioLabs); GST or His-tagged fusion proteins were purified with GST beads (Novagen) or Ni-NTA His-Bind Resin (Novagen) respectively. Purified proteins were dialyzed with dialysis buffer described above. The concentrations of proteins were evaluated visually on SDS-PAGE gels stained with Coomassie Brilliant blue (Sigma) in comparison with protein standards (Invitrogen).

In vitro binding assays have been described [102]. In brief, equal amounts of MBP or MBP-Cby fusion protein was incubated with GST, GST-importin α3 or

His-βcatR10-C at 4°C for 1 hour in 30 μl of protein binding buffer (PBB) containing 20 mM Hepes, pH 7.9, 20% glycerol, 0.5 mM EDTA, 100 mM NaCl, 6 mM $MgCl_2$ and 0.1% NP40. After incubation 10 μl packed volume of amylose resin resuspended in PBB was added and incubated at 4°C for 1 hour. The beads were collected and washed with PBB three times and subjected to 12% SDS-PAGE. GST-Importin α3 and His-βcatR10-C were detected using anti-GST antibody, 1:5,000 dilutions (Novagen) and anti-β-catenin antibody, 1:1,000 dilutions (Sigma), respectively.

Immunofluorescence microscopy

COS7 cells were grown on glass coverslips and transfected as described above. After 24 hours of transfection, the cells were fixed with methanol-acetone (1:1, v/v), permeabilized with 0.2% Triton X-100 and blocked with 1% BSA in PBS. Cells were immunostained with mouse anti-Flag M2 antibodies (Sigma) at 1:400 dilutions for 1 hour at room temperature, and then washed thrice with PBS, followed by TRITC-labeled goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at 1:300 dilutions for 1 hour. Cells were washed thrice with PBS and nuclei were counterstained with DAPI (Sigma) for 1 min. A Leica DM500 fluorescent microscope was used to analyze stained cells. To quantify subcellular localization, independent transfections were performed at least three times and a minimum of 100 cells was counted for each transfection.

Chapter 3: Results

Chapter 3.1 Cby interacts with itself in a stable complex

Cby has been shown to interact with itself using a conventional yeast two-hybrid system [85]. However, the nature of Cby homoligomers remains to be elucidated. In order to confirm and extend this observation, Cby's ability to interact with itself was examined using two independent approaches. Additionally, the type of complex Cby forms was also investigated.

To confirm that Cby interacts with itself, coimmunoprecipitation experiments were performed (Figure 3.1-1). Expression plasmids for Myc-tagged Cby and Flag-tagged Cby were transiently transfected singly or together into human embryonic kidney (HEK) 293T cells. The cell lysates were immunoprecipitated with anti-Myc antibody and following SDS-PAGE Western blot analyses were performed with anti-Flag antibody. As shown in Figure 3.1-1, Flag-Cby was detected only when co-expressed with Myc-Cby. Immunoprecipitates were also subjected to immunoblotting with anti-Myc antibody to demonstrate that equal amounts of Myc-Cby were pulled down.

Next, spilt synthetic Renilla luciferase (hRluc assays) [104] were employed as an independent means to verify the Cby-Cby interaction. To determine if two proteins for example protein X and protein Y interact using hRluc protein X is linked to the N-terminal region of Renilla luciferase and protein Y is linked to the C-terminal portion of Renilla luciferase (Figure 3.1-2). If these fusion proteins physically interact with each other, they would bring the N- and C- terminal portions of Renilla luciferase together and produce measurable Renilla luciferase

activity [104]. Cby or negative control GFP was fused in-frame to the N-terminal portion of hRluc (Cby-RN or GFP-RN) or to the C-terminal portion of hRluc (Cby-RC or GFP-RC). The constructs were transfected into HEK293T cells in various combinations and hRluc activities were measured. As seen in Figure 3.1-3, co-transfection of empty vectors (RN and RC) or Cby- and GFP-hRluc fusion plasmids (Cby-RN and GFP-RC or GFP-RN and Cby-RC) produced only a basal level of hRLuc activity. On the other hand, high Renilla activity was observed when Cby-RN and Cby-RC were co-expressed. Western blot analyses with anti-Cby antibody clearly demonstrated that these Cby fusion proteins were similarly expressed.

Cby-Cby interactions were further confirmed in live cells in real time. A cell-permeable substrate for Renilla luciferase, ViviRen was utilized to detect protein-protein interactions in live cells. Transient transfection of Cby-RN and Cby-RC expression vectors resulted in a high luminescence value, whereas either vector in combination with an empty vector (RN or RC) generated only low background luminescence (Figure 3.1-4). Interactions between Cby and well-established basic leucine zipper coiled-coil transcription factors, Jun and Fos, were also tested using the ViviRen substrate. Remarkably reduced luminescence was detected when Cby-RN and Fos-RC or Jun-RN and Cby-RC were coexpressed in HEK293T cells, compared to that of Jun-Fos and Cby-Cby interactions, suggesting that the Cby-Cby interaction is specific.

Furthermore, the stability of the Cby oligomer was examined Figure 3.1-5. Flag-Cby and HA-Cby expression plasmids were cotransfected into HEK293T

cells. Whole cell lysates were immunoprecipitated with anti-HA antibody. The immunoprecipitates were washed with lysis buffer containing varying concentrations of NaCl or urea as indicated, resolved by SDS-PAGE and analyzed by immunoblotting using anti-Flag antibody. Essentially, similar levels of Flag-Cby were detected in the presence of up to 2 M NaCl or 2 M urea. These results suggest that Cby-Cby interaction is very stable and that both hydrophobic and electrostatic interactions contribute to the stability of Cby-Cby complex formation. Taken together, the above results show that Cby forms a stable complex with itself.

Cby is present as a homodimer

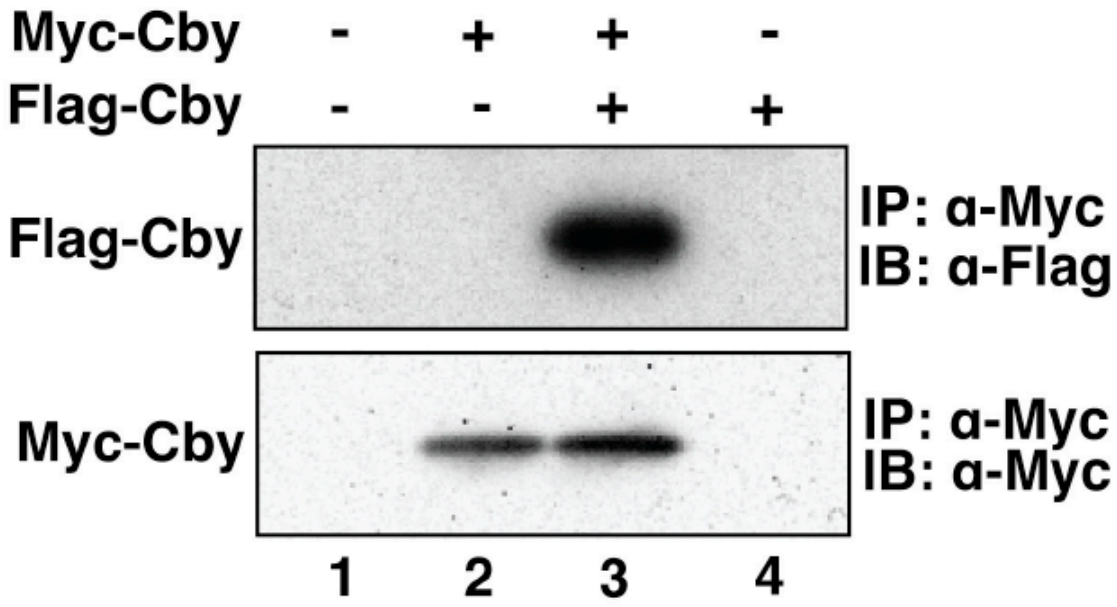
In order to gain further insights into the oligomeric nature of Cby, analytical gel chromatography was performed. His-tagged Cby was purified from stable HEK293T cells using nickel beads. Purified His-Cby sample was applied to a Superdex 75 pre-calibrated gel filtration column. Following fractionation, each fraction was analyzed by immunoblotting with anti-Cby antibody. As shown in (Figure 3.1-6), Cby was eluted as a broad single peak centered at fraction 34, corresponding to a molecular weight of about 45 kDa. Since the molecular weight of His-Cby monomer is 22 kDa Cby is most likely present as a homodimer.

Chemical cross-linking analyses were conducted to confirm homodimerization of Cby. Glutaraldehyde and dimethyl suberimidate (DMS) were used. Both chemicals are homobifunctional cross-linking agents that covalently link amine groups in proteins, though DMS has a longer spacer arm [105-107]. His-tagged Cby was transiently expressed in HEK293T cells and purified using

nickel beads. The purified Cby protein was then incubated in the absence or presence of glutaraldehyde for 5 or 20 min. Cross-linked proteins were resolved by SDS-PAGE, followed by Western blotting with anti-Cby antibody (Figure 3.1-7). As expected, in the absence of glutaraldehyde, His-Cby had a molecular size of 22 kDa, but after incubation with glutaraldehyde, large bands of approximately 42 kDa were observed, suggesting that His-Cby forms a dimer under these conditions. Furthermore, similar results were obtained after incubation of purified His-Cby protein with DMS for 30 minutes (Figure 3.1-8).

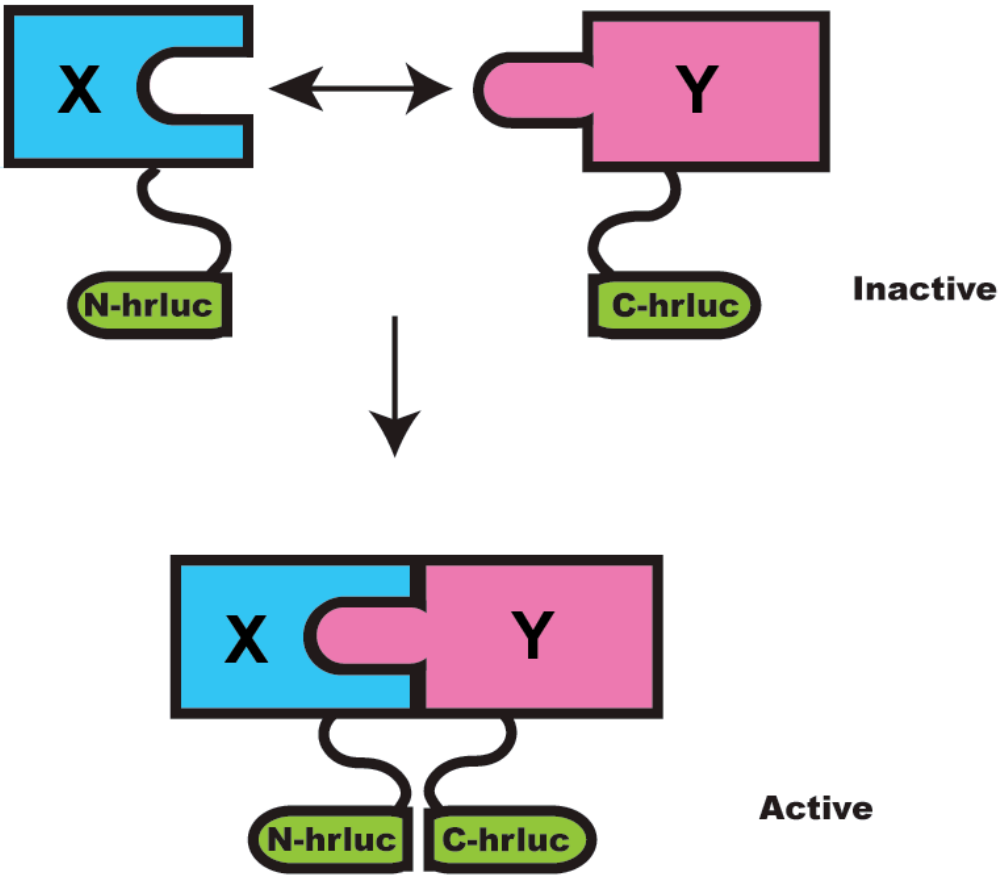
Collectively, these findings clearly demonstrate that under these conditions, Cby interacts with itself predominantly as a dimer in a very stable complex.

Figure 3.1-1



Coimmunoprecipitation of Myc-Cby and Flag-Cby. HEK293T cells were transfected with the indicated constructs, and cell lysates were immunoprecipitated with anti-Myc antibody. The immunoprecipitates were separated by SDS-PAGE and subjected to Western blotting with anti-Flag or anti-Myc antibody.

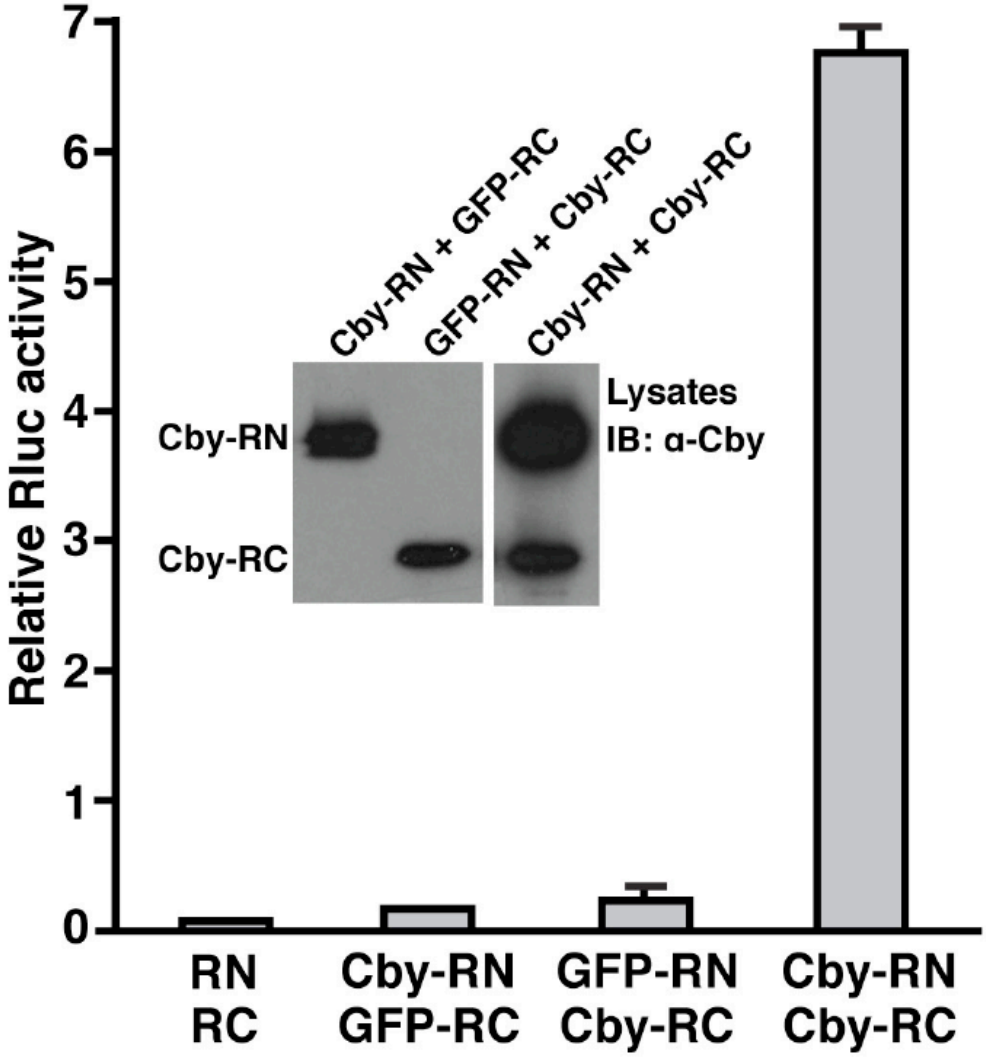
Figure 3.1-2



Paulmurugan R., 2003

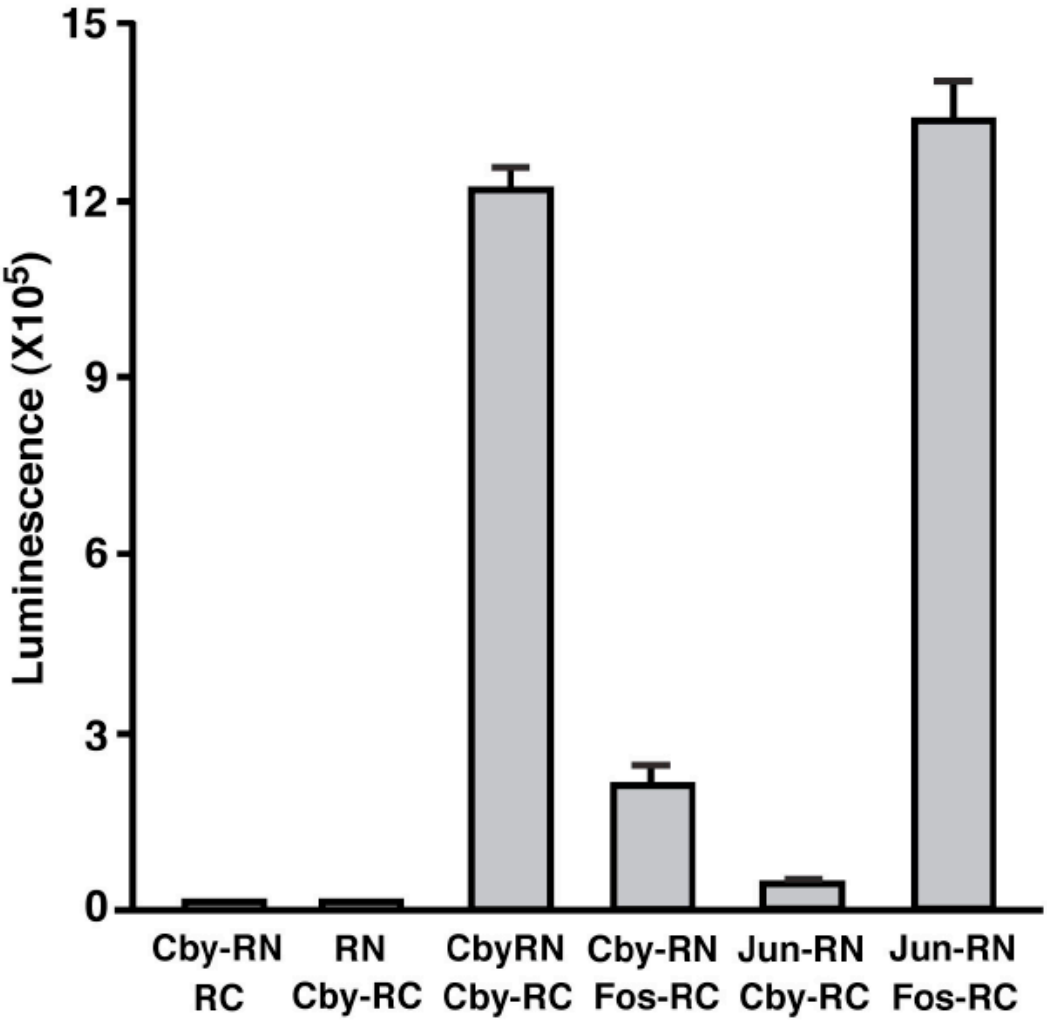
Schematic diagram of the split synthetic Renilla luciferase (hRluc) protein-fragment-assisted complementation assay used to monitor protein-protein interactions. In the split Renilla complementation assay, N-terminal portion of Renilla luciferase is attached to protein X and the C-terminal portion of Renilla luciferase is attached to protein Y. Interactions of proteins X and Y recovers Renilla luciferase activity through protein complementation [104].

Figure 3.1-3



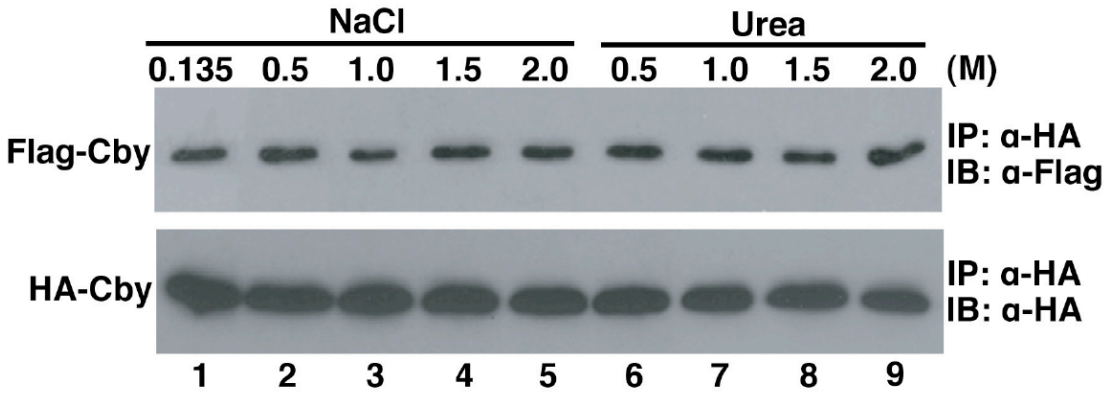
Cby self-interaction was detected by split (hRluc) protein-fragment-assisted complementation. Cby or negative control GFP was fused in-frame to the N-terminal portion (RN) and C-terminal portion (RC) of hRluc. These expression plasmids (400 ng each) were transfected into HEK293T cells as indicated, and Renilla luciferase (Rluc) activities were measured 24 hours post-transfection in cell lysates. A firefly luciferase plasmid (5 ng) was co-transfected to normalize transfection efficiency. Transfections were carried out in triplicate and the means ± SD are shown. Immunoblotting with anti-Cby antibody showed that the fusion proteins were stably expressed.

Figure 3.1-4



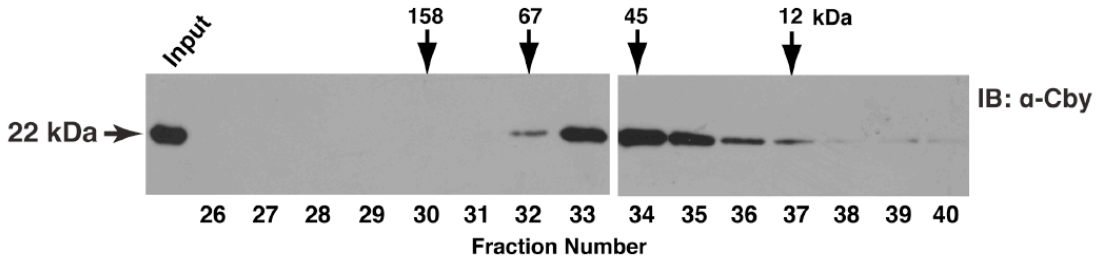
Detection of Cby self-interaction *in vivo*. Cby-Cby interactions were detectable *in vivo* in real time using cell-permeable ViviRen live cell substrate. Cby-RN, Cby-RC, Fos-RC and Jun-RN were transiently expressed in HEK293T cells as shown, and ViviRen was added to the tissue culture media 24 hours post-transfection for luminescence measurements. Transfections were carried out in triplicate and the means ± SD are shown.

Figure 3.1-5



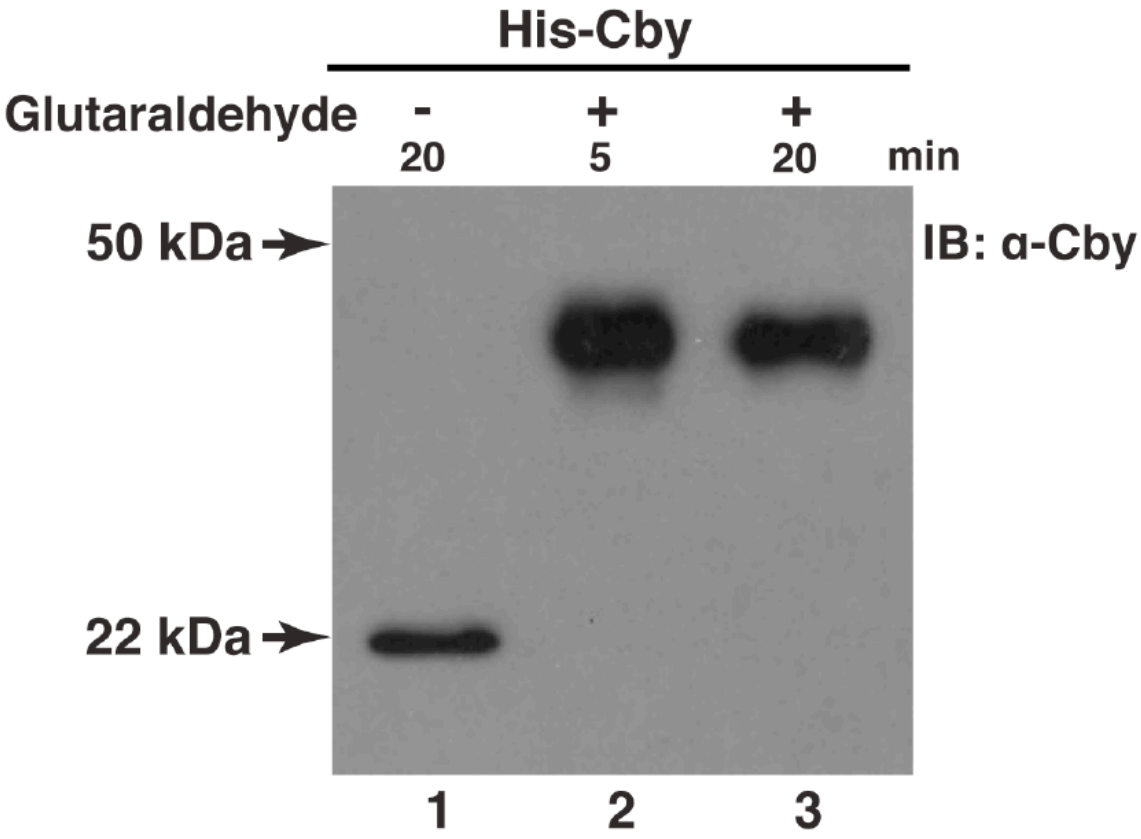
Cby forms a stable complex with itself. Cby complex is highly stable. HEK293T cells were co-transfected with Flag-Cby and HA-Cby, and cell lysates were immunoprecipitated with anti-HA antibody. The immunoprecipitates were subsequently washed three times with wash buffer containing 0.135, 0.5, 1.0, 1.5 or 2.0 M NaCl, or 0.5, 1.0, 1.5 or 2.0 M urea as shown. The experiments with urea were performed in the presence of 0.135 M NaCl. The immunoprecipitates were then separated by SDS-PAGE and immunoblotted with anti-Flag or anti-HA antibody.

Figure 3.1-6



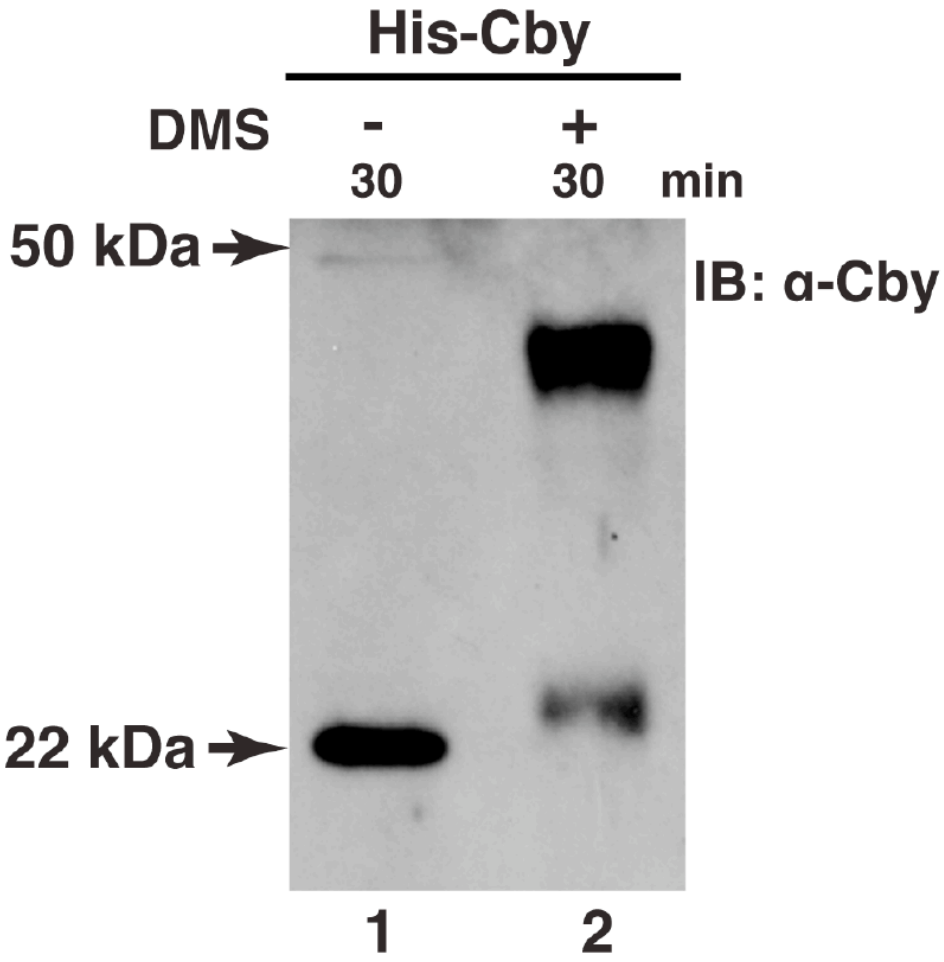
Gel Filtration Analysis of His-CbyWT. His-tagged Cby purified from stably transfected HEK293T cells using Ni-NTA beads was loaded on to a previously calibrated Superdex 75 gel filtration column and run via fast protein liquid chromatography (FPLC) with buffer containing 1.0 M NaCl. Fractions of approximately 500 μL were collected and analyzed by Western blot with anti-Cby antibody. The arrows indicate the elution positions of protein standards: aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa) and cytochrome C (12 kDa).

Figure 3.1-7



Glutaraldehyde crosslinking of His-CbyWT. His-tagged Cby was purified from transiently transfected HEK293T cells were subjected to cross-linking with glutaraldehyde for 5 or 20 min. The samples were then resolved by SDS-PAGE, followed by Western blotting with anti-Cby antibody.

Figure 3.1-8



DMS crosslinking of His-CbyWT. His-tagged Cby was purified from transiently transfected HEK293T cells were subjected to cross-linking with dimethyl suberimidate (DMS) for 30 min. The samples were then resolved by SDS-PAGE, followed by Western blotting with anti-Cby antibody.

Chapter 3.2: Chibby homodimerization is mediated through its coiled-coil domain

Cby has been demonstrated to interact with itself. Since it contains a putative coiled-coil domain in its C-terminal region, the functional importance of the putative coiled-coil motif in Cby was investigated.

Human Cby amino acid sequence was analyzed using COILS program, which calculates the probability that a protein sequence will adopt a coiled-coil conformation [108, 109]. The program predicted a high probability that the C-terminal region of human Cby from amino acid 68 to 102 could form an α-helical coiled-coil structure (Figure 3.2-1). Further examination of this region revealed four leucine residues that appear every seventh position. This is characteristic of a leucine zipper motif, which mediates dimerization [95]. Sequence alignments of Cby coiled-coil domain across species show that these four leucine residues are perfectly conserved in vertebrate Cby homologues (Figure 3.2-2). In contrast, only two of these leucine residues (L77 and L84) are conserved in *D. melanogaster* Cby. However, the corresponding region of *D. melanogaster* Cby was also predicted to form a coiled-coil structure by the COILS program (data not shown), signifying the functional importance of this domain.

A typical leucine zipper sequence is made up of 7-residue repeats called “heptads”. The 7 positions in a heptad are denoted a-g, positions a and d contain hydrophobic residues and form the helix interface while positions b, c, e and f contain hydrophilic residues and form the solvent-exposed part of the coiled-coil

[94, 110, 111]. The coiled-coil domain of human Cby comprises four consecutive heptad repeats with amino acid positions labeled as a-g (Figure 3.2-3) The helical wheel projection illustrates that the conserved leucine residues are located at position d, which is typically observed in well-characterized basic leucine zipper transcription factors [96, 112]. The presence of four conserved heptad leucines suggests that they might be necessary for mediating Cby homodimerization. Having demonstrated that Cby forms a stable dimer, the role of these leucine residues in mediating this dimerization was extensively investigated as detailed below.

Site-directed mutagenesis was performed to mutate each leucine residue (L77, L84, L91 and L98) to alanine in all possible combinations. Single, double, triple and quadruple leucine zipper mutants were generated. These mutations would not be expected to disrupt the α-helical structure of Cby, but the shorter alanine side chain would not be able to form a leucine zipper-mediated coiled-coil structure. The N-terminally Flag-tagged wild-type (CbyWT) and mutants were transiently transfected into HEK293T cells and cell lysates were subjected to SDS-PAGE, followed by immunoblotting with anti-Flag antibody. All Cby mutants were detected at appreciable levels, showing that mutants were stably expressed (Figure 3.2-4). Next the Cby mutants were evaluated for their ability to form a complex with (CbyWT) by coimmunoprecipitation assays. Flag-tagged CbyWT or Cby mutants were coexpressed with HA-tagged CbyWT in HEK293T cells. The cell lysates were immunoprecipitated with anti-HA antibody and following SDS-PAGE, Western blot analyses were carried out with anti-Flag antibody.

Interactions were detected when HA-CbyWT was coexpressed with Flag-CbyWT and Flag-tagged Cby single point mutations (CbyL77A, CbyL84A, CbyL91A and CbyL98A), even though reduced interactions were noted for CbyL84A. Remarkably, Cby mutants with two or more alanine substitutions completely lost interaction with CbyWT. As a control, cell lysates were subjected to Western blotting with anti-Flag antibody to establish that Flag-tagged Cby WT and Cby mutants were expressed at similar levels (Figure 3.2-5).

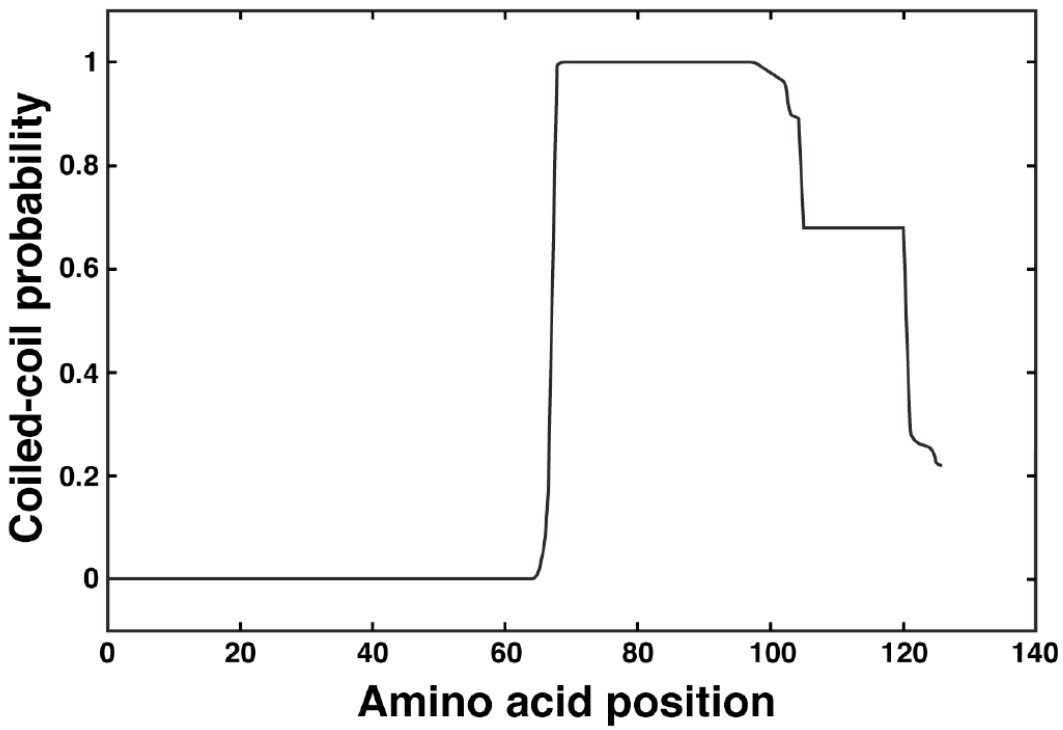
Spilt hRluc assays for protein-protein interactions were performed to confirm the coimmunoprecipitation results. HEK293T cells were cotransfected with expression plasmids for Renilla fusions with wild-type or mutant Cby. Renilla luciferase activities were measured and firefly luciferase control was used to normalize transfection efficiency. Coexpression of CbyWT-hRluc fusion proteins yielded high Rluc activity. Consistent with the coimmunoprecipitation experiments, reduced yet significant Rluc activities were observed for Cby single point mutants, whereas all Cby double mutants or the quadruple mutant, Cby4A, displayed only basal Rluc activities (Figure 3.2-6).

To establish that the Cby mutants incapable of self-interacting are comprised in homodimer formation, glutaraldehyde cross-linking experiments were performed (Figure 3.2-7). His-tagged CbyWT, Cby double mutant (CbyL77A/L91A) and Cby quadruple mutant (Cby4A) were transiently transfected into HEK293T cells and purified using nickel beads. Purified Cby proteins were incubated with glutaraldehye for 1 min at 37°C. Cross-linked proteins were

subjected to SDS-PAGE and immunoblotted with anti-Cby antibody. As expected, CbyWT migrated as a dimer after incubation with glutaraldehyde (lane 2) whereas CbyL77A/L91A and Cby4A incapable of interacting with itself, migrated as a monomer after incubation with glutaraldehyde (lanes 4 and 6, respectively).

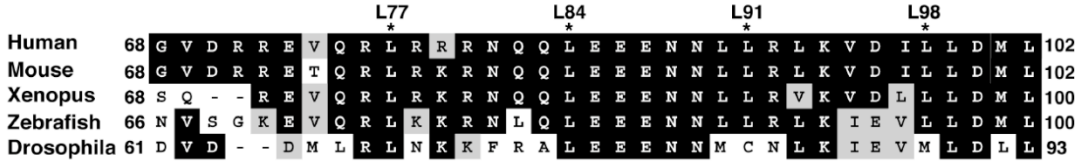
In conclusion, Cby contains a functional coiled-coil motif in its C-terminal region. The conserved leucines (L77, L84, L91 and L98), located at position d of the helical wheel diagram are crucial for mediating Cby-Cby interactions. Alanine substitutions of these leucines completely abrogate Cby self-assembly. Glutaraldehyde cross-linking assays clearly demonstrated that Cby mutants incapable of self-interacting migrated as a monomer on SDS-PAGE after incubation with glutaraldehyde.

Figure 3.2-1



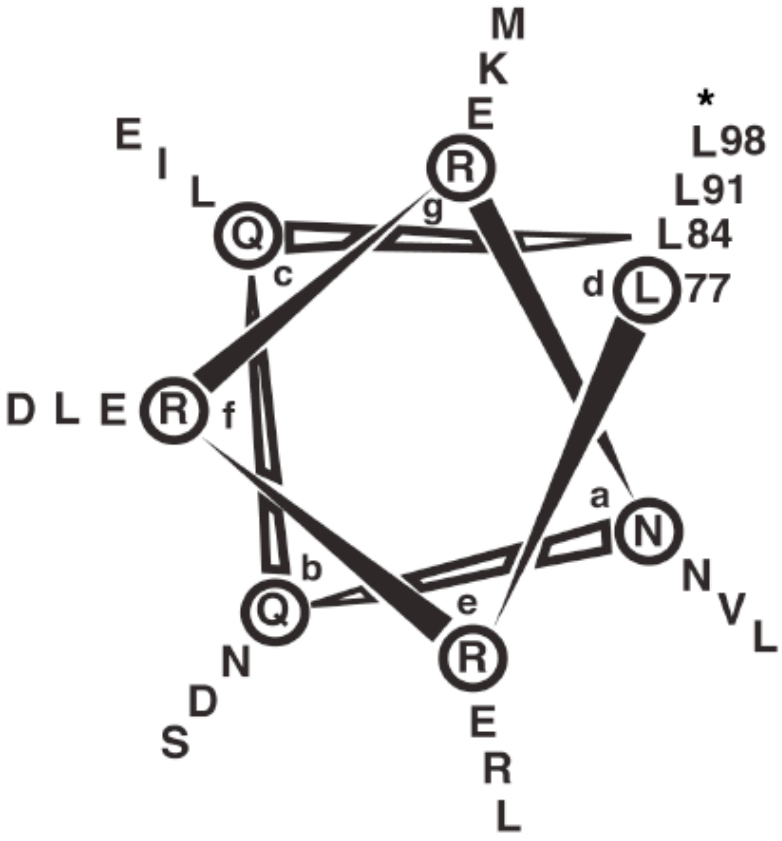
COILS program analysis of human Cby protein sequence. The human Cby protein sequence (126 amino acid residues) was analyzed by the COILS program at a window size of 21 residues. Note that high coiled-coil probabilities exceeding 0.9 were evident from amino acid 68 to 102.

Figure 3.2-2



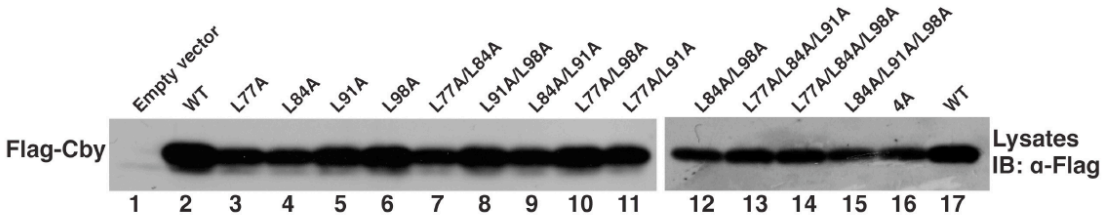
The C-terminal region of Cby depicting the putative coiled-coil domain. Sequence alignment of the Cby coiled-coil domain across species. Four leucines, indicated by asterisks, appear at every seventh position, indicative of a leucine-zipper motif. These leucines are highly conserved across species. *Drosophila* Cby has only two of the conserved leucines.

Figure 3.2-3



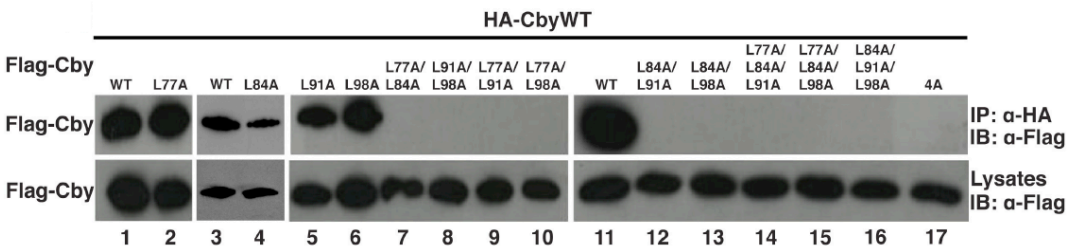
The helical wheel presentation of the putative coiled-coil motif of human Cby. The putative coiled-coil motif of human Cby is depicted as heptad repeats of seven amino acids. The letters a through g designate the positions of residues within the heptad with hydrophobic residues generally found at the a and d positions. The four consecutive heptads are numbered 1 through 4. Note that the d positions are occupied by leucine residues. Helical wheel diagram of the coiled-coil domain of human Cby (amino acids 77-98).

Figure 3.2-4



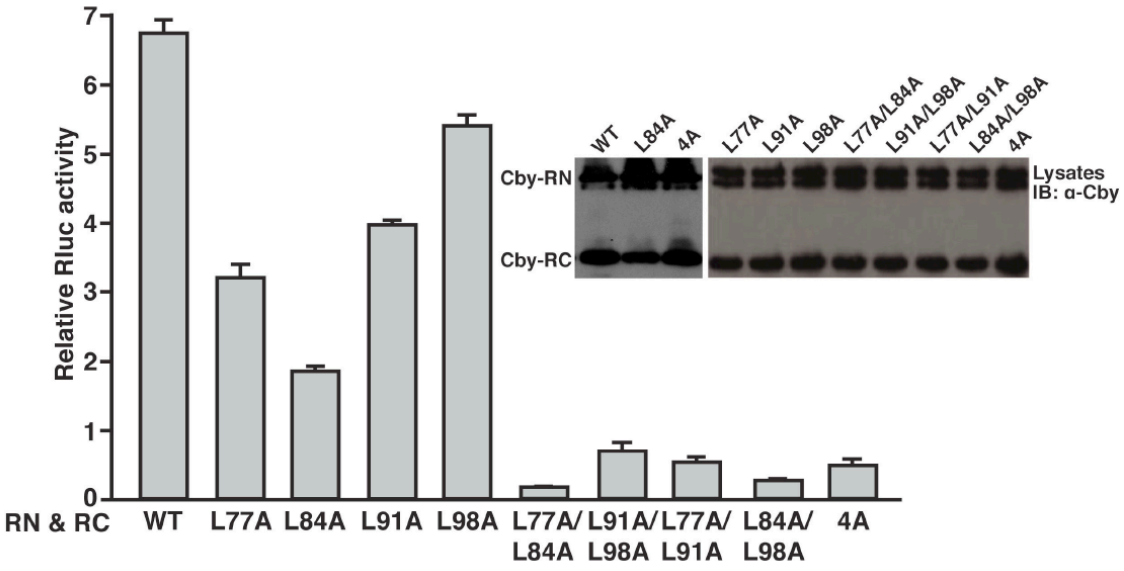
Expression levels of Flag-tagged wild-type Cby (CbyWT) and Flag-Cby coiled-coil motif point mutants. Lysates from HEK293T cells transfected with an equal amount (600 ng) of an expression plasmid for Flag-tagged WT or mutant Cby were subjected to Western blotting with an anti-Flag antibody.

Figure 3.2-5



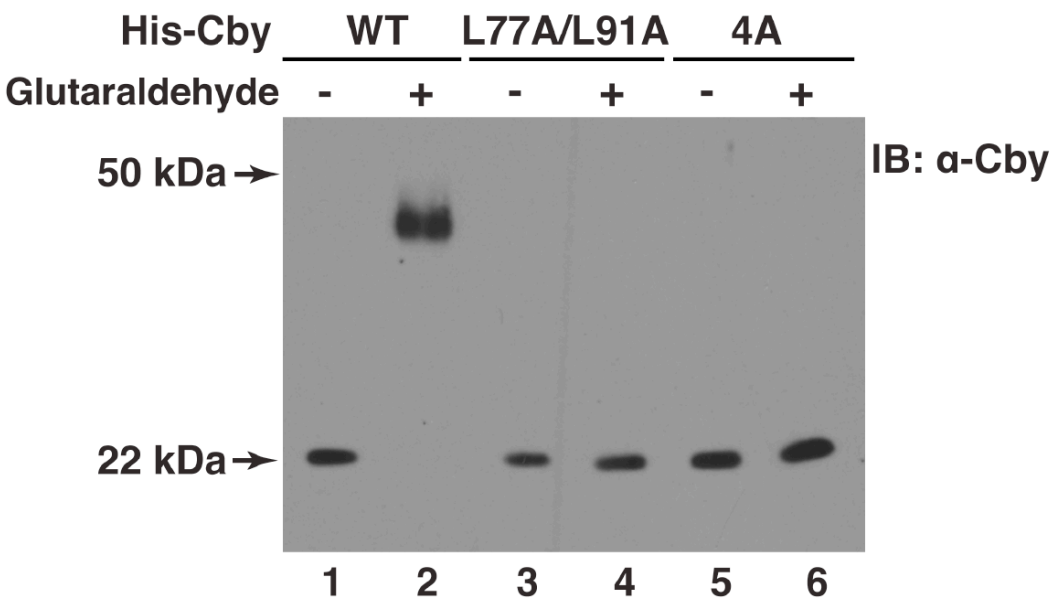
Coimmunoprecipitation of HA-CbyWT and Flag-CbyWT or Flag-Cby coiled-coil motif point mutants. Cell lysates were prepared from HEK293T cells transiently co-transfected with HA-CbyWT and Flag-CbyWT or individual Cby variants with all possible combinations of leucine-to-alanine mutations [single, double, triple and quadruple (4A)], and subjected to immunoprecipitation with anti-HA antibody. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-Flag antibody. To ensure sufficient protein expression levels, the amounts of plasmid DNA expressing Flag-tagged Cby mutants were increased by 2-fold for transfection, compared to those of Flag-CbyWT plasmid.

Figure 3.2-6



Split hRluc protein-fragment-assisted complementation assays of Cby coiled-coil motif mutants. CbyWT or each of the indicated Cby mutants was fused in-frame to RN and RC. These expression plasmids (400 ng each) were transfected into HEK293T cells, and Rluc activities were measured 24 hours post-transfection. A firefly luciferase plasmid (5 ng) was co-transfected to normalize transfection efficiency. Transfections were carried out in triplicate and the means ± SD are shown. Immunoblotting with anti-Cby antibody showed that the fusion proteins were stably expressed. Cby-RN fusion proteins appear as a doublet probably due to degradation.

Figure 3.2-7



Glutaraldehyde cross-linking of dimerization-deficient Cby mutants. His-tagged Cby WT, L77A/L91A or 4A was transiently expressed in HEK293T cells, and purified using Ni-NTA beads. The purified Cby protein was incubated in the absence or presence of glutaraldehyde for 1 min, and resolved on SDS-PAGE, followed by immunoblotting with anti-Cby antibody.

Chapter 3.3: Biological significance of the Cby-Cby interaction

Cby inhibits β-catenin–mediated gene activation [58]. The C-terminal region of Cby that contains the coiled-coil domain is required for interacting with β-catenin. We investigated whether Cby homodimerization is a prerequisite for Cby interaction with β-catenin and inhibiting β-catenin-dependent transcription activation.

In vitro pull-down assays were performed to analyze interactions between Cby dimerization-deficient mutants and β-catenin. Bacterially expressed and purified maltose-binding protein (MBP) or MBP-CbyWT, MBP-CbyL77A, MBP-CbyL91A, MBP-CbyL77A/L91A or MBP-Cby4A was incubated with His-tagged β-catenin C-terminal region (Arm repeat 10 to C terminus, β-catR10-C) and pulled down with amylose beads. The bound proteins were resolved by SDS-PAGE and analyzed by immunoblotting with anti-β-catenin antibody (Figure 3.3-1). Intriguingly, all the Cby point mutants tested, including the mutants that were dimerization-deficient (CbyL77A/L91A and Cby 4A) were still able to bind efficiently to β-catenin. Each pull-down sample was run on a separate SDS-PAGE and immunoblotted with anti-MBP antibody, showing that similar amounts of MBP-Cby proteins were pulled down.

Tcf/Lef luciferase reporter (TOPFLASH) assays [92] were performed to examine if Cby dimerization- deficient mutants would be capable of inhibiting β-catenin-mediated transcription activation. The TOPFLASH plasmid contains

three optimal Tcf/Lef binding sites driving luciferase activity. TOPFLASH plasmid, stabilized β-catenin-Myc and Flag-CbyWT or mutants were transfected into HEK293T cells. Transfection of stabilized β-catenin activated TOPFLASH activity near 25-fold (Figure 3.3-2). Cotransfection of CbyWT potently repressed β-catenin-mediated transcription activation in a dose-dependent manner. In agreement with the pull-down assays, all Cby point mutants inhibited TOPFLASH activation by β-catenin to an extent similar to CbyWT. In all cases, little or no changes were observed with the negative control reporter FOPFLASH carrying mutated Tcf/Lef binding sites. Taken together, these observations suggest that Cby does not have to homodimerize to inhibit β-catenin-dependent transcription activation and that monomeric Cby is sufficient for binding to β-catenin and for inhibiting β-catenin signaling activity.

To begin to understand the biological significance of Cby homodimerization, we assessed the subcellular localization of Cby mutants, using C-terminally Flag-tagged Cby variants (Cby-Flag). These constructs were transfected into COS7 cells and stained with anti-Flag antibody. C-terminally Flag-tagged Cby was used because it showed complete nuclear localization, most likely due to a conformational change that exposes the C-terminal NLS in contrast to the cytoplasmic and nuclear localization of N-terminally Flag-tagged CbyWT [61] or untagged CbyWT (data not shown). Cby mutants, CbyL77A-Flag and Cby L91A-Flag that were capable of self-interaction, displayed nuclear localization. Notably, the dimerization-deficient mutant CbyL77A/L91A-Flag exhibited increased cytoplasmic localization (Figure 3.3-3). To confirm these

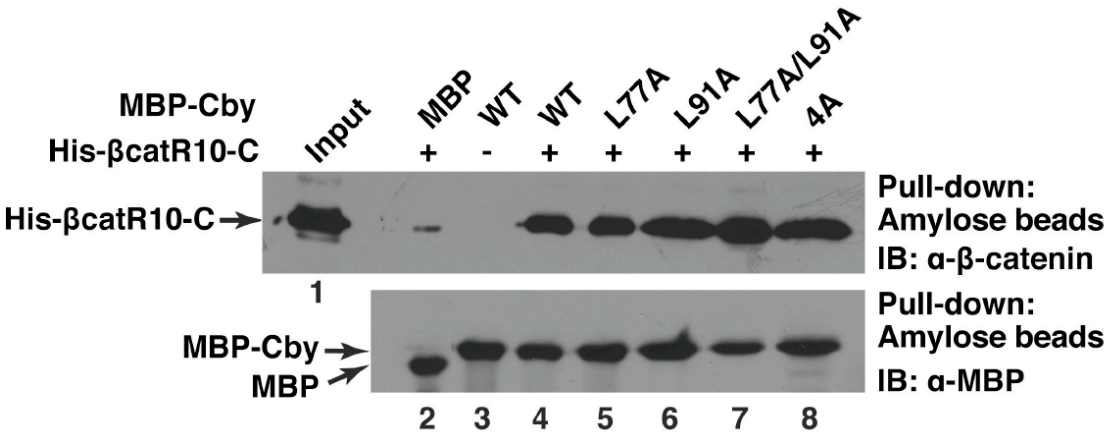
results, the subcellular localization of N-terminally Flag-tagged Cby (Flag-Cby) was evaluated. Consistent with previous observation, Flag-CbyWT localized primarily to the cytoplasm or both nucleus and cytoplasm, especially in cells that have high levels of Cby expression (Figure 3.3-4). In contrast, Cby mutant defective in dimerization, Flag-CbyL77A/L91A showed a subtle yet significant shift towards cytoplasmic localization. This trend became more obvious when cells were treated with LMB, a potent inhibitor of the nuclear export receptor CRM1 [113-115]. LMB treatment caused enrichment of Flag-Cby WT in the nucleus, but a significant fraction of Flag-CbyL77A/L91A was still found in the cytoplasmic compartment. Similar results were observed with C-terminally Flag-tagged Cby proteins (Figure 3.3-5). Even after LMB treatment, a significant fraction of CbyL77A/L91A-Flag was still found in the cytoplasmic compartment. These results suggest that there is a defect in nuclear import of Cby dimerization-deficient mutants.

Our recent data suggest that the C-terminal classical NLS of Cby physically interacts with the nuclear import receptor, importin-α3 (F.-Q. Li *et al*., manuscript under revision). In order to explore the molecular basis underlying the nuclear import defect of Cby dimerization defective mutants, interactions between Cby mutants and importin-α3 were investigated using *in vitro* binding assays. Bacterially expressed and purified MBP, MBP-CbyWT and MBP-Cby mutants were incubated with GST-importin-α3 and pulled down using amylose resin. After extensive washing, the bound proteins were separated on an SDS-PAGE and immunoblotted with anti-GST antibody. As shown in Figure 3.3-6, Cby

single point mutants L77A and L91A interacted with importin-α3, albeit at a reduced interaction compared with CbyWT. On other hand, the dimerization-deficient CbyL77A/L91A exhibited a significant reduction in its ability to interact with importin-α3. Each pull-down sample was run on a separate SDS-PAGE and immunoblotted with anti-MBP antibody, showing that similar amounts of MBP-Cby proteins were pulled down.

Collectively, these data suggest that Cby homodimerization is required for its efficient nuclear import. In the cytoplasm, Cby assembles into dimers through its coiled-coil domain, thus it is able to interact efficiently with importin-α proteins and translocates into the nucleus, whereas Cby dimerization-deficient mutants are compromised for their interaction with importin-α proteins and thus migrate into nucleus less efficiently (Figure 3.3-7).

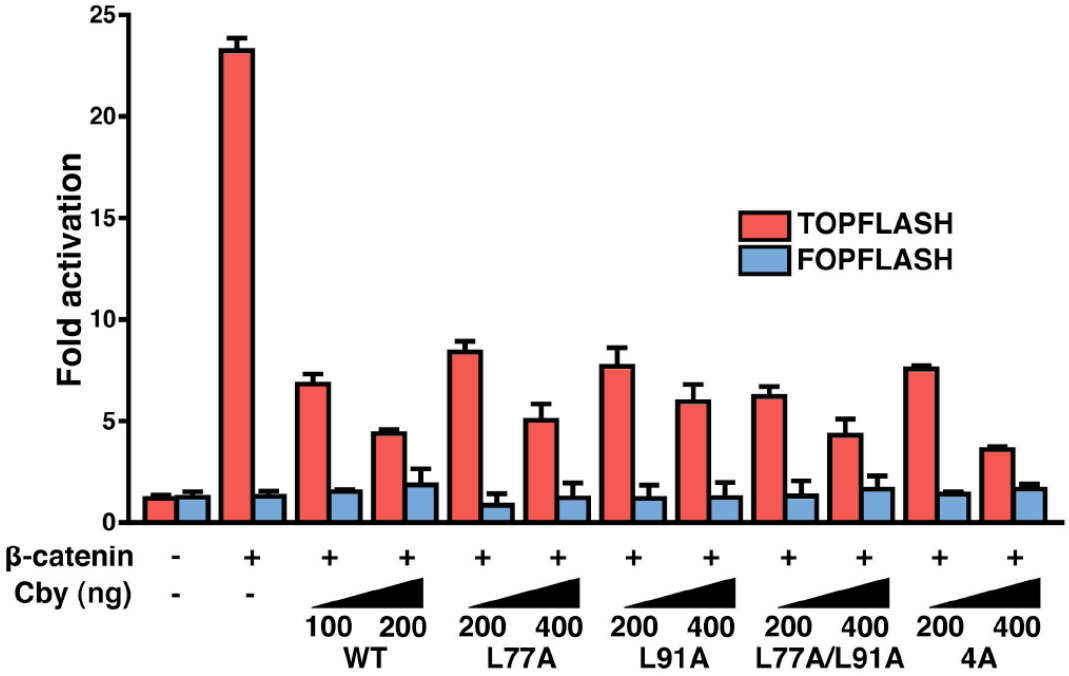
Figure 3.3-1



In vitro binding assays of Cby coiled-coil point mutants and β-catenin. Binding of Cby point mutants to β-catenin was evaluated by MBP pull-down assays. Bacterially expressed and purified wild-type or mutant MBP-Cby fusion proteins was incubated with bacterially expressed and purified His-β-catenin R10-C, pulled down with amylose resin and followed by Western blotting with anti-β-catenin antibody or anti- MBP antibody.

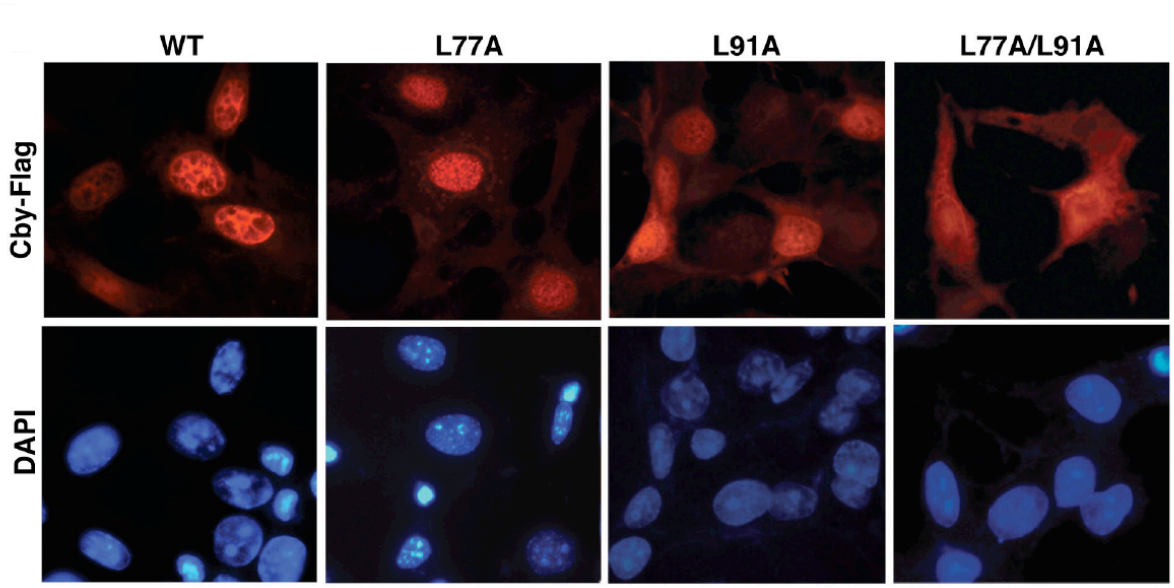
Figure 3.3-2

The effect of Cby's coiled-coil point mutants on TOPFLASH activity.



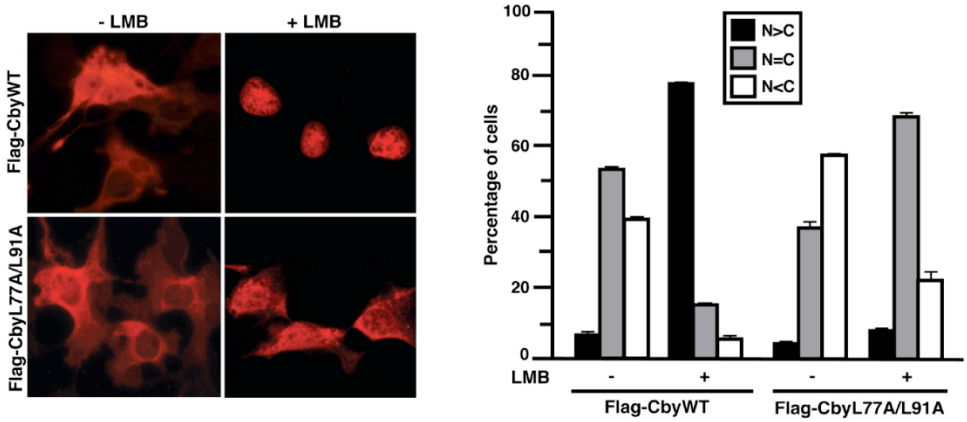
The ability of Cby mutants to repress *β*-catenin signaling was tested by TOPFLASH assays. HEK293T cells were transfected with 60 ng of TOPFLASH or mutant FOPFLASH luciferase reporter, with or without 40 ng of an expression vector for stabilized β-catenin (β-catenin-Myc), and the indicated amounts of a Flag-tagged Cby expression vector. Luciferase activity was measured 24 hours post-transfection, and normalized to Renilla luciferase activity used as an internal control. Transfections were carried out in triplicate and the means ± SD are shown. Note that, to compensate protein levels, higher amounts of plasmid DNA for the Cby mutants were used for transfection.

Figure 3.3-3



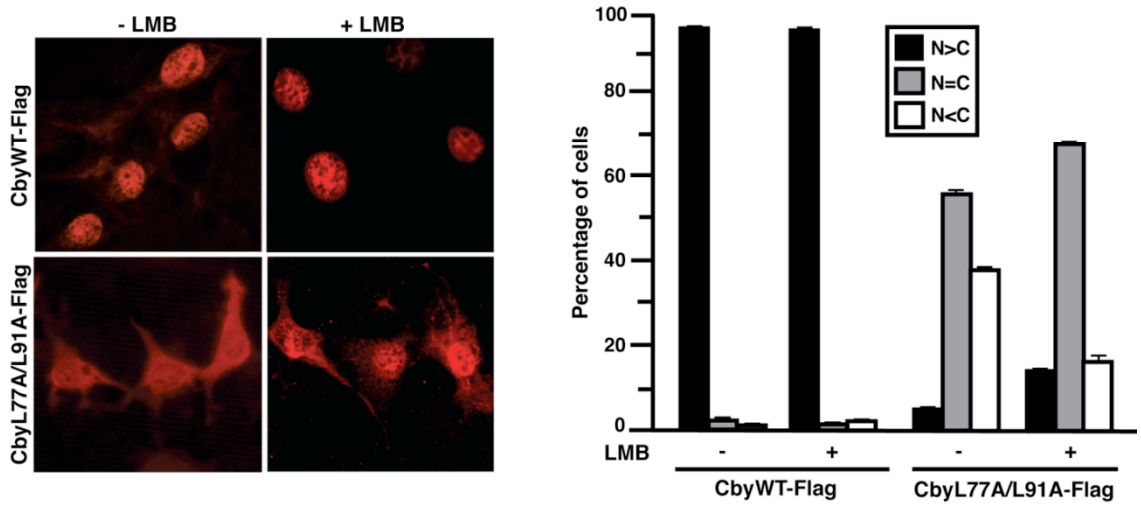
Cby dimerization-deficient mutants localize mainly in the cytoplasm.
COS7 cells were transiently transfected with an expression vector encoding C-terminally tagged Cby WT or Cby mutants. Cells were fixed 24 hours after transfection and immunostained with an anti-Flag antibody. Nuclei were stained with DAPI.

Figure 3.3-4



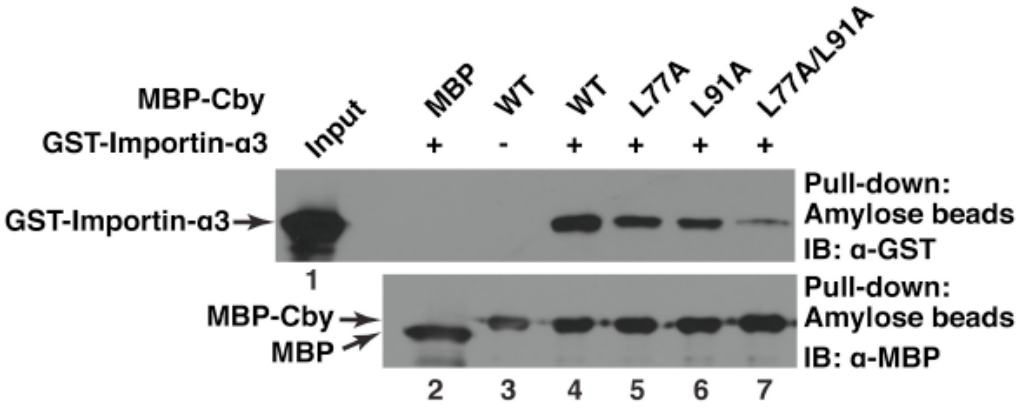
N-terminally Flag-tagged Cby dimerization mutant display reduced nuclear entry. COS7 cells were transiently transfected with an expression vector for N-terminally flagged Cby WT (Flag-CbyWT) The cells were exposed to methanol (control) or 40 nM LMB 17 hours post transfection before fixation and immunostained with an anti-Flag antibody (left panel). Quantitative analysis of the subcellular localization of CbyWT or mutant. Subcellular localization 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.

Figure 3.3-5



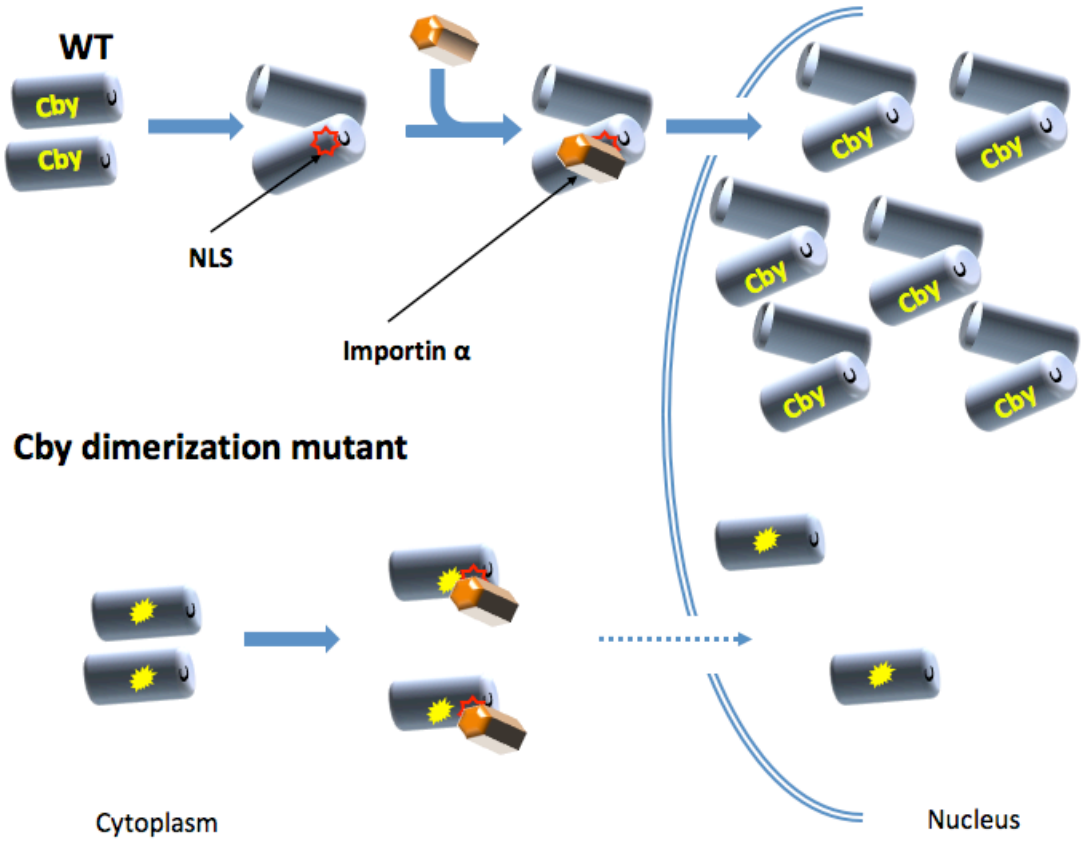
C-terminally Flag-tagged Cby dimerization mutant display reduced nuclear entry. COS7 cells were transiently transfected with an expression vector for C-terminally flagged Cby WT (CbyWT-Flag) The cells were exposed to methanol (control) or 40 nM LMB 17 hours post transfection before fixation and immunostained with an anti-Flag antibody (left panel). Quantitative analysis of the subcellular localization of CbyWT or mutant. Subcellular localization 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.

Figure 3.3-6



Cby dimerization-deficient mutant interact weakly with importin-α3. Binding of Cby point mutants to Importin α3 was evaluated by MBP pull-down assays. Bacterially expressed and purified wild-type or mutant MBP-Cby fusion protein was incubated with purified GST-importin α3, pulled down with amylose resin and followed by Western blotting with anti GST antibody or anti-MBP antibody.

Figure 3.3-7



Model for Cby dimerization and nuclear import. In the cytoplasm, Cby assemble into dimers, thereby constituting a fully functional NLS or unmasking an NLS that interacts with importin-α proteins for efficient nuclear import. Dimerization-deficient Cby mutants are compromised for its interaction with importin-α proteins and thus migrate into the nucleus less efficiently.

Chapter 4: General Discussion

The canonical Wnt/ β-catenin signaling pathway is essential for numerous cellular processes during embryonic development, tissue homeostasis and tumorigenesis. Upon Wnt binding to its receptors, β-catenin, the central component of this pathway enters the nucleus and acts as a transcriptional coactivator to upregulate target gene expression. Cby is an evolutionarily conserved protein that directly interacts with the C-terminal activation domain of β-catenin [58]. Cby antagonizes β-catenin signaling activity by competing with Tcf/Lef family of transcription factors for binding to β-catenin and then in concert with 14-3-3 proteins acts to relocate β-catenin into the cytoplasm [61]. Cby has been shown to interact with itself through its C-terminal region, which contains a putative coiled-coil motif using the yeast two-hybrid system [85]. However the precise residues required for the Cby self-interaction and the biological significance of this interaction has remained unknown.

Coiled-coil domains have been shown to be necessary and sufficient for mediating dimerization in a variety of proteins. The ability to form dimers has been demonstrated to have an impact on their function. Coiled-coil domain mutants usually lose oligomerization and are unable to carry out their function. Mixed lineage kinase-3 (MLK-3) is a mitogen-activated kinase kinase kinase that mediates stress-activating protein kinase (SAPK)/c-Jun kinase activation [101]. MLK-3 has a leucine/isoleucine zipper coiled-coil motif required for

homodimerization, which is a prerequisite for its autophosphorylation [101]. Leucine zipper mutants failed to form homodimers, were unable to undergo autophosphorylation and did not activate a downstream target, SAPK. In essence, MLK-3 forms dimers, followed by activation by autophosphorylation and then in turn it is able to phosphorylate and activates its downstream targets [101].

Quiescent cell proline dipeptidase (QPP), isolated from human T-cells is an intracellular serine protease that cleaves dipeptides off the N-terminal region of proteins when the penultimate amino acid is a proline or an alanine [100, 116]. Active QPP, a 58-kDa protein was eluted from a gel filtration column at a molecular weight of 130 kDa, indicating that it could form a homodimer. A leucine zipper coiled-coil motif N-terminal to its catalytic region was found to be responsible for dimerization. Leucine zipper mutants showed a complete lack of enzymatic activities suggesting that homodimerization is important for QPP proteolytic function [100].

c-Cbl is a 120-kDa proto-oncogenic, multidomain adaptor protein phosphorylated in response to the stimulation of a broad range of cell surface receptors such as epidermal growth factor (EGF) and participates in the assembly of signaling complexes formed as a result of the activation of various signal transduction pathways [117]. c-Cbl contains a leucine zipper, which mediates homodimerization. A leucine zipper deletion mutant caused a decrease in phosphorylation of Cbl and its association with the EGF receptor, following stimulation with EGF, thus demonstrating the critical role of the leucine zipper in c-Cbl's signaling functions [118]. Thus the leucine zipper enables c-Cbl to

homodimerize and this homodimerization influences c-Cbl's signaling function and affects its associations with other signaling proteins in the cell [118].

Tyrosine hydroxylase is a neuronal enzyme that catalyzes biosynthesis of the catecholamine neurotransmitters and hormones [119]. Rat tyrosine hydroxylase exists as a tetramer in its native state. Mutational analyses performed on bacterially expressed enzyme showed that the leucine zipper motif mediated tetrameric formation. Mutants that convert the enzyme from a tetrameric form to a dimer, led to a reduction in activity of the enzyme [119].

Furthermore, coiled-coil domains have also been shown to be required for proper subcellular localization. Coronin is an actin-binding protein, which plays a role in regulating and organizing actin cytoskeletal network [120]. It localizes to the cell periphery and has been implicated in cytokinesis, cell motility and phagocytosis. It has a coiled-coil domain in its C-terminal region that is evolutionarily conserved. The coiled-coil domain was shown to be necessary and sufficient for dimerization of *Xenopus* coronin (Xcoronin) [99]. Mutations in the coiled-coil motif generated dimerization deficient mutants that failed to localize to the cell periphery, implying that dimerization via its coiled-coil domain is important for proper subcellular localization and function of Xcoronin [99].

Coiled-coil domains have also been implicated in oligomerization of viral envelope glycoproteins of many retroviruses. The envelope glycoprotein of the human immunodeficiency virus type 1 (HIV-1) is responsible for the binding of the virus to the cellular receptor CD4 and participates in the CD4-dependent cell fusion exhibited by HIV [121, 122]. The envelope glycoprotein, gp41 contains a

coiled-coil domain, through which it oligomerizes and this oligomerization is required for viral fusion and viral entry into host cells. Mutational analyses of the coiled-coil domain of gp41 showed that mutants that did not oligomerize were unable to enter host cell or initiate infection [123].

In this study, we have shown that Cby contains a functional coiled-coil motif in its C-terminal region. Cby's coiled-coil motif is evolutionarily conserved from fly to human, implying the biological importance of this domain. Consistent with the presence of the α-helical coiled-coil structure, circular dischroism (CD) studies showed that Cby protein has a high α-helical content [124]. The results presented here show that Cby forms a homodimer through the leucine zipper coiled-coil motif. This complex is highly stable even in the presence of 2 M NaCl or 2 M urea (Figure 3.1-5). The four leucines (L77, L84, L91 and L98) located in position d of the helical wheel are crucial in mediating Cby-Cby interactions (Figure 3.2-3). Alanine substitution of two or more leucines completely abrogated Cby dimerization (Figure 3.2-5 and 3.2-6). Gel filtration and cross-linking data (Figures 3.1-6, 3.1-7 and 3.1-8) suggest that Cby predominantly exists as a dimer under these conditions. However, it is possible that Cby can exist in larger oligomeric state as seen in some other coiled-coil proteins, like tyrosine hydroxylase that forms a tetramer [93, 119]. In order to gain further insights into the molecular basis and functional significance of Cby-Cby interaction, it would be of interest to determine the three- dimensional crystal structure of Cby. However, so far, Cby protein is insoluble and forms inclusion

bodies when expressed in bacterial and baculoviral expression systems (data not shown), therefore impeding further biochemical and structural analyses.

Our results clearly demonstrate that Cby-Cby interaction is not required for binding to β-catenin and for suppressing its signaling activity in TOPFLASH assays (Figures 3.3-1 and 3.3-2). Instead Cby homodimerization is a prerequisite for its efficient binding to importin-α and subsequent nuclear import (Figure 3.3-6). Recent data suggests that Cby contains functional NLS and NES motifs and constantly shuttles between the cytoplasm and nucleus. Thus, it appears that Cby intracellular localization at steady state is determined by a dynamic balance between its nuclear import and export. In this aspect, Cby homodimerization is a prerequisite for its efficient binding to importin-α and subsequent nuclear import.

A variety of studies have shown that dimerization is a prerequisite process for nuclear import. Sterol regulatory element-binding protein 2 (SREBP-2) is a transcription factor of the basic helix-loop-helix-leucine zipper (bHLH-Zip) family. Studies have shown that SREBP-2 interacts directly with importin-β for its nuclear import, without the adaptor protein importin-α [125]. SREBP-2 exists as a dimer and alanine substitution of leucine residues in the leucine zipper coiled-coil motif disrupted dimerization and the mutant protein was unable to enter the nucleus, demonstrating that dimerization is a prerequisite process for nuclear import of SREP-2 by importin-β [90].

Viral IE1A transcription factor is another protein that has shown that dimerization is a prerequisite for nuclear import. A helix-loop-helix (HLH) domain

in the C-terminal region of IE1 mediates homooligomerization [126]. Amino acid substitutions adjacent to this domain disrupted oligomerization, and mutants had impaired nuclear import. Furthermore, a dimerization-deficient mutant when coexpressed with wild-type IE1 also prevented nuclear import of wild-type IE1. Findings suggest a novel nuclear localization element that functions only upon dimerization of IE1. Hence, IE1 has to oligomerize in the cytoplasm before it is imported into the nucleus [127].

Signal transducers and activators of transcription (STATs) are latent transcription factors that are activated by cytokines. When phosphorylated, STATs dimerize and enter into the nucleus, where they activate transcription. Only homodimeric STAT-1 or heterodimeric STAT1-STAT2 is able to bind to importin-α5 [128]. Interestingly, binding of importin-α5 to STAT dimers strictly requires two intact NLS elements, one in each STAT monomer [128].

Therefore, Cby self-assembly might create a fully functional NLS probably by juxtaposing the NLS of each monomer, allowing its efficient binding to importin-α and subsequent nuclear import. Alternatively, Cby dimerization may induce a conformational change that unmasks the NLS.

Cby dimerization-deficient mutants are capable of binding efficiently to β-catenin, and repressing its signaling activity to a similar extent as CbyWT, despite the fact that these mutants localize mainly in the cytoplasm. This is consistent with the dual mechanism model by which Cby inhibits β-catenin signaling activity [61]. In the nucleus, Cby competes with Tcf/Lef transcription factors for binding to β-catenin and then Cby in conjunction with 14-3-3 proteins,

relocates β-catenin to the cytoplasm. Thus monomeric Cby is able to inhibit β-catenin signaling perhaps by trapping β-catenin in the cytoplasm. Alternatively, since Cby dimerization-deficient mutants are able to enter the nucleus at reduced rate, so a small portion of nuclear Cby might be sufficient to repress β-catenin transcriptional activation.

In sum, Cby forms a stable dimer through its C-terminal coiled-coil motif, which consists of a heptad repeat of four conserved leucine residues. These leucines are critical for mediating Cby homodimerization. Cby-Cby interaction is dispensable for inhibiting β-catenin signaling but it is required for efficient nuclear import.

Chapter 5: Future Directions

The data presented here are mostly based on overexpression studies and *in vitro* situations. The *in vivo* significance of Cby homodimerization in inhibiting β-catenin signaling requires further experiments. Activation of the Wnt/β-catenin pathway suppresses adipogenesis and cardiomyocyte differentiation [65, 66]. Consistent with this, Cby has been shown to be required for adipogenesis and for cardiomyocyte differentiation [60, 64]. Ectopic expression of Cby in 3T3-L1 preadipocytes causes spontaneous differentiation into mature adipocytes whereas depletion of Cby by RNAi blocks adipogenesis, implying that Cby promotes adipogenesis in part by inhibiting β-catenin signaling [60].

Mouse 3T3-L1 preadipocytes are used as an *in vitro* model of adipocyte differentiation since they can differentiate into mature fat cells in the presence of FBS with methylisobutylxanthine, dexamethasone and insulin (MDI) [129]. Embryonic stem (ES) cells are known to differentiate into a number of cells types, including beating cardiomyocytes [64]. Cby is ubiquitously expressed during the early stages of ES cell differentiation but its expression is later restricted to cardiomyocytes. Cby overexpression increases cardiac differentiation of murine ES cells while Cby knockdown by RNAi severely impairs the differentiation process [64]. It would be interesting to investigate if Cby dimerization-deficient mutants are able to induce spontaneous adipogenesis of 3T3-L1 cells and

cardiomyocyte differentiation of murine ES cells. The ability of Cby dimerization-deficient mutants to rescue Cby knockdown in Cby RNAi 3T3-L1 cells can also be performed. Since knockdown of endogenous Cby impairs the differentiation of these cells into adipocytes, Cby mutants can be reexpressed to examine if they can rescue adipocyte differentiation. Since TOPFLASH assays were performed in HEK293T cells in which relatively high levels of endogenous Cby is present, TOPFLASH assay can be done in Cby RNAi 3T3-L1 cells to directly test the potential of Cby dimerization-deficient mutants to inhibit β-catenin-mediated activation of TOPFLASH activity without concerns of the activity of endogenous Cby. If Cby homodimerization is not required to inhibit β-catenin-mediated activation, Cby dimerization-deficient mutants would be capable of inducing spontaneous adipogenesis of 3T3-L1 cells and cardiomyocyte differentiation of murine ES cells.

It is possible that Cby might play a role in other cellular contexts apart from the canonical Wnt pathway. Cby has been shown to interact with TC1 and PC2 proteins [77, 85] and seems to modulate their intracellular localization. Cby also interacts with 14-3-3 proteins to relocate β-catenin into the cytoplasm [61], so a role of Cby in modulating subcellular localization of other intracellular proteins can be easily envisioned. Whether Cby relocates PC2 and TC1 through its interaction with 14-3-3 proteins needs to be further illuminated. It might be worthwhile to isolate and identify other Cby-interacting proteins to uncover other uncharacterized roles of Cby.

Besides, β-catenin, the significance of Cby homodimerization in mediating Cby interactions with other binding partners needs to be studied. 14-3-3 proteins assemble into dimers to interact with their targets [71]. It is plausible that 14-3-3 dimers associate with Cby dimers in the nucleus to relocate β-catenin into the cytoplasm. Thus, it would be interesting to determine if Cby homodimerization is required for efficient interaction with 14-3-3 proteins. The C-terminal half of Cby containing the coiled-coil motif is required for the interaction with TC1 [77]. The coiled-coil domain of Cby (amino acids 60-112) is required for interaction with PC2 [85]. The putative coiled-coil domain of TC1 remains to be characterized, while the coiled-coil domain of PC2 (amino acids 769-796) is required for interaction with itself and with polycystin 1 (PC1) [80]. PC1 is the second protein that is also mutated in patients with PKD [80-82]. Formation of PC1-PC2 heterodimers and PC2-PC2 dimers is essential for normal renal tubulogenesis [80]. The coiled-coil domain of PC2 is not necessary for interaction with Cby [85]. Hence the functional significance of Cby homodimerization in its binding to PC2, TC1 and 14-3-3 proteins can be investigated using coimmunoprecipitations and spilt hRluc protein complementation assays. In addition, Cby dimerization-deficient mutants can be coexpressed with PC2 and TC1 to determine if Cby homodimerization is required for modulating localization of its binding partners-PC2 and TC1.

PC2 is essential for proper function of the cilia and localizes to the membrane of motile cilia, suggesting that it might be important for cilia function [84, 130]. Cilia are membrane-bounded, centriole-derived projections and play

critical roles in cell motility, development and sensory perception [130, 131]. Motile cilia are important in clearing mucus and debris from airways and defects in motile cilia lead to a wide spectrum of abnormalities including respiratory infections [132]. Cby KO mice, among other phenotypes, bear striking similarities to primary ciliary dyskinesia (PCD) [132]. PCD represents a set of human diseases caused by mutations in ciliary proteins leading to immotile or discoordinated movement, resulting in impaired mucociliary clearance [130]. Consistently, Cby KO mice develop rhinitis and sinusitis and are unable to clear bacteria from their nasal cavity, when challenged with *Pseudomonas aeruginosa* isolates and show a complete absence of mucociliary transport caused by a marked paucity of motile cilia in the nasal epithelium [132]. Ultrastructural experiments revealed impaired docking of basal bodies to the apical membrane in Cby KO mice. Since cilia extend from basal bodies, the compromised basal body docking seem to explain defective ciliogenesis in Cby KO mice [132]. Furthermore, endogenous Cby was shown to localize to the base of the cilia in cultured MDCK2 cells and in the nasal epithelial tissue, suggesting Cby might play a direct role in regulating cilia formation and/or function [132].

C. elegans Dyf-11 encodes an evolutionarily conserved protein that is required for cilium biogenesis [131]. It is expressed in most ciliated neurons and was shown to localize to the cilia. Dyf-11 mutants exhibit stunted cilia and rescue experiments indicate that Dyf-11 is required for ciliary formation. Domain analysis using Dyf-11 deletion constructs revealed that the coiled-coil domain is required for proper localization and its function in ciliogenesis [131]. Since Cby

localizes to the cilia and has been implicated in ciliogenesis [132], it would be of great interest to investigate if the coiled-coil domain of Cby is required for its localization at the base of the cilia or its function in ciliogenesis using the Cby coiled-coil mutants.

Cby was also shown to colocalize with the centrosomal marker, γ-tubulin [132]. Oral-facial-digital (OFD) syndromes are a group of developmental disorders that feature malformations of the face, cavity and digits [133]. OFD type 1 (OFD1) is most common and its distinguishing clinical feature, from other OFD syndromes, is the occurrence of cystic renal disease [133]. OFD1 is a core component of the centrosome and colocalizes with γ-tubulin [133]. OFD1 contains 5 coiled-coil motifs. Deletion constructs showed that the coiled-coil domains are critical with at least two being absolutely necessary for centrosomal targeting [133]. More importantly, most reported OFD1 mutations are predicted to cause protein truncation with loss of coiled-coil domains, which leads to loss of centrosomal localization [133]. So the importance of the Cby's coiled-coil motif in targeting Cby to the centrosome needs to be investigated.

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