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Dual Role of Chibby in Regulation of Cell Growth and Mesenchymal-to-Epithelial Transition-like Processes

A Dissertation Presented

by

Victoria Fischer

to

The Graduate School

in Partial Fulfillment of the

Requirements

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Abstract of the Dissertation

Dual Role of Chibby in Regulation of Cell Growth and

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The Wnt/β-catenin signaling pathway is well known for its prominent role in tumorigenesis of colon cancers, but also of some other tumors. β-Catenin, the downstream mediator of canonical Wnt-signaling, is continuously degraded in the absence of Wnt signaling but stabilized upon activation of the pathway. β-Catenin then activates transcription of target genes in the nucleus, leading e.g. to proliferation, invasiveness and anoikis resistance, and can induce epithelial-to-mesenchymal transition (EMT). Apart from its function as a transcriptional coactivator, β-catenin forms part of adherens junctions where it plays a pivotal role mediating the connection between the adherens junction protein E-cadherin and the actin cytoskeleton, and its loss from the membrane entrails reduced cell-cell adhesion. This role contrasts β-catenin's function in the nucleus since formation of E-cadherin-mediated adherens junctions reverses a malignant phenotype, effectuating mesenchymal-to-epithelial transition (MET), in a variety of tumor cell lines.

The small, evolutionarily conserved protein Chibby was initially discovered as β-catenin binding partner. Our lab has shown that Chibby shuttles β-catenin out of the nucleus, in cooperation with

14-3-3 proteins. By this mechanism and by competing with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors for β-catenin binding, Chibby inhibits nuclear β-catenin signaling. We show here that Chibby counteracts both of β-catenin's opposing roles in that

a) Chibby can reduce β-catenin nuclear signaling and cell proliferation in human colon cancer cells bearing stabilized β-catenin by reducing nuclear levels of β-catenin, and that

b) Chibby knock-down leads to increased proteins levels of E-cadherin and β-catenin at the plasma membrane, to the point of inducing mesenchymal-to-epithelial reversion with reduced tumor characteristics in human embryonic kidney and human colon cancer cells, and that this is due at least in part to increased transcription of the *E-cadherin* gene.

These findings are relevant to further development of treatment options for Wnt/β-catenin-dependent tumors.

Dedication Page

This dissertation is dedicated to my husband, Wolfram Fischer, who let me enter the graduate program in full awareness of the consequences to our lifestyle, and not only accepted those consequences, but never tired to encourage me to keep working. His constant support made completion of this dissertation possible.

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List of Abbreviations

Cby	Chibby
Cby sh cells	Cby knock-down cells
CbyWT	Chibby wild-type
Cdc42	Cell division control protein 42
cDNA	complementary deoxyribonucleic acid
CKIa	casein kinase Ia
CRM1	Chromosome region maintenance 1
DAPI	4',6-diamidino-2-phenylindole
DSH	dishevelled protein
EMT	epithelial-to-mesenchymal transition
Fz	Frizzled
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSK3β	glycogen synthase 3β
HEK293	human embryonic kidney cells 293
HRP	horseradish peroxidase
Lef	lymphoid enhancer factor
LRP-5/6	lipoprotein-related proteins 5 and 6
MET	mesenchymal-to-epithelial transition
mRNA	messenger ribonucleic acid
NES	nuclear export signal
NLS	nuclear localization signal
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PP2A	protein phosphatase 2A
RT-qPCR	reverse transcription (followed by) quantitative polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tcf	T-cell factor
ZO-1	zonula occludens protein 1

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importantly, Dr. Susan Ettinger, who did not let up in her encouragement for me to take on a research career. I truly appreciate the kindness and support she has offered me, giving me the opportunity to present at conferences, and creating multiple invaluable memories of exploring in the lab and in the literature, as well as playing Dominos and practicing healthy nutrition, and thereby showing me the beauty of life in research.

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Chapter 1 General Introduction

The canonical Wnt signaling pathway

Wnt signaling counts among the major signaling pathways regulating cellular behavior from early on in development through adult life. Mammals express at least 19 different Wnt ligands that can elicit a response along one of three known downstream signaling pathways (Prosperi and Goss 2010), the Wnt/Calcium pathway, the planar cell polarity pathway and the canonical Wnt signaling pathway. The latter is activated when Wnt-ligand binds to both a Frizzled (Fz) receptor and lipoprotein-related proteins 5 and 6 (LRP-5/6) (Nelson and Nusse 2004). The downstream mediator of the canonical Wnt signaling pathway is β-catenin. In the absence of Wnt signaling, levels of cytoplasmic β-catenin are kept low by continuous degradation of β-catenin. β-Catenin is recruited to the so-called destruction complex, composed of the scaffold protein axin, the tumor suppressor adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), casein kinase Ia (CKIα), and protein phosphatase 2A (PP2A). β-Catenin is sequentially phosphorylated in this complex, first by CK1α at serine 45, then by GSK3β at threonine 41, and serines 37 and 33. This phosphorylation allows recognition by β-TrCP (β-transducin-repeat-containing protein) within an E3 ubiquitin ligase complex, ubiquitination and subsequent degradation by the 26S proteasome (Herbst and Kolligs 2007). UponWnt-ligand binding, β-catenin is no longer phosphorylated and accumulates in the cytoplasm. Additional regulation of β-catenin levels at the level of mRNA has been reported (Bikkavilli and Malbon 2010). It translocates to the nucleus, where it acts as transcriptional coactivator in conjunction with T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factors (Fig. 1-1).

This signaling pathway controls multiple processes during development and in adult life, and the exact processes activated depend on the cellular context (Clevers 2006). In mice, the canonical Wnt-signaling pathway is essential for gastrulation (Moustakas and Heldin 2007) and the establishment of the primary body axis. In adult mammals, Wnt signaling controls, for example, cell fate decisions in the renewal of intestinal epithelial cells, formation of hair, regulation of bone mass and self-renewal of hematopoetic stem cells (Clevers 2006). The signaling pathway is tightly regulated, including autoregulatory mechanisms, in that positive and negative regulators are controlled by the β-catenin/TCF complex (Clevers 2006). However, in accordance with the central role of the Wnt signaling pathway, multiple diseases are associated with dysregulation of this pathway, e.g. idiopathic pulmonary fibrosis marked by proliferating fibroblasts. Malignancies rank high among those diseases, including colorectal adenocarcinomas and hair follicle tumors (Moon, Kohn et al. 2004; Clevers 2006).

Epithelial-to-Mesenchymal and Mesenchymal-to-Epithelial Transitions

One of the processes that canonical Wnt-signaling can elicit is epithelial-to-mesenchymal transition (EMT), a process defined as a phenotypic switch from epithelial to mesenchymal characteristics. These characteristics include reduction of cell-cell adhesion via adherens junctions, tight junctions and desmosomes, loss of apical-basal polarity, replacement of the cortical actin ring by a network of stress fibers, expression of mesenchymal filament protein vimentin, and expression of matrix metalloproteases that ultimately allow degradation of basement membrane and an invasive phenotype (Xie, Law et al. 2003; Lee, Dedhar et al. 2006; Aroeira, Aguilera et al. 2007; Hugo, Ackland et al. 2007).

EMT takes place for example during gastrulation (Moustakas and Heldin 2007) and allows neural crest cell migration (Palacios, Tushir et al. 2005). It is required for formation of pancreatic Langerhans islets, where pancreatic endocrine cells undergo secondary EMT, migrate through the mesenchyme and undergo the reverse process of mesenchymal-to-epithelial transition (MET) at their target location (Johansson and Grapin-Botton 2002). In adult life, EMT and MET are important for wound healing, but EMT also forms part of the pathogenic processes in fibrosis, e.g. renal fibrosis, pulmonary fibrosis or liver cirrhosis, and in carcinogenesis (Thiery, Acloque et al. 2009). In colon carcinomas, for example, EMT has been observed at the invasive front where single migratory cells appear that do not show E-cadherin expression and display nuclear accumulation of β-catenin (Brabletz, Jung et al. 2001). In metastases however, epithelial characteristics are observed, supporting that MET occurs at the site of metastases (Moustakas and Heldin 2007; Chao, Shephard et al. 2010). One concern with this shift back to an epithelial phenotype in metastases is that the re-formed epithelial junctions may restrict access, and thereby reduce efficacy, of drugs or antibodies used to eliminate the tumor cells (Christiansen and Rajasekaran 2006).

Tumorigenesis

Cancer is the second leading cause of death in the US, and although some progress has been made to increase the five-year survival rates for many cancers, the death toll remains high (American Cancer Society 2010). Our current understanding of the disease process reveals a multitude of pathways and causes for the pathologic process but the disease progression shares common properties among the many types of cancer. The disease is marked by uncontrolled proliferation of cells. In the case of carcinomas, cancers arising from epithelial cells, the tumor of proliferating cells is considered malignant, cancerous, when it breaks through a basement membrane and invades adjoining tissues. Tumor cells may additionally leave the primary tumor site and establish new daughter tumors in different tissues, metastases (Thiery 2002; Hanahan and Weinberg 2011). Several properties of malignant tumors add particular difficulty to the task of eliminating tumor cells in attempts to cure the disease. Firstly, the tumor is composed of and supported by multiple cell types, not all of which proliferate rapidly. Specifically so-called tumor stem cells are difficult to target because they are quiescent but have the propensity to become

active and cause recurrence of the disease, secondly, malignant tumors metastasize to new host tissues where they are difficult to detect and may acquire more mutations making them sufficiently different from the primary tumor to escape treatment (Weinberg 2007)(esp. chapter 16).

In order to form malignant, metastatic tumors, cells have to acquire numerous properties that enable them to evade the multiple checks and balances in existence to coordinate growth and homeostasis of tissues. These properties include, though they are not restricted to, the ability for unlimited proliferation and evasion of cell death and growth suppressing signals as well as the abilities to invade adjoining tissues, which requires degradation of extracellular matrix, and the ability to survive as single cells or small groups of cells, traveling to new target sites and establishing new growth sites in distant host tissues (Thiery 2002; Kalluri 2009; Hanahan and Weinberg 2011). Many of the acquired characteristics of tumor cells can be a result of dysregulated canonical Wnt-signaling (Clevers 2006). It is estimated that 90% of spontaneous colon carcinomas are caused by aberrant Wnt-signaling (Herbst and Kolligs 2007), mediated by stabilized β-catenin (Clevers 2006). Such stabilization can be achieved by various mutations. Most frequent are mutations in APC that prevent the phosphorylation-dependent degradation of β-catenin, but mutations in axin and β-catenin itself also occur (Clevers 2006).

E-cadherin

E-cadherin is a type I cadherin, a single transmembrane protein of 120kD in its mature form (Hinck, Nathke et al. 1994) that binds several other proteins to exert its functions. It binds β-catenin early in biosynthesis in the endoplasmic reticulum (Nakamura, Hayashi et al. 2008) and is transported to the plasma membrane via the Rab11-positive recycling endosome (Lock and Stow 2005). At the plasma membrane, it forms homodimers and binds to the extracellular domain of E-cadherin homodimers of adjacent cells, in a calcium-dependent manner (Wheelock and Johnson 2003), to form adherens junctions. Once an initial contact is established, E-cadherin dimers form clusters that are more stable, mature adherens junctions. Intracellularly, E-cadherin binds p120 catenin, and β-catenin or γ-catenin, either of the latter linking the adherens junction to the actin cytoskeleton by binding α-catenin (Aberle, Butz et al. 1994), which provides an indirect connection to the actin filaments (Wheelock and Johnson 2003; Cavey and Lecuit 2009).

E-cadherin is constantly turned over at the plasma membrane, being internalized and recycled to the membrane, or lysosomally degraded (D'Souza-Schorey 2005; Palacios, Tushir et al. 2005; Ulrich, Krieg et al. 2005; Yap, Crampton et al. 2007; Shen, Hirsch et al. 2008; Hong, Troyanovsky et al. 2010), with an estimated half life of 2-5 hours at the membrane (Hinck, Nathke et al. 1994; Gottardi and Gumbiner 2004), though other experiments suggest a much shorter half-life (Hong, Troyanovsky et al. 2010). Several mechanisms have been described by which internalization can be achieved, via the endosomal system (Bryant and Stow 2004) in a clathrin-dependent (Xiao, Oas et al. 2007) or independent manner (Paterson, Parton et al. 2003), or by macropinocytosis (Bryant, Kerr et al. 2007). This recycling mechanism allows for

plasticity of adherens junctions required for fine-tuning of adhesion and morphology (Bryant and Stow 2004). There is some evidence that E-cadherin not engaged in adherens junctions is recycled more frequently (Yap, Crampton et al. 2007; Hong, Troyanovsky et al. 2010).

E-cadherin is regulated with respect to its plasma membrane localization in various ways. Its immediate binding partner p120 catenin is thought to regulate adherens junctions positively and negatively (Oyama, Kanai et al. 1994; Bryant, Kerr et al. 2007; Xiao, Oas et al. 2007; Yap, Crampton et al. 2007).Tyrosine-phosphorylation and ubiquination of E-cadherin, mediated by the ubiquitin ligase Hakai are essential for its sorting to the lysosome (zum Bueschenfelde, Hoschuetzky et al. 2004; Palacios, Tushir et al. 2005). Adhesive activity is additionally regulated by N-glycosylation of E-cadherin (Zhou, Su et al. 2008), affecting tyrosine-phosphorylation of β-catenin (Zhao, Liang et al. 2008). Notably, loss of β-catenin leads to internalization and degradation of E-cadherin (Miyashita and Ozawa 2007). E-cadherin binding affinity to β-catenin can be affected by phosphorylation of either molecule. E-cadherin tyrosine-phosphorylation leads to enhanced β-catenin binding (Wheelock and Johnson 2003; Gottardi and Gumbiner 2004), while β-catenin phosphorylation at tyrosine 654 reduces its affinity for E-cadherin. Known regulators of adherens junction stability include Cdc42, atypical protein kinase C and Par6 (Cavey and Lecuit 2009). Apart from regulation of E-cadherin's stability at the membrane, regulation at the transcriptional level is expectedly a mechanism to control expression and function of the protein (Peinado, Portillo et al. 2004). The *cdh1* gene that codes for E-cadherin is subject to complex regulation. Among the known gene repressors are snail and slug, which are themselves gene targets of Wnt signaling.

E-cadherin's function at the plasma membrane is not merely restricted to establishment of physical contact with adjacent cells though. Formation of E-cadherin-mediated adherens junctions triggers subsequent formation of other cell-cell junction types, specifically tight junctions, gap junctions and desmosomes (Gottardi, Wong et al. 2001). Additionally, E-cadherin is required for polarity establishment and cilium formation (Wheelock and Johnson 2003; Maher, Flozak et al. 2009) and effectively causes epithelialization of the cell (Moustakas and Heldin 2007). It activates additional signaling pathways, participating in the regulation of migration, proliferation (Perrais, Chen et al. 2007) and differentiation (Theard, Raspe et al. 2008). Binding of β-catenin by E-cadherin is required for inhibition of invasion (Wong and Gumbiner 2003). Formation of adherens junctions leads to rearrangements of actin and microtubule cytoskeletons (Desai, Gao et al. 2009). Interestingly, binding of E-cadherin to β-catenin decreases β-catenin signaling, even when cytosolic and nuclear levels β-catenin are not decreased, possibly because E-cadherin and Tcf/Lef transcription factors bind the same pool of β-catenin (Gottardi, Wong et al. 2001). Accordingly, E-cadherin is involved in MET-processes, e.g. during kidney morphogenesis during development (Peinado, Portillo et al. 2004).

These functions may explain E-cadherin's prominent role as tumor suppressor (Wheelock and Johnson 2003). Loss of E-cadherin correlates with a poor prognosis for the patient (Syed, Mak et al. 2008) and is thought to be a decisive, though not irreversible event during tumor

progression (Peinado, Portillo et al. 2004). While loss of E-cadherin correlates with increased invasiveness and metastasis (Wheelock and Johnson 2003*)*, E-cadherin reexpression is observed in metastatic lesions (Natalwala, Spychal et al. 2008). In agreement with these observations, forced expression of E-cadherin reduces proliferation, migration and invasion of tumor cells in *in vitro* assays (Oyama, Kanai et al. 1994; Hirohashi 1998; Gottardi, Wong et al. 2001; Nawrocki-Raby, Gilles et al. 2003; Seidel, Braeg et al. 2004), underlining E-cadherin's importance in the disease process.

β-Catenin

β-catenin is a 781 amino acid protein characterized as a proto-oncogene (Herbst and Kolligs 2007). It contains 12 armadillo repeats (residues 138-664) that are required for interaction with multiple proteins that define β-catenin's diverse functions at the cell membrane, in the nucleus and at the centrosome (Fuentealba, Eivers et al. 2008; Xing, Takemaru et al. 2008). Binding with cadherins, APC and Tcf/Lef transcription factors takes place at β-catenin's armadillo repeats, in overlapping regions (Sadot, Simcha et al. 1998). The N-terminal and C-terminal domains of β-catenin are less structured and enable interactions with a different set of proteins. The N-terminus interacts, for example, with α-catenin, enabling β-catenin to connect the adherens junction protein E-cadherin with α-catenin as a key regulator of the actin cytoskeleton in a dynamic manner (Drees, Pokutta et al. 2005; Yamada, Pokutta et al. 2005). The first armadillo repeat is required for binding BCL-9 in the nucleus where β-catenin functions as transcriptional coactivator with Tcf/Lef transcription factors. BCL-9 then recruits an additional coactivator, Pygopus (Kramps, Peter et al. 2002) to allow Wnt-signaling mediated gene transcription. The C-terminal domain, starting in armadillo repeat 11, interacts with various transcriptional coactivators, but also with inhibitors like Cby (Takemaru, Yamaguchi et al. 2003).

In addition to its function as a transcriptional coactivator, an apparently contradictory function is known for β-catenin. It binds to E-cadherin at adherens junctions where it contributes to the connection between adherens junctions and actin cytoskeleton, and contributes to epithelialization of cells even independent of E-cadherin's adhesive function (Gottardi, Wong et al. 2001) (see E-cadherin above). Furthermore, β-catenin is found at the mother centriole, in a form phosphorylated at serines 33 and 37 (Fuentealba, Eivers et al. 2008) where is involved in spindle separation during mitosis (Davidson, Shen et al. 2009). It is possible that this N-terminally phosphorylated β-catenin additionally localizes to the basal body of the primary cilium because of the centrosme's function as the proteasomal center of the cell (Fuentealba, Eivers et al. 2008).

Given the diverse and partially contradictory functions of β-catenin, contributing to an epithelial phenotype at the plasma membrane on one side but mediating Wnt-signaling that can induce EMT on the other, regulation of β-catenin is crucial. Numerous regulating modifications of β-catenin have been described, mainly in the form of phosphorylations. Cytoplasmic levels of

β-catenin are regulated, depending on presence or absence on Wnt-signaling, by phosphorylation of β-catenin at serines 33, 37, 45 and at threonine 41 (see Wnt-signaling). β-catenin not phosphorylated at serines 33, 37 and threonin 41 is transcriptionally active (Maher, Flozak et al. 2009). While β-catenin unphosphorylated at serine 33 and 37, thus potentially signaling active, can bind E-cadherin (Gottardi and Gumbiner 2004; Maher, Mo et al. 2010), binding of β-catenin with E-cadherin can be regulated by phosphorylation at tyrosine 654 of β-catenin where tyrosine 654 phosphorylation reduces interaction with E-cadherin (Castano, Raurell et al. 2002). Additionally, tyrosine-142 phosphorylation, e.g., precludes β-catenin interaction with α-catenin at adherens junctions but enables interaction with BCL9, promoting transcriptional function and inhibiting adhesive function of β-catenin (Brembeck, Schwarz-Romond et al. 2004). Activation of tyrosine kinases has been shown to mediate loss of cadherin-mediated cell-cell adhesion by release of β-catenin into the cytoplasm or by activating cadherin endocytosis (Hirohashi 1998; Nelson and Nusse 2004), and tyrosine kinase inhibitor imatinib downregulates β-catenin-mediated signaling in colon cancer cells (Herbst and Kolligs 2007), in accordance with the notion that binding of β-catenin by E-cadherin reduces β-catenin's nuclear activity. These examples demonstrate the complexity of the regulation of β-catenin's functions in the cell by post-tranlational modifications. Additional regulation of β-catenin's functions is achieved by interacting proteins, including Cby.

Chibby

The evolutionarily conserved protein Chibby (Cby) was discovered as a Wnt/β-catenin signaling inhibitor protein, binding to the transactivation domain of β-catenin at β-catenin's C-terminus (Takemaru, Yamaguchi et al. 2003). Cby is a small protein of 126 amino acids with a C-terminal coiled-coil domain. This coiled-coil is involved in homodimerization of Cby that enables interaction with importin-α and thereby entry into the nucleus (Mofunanya, Li et al. 2009). Cby furthermore harbors a NLS at residues 123-126, and a functional NES at residues 21-29 that are necessary for entry into and exit from the nucleus, respectively (Li, Mofunanya et al. 2010). Among several predicted phosphorylation sites, serine 20 has been shown functionally relevant, as phosphorylation at this site is required for binding to 14-3-3 chaperone proteins (Li, Mofunanya et al. 2008).

Functionally, Cby reduces canonical Wnt signaling in various settings. Cby facilitates differentiation of embryonic stem cells into cardiomyocytes in mice ES cells (Singh, Li et al. 2007), and promotes differentiation of pre-adipocytes into adipocytes by inhibition of Wnt/β-catenin-signaling (Li, Singh et al. 2007). Mechanistically, Cby inhibits β-catenin-mediated transcriptional activation by competing with Tcf/Lef transcription factors for β-catenin binding (Takemaru, Yamaguchi et al. 2003) and by shuttling β-catenin out of the nucleus in cooperation with 14-3-3 proteins. This function depends on phosphorylation of Cby in the serine-20 position (Li, Mofunanya et al. 2008) (Fig. 1-2).

Recently, a new function of Cby in ciliogenesis has been defined. Cby localizes to the basal body, from which cilia emerge. In Cby knock-out mice, defects in ciliogenesis are observed, leading to severe defects in mucociliary transport in nasal passages in mice (Voronina, Takemaru et al. 2009). In this respect, Cby is required for protein transport into and out of cilia in *Drosophila melanogaster* (Enjolras, Thomas et al. 2012).While β-catenin localizes to the base of cilia as well (Fuentealba, Eivers et al. 2008), there is no evidence at this point that this function of Cby is linked to its antagonistic effect on β-catenin, but it may be connected to a function of Cby at the golgi, where it colocalizes with golgi matrix component GM130 (Hidaka, Koenecke et al. 2004), and interacts with polycystin-2, a protein that ultimately localizes to the primary cilium (Hoffmeister, Babinger et al. 2011). Since primary cilia are considered essential for transduction of Wnt signaling (Hoffmeister, Babinger et al. 2011), an indirect effect of Cby on Wnt signaling appears possible, where loss of Cby would lead to reduced signaling, due to defects in the primary cilium, and is subject to further research.

The present project investigates a novel function of Cby at the plasma membrane using various molecular and cellular biology techniques. Despite the fact that β-catenin has been known to form a tight complex with the cytoplasmic domains of membrane-bound E-cadherin at the adherens junctions more than a century ago (Huber, Stewart et al. 2001), it remains largely unclear how β-catenin dissociates from E-cadherin, and what the underlying mechanisms of cytoslic accumulation of β-catenin are. Recently, we found that knockdown or overexpression of Cby dramatically alters the cell shape of human embryonic kidney (HEK) 203 cells, as well as SW480 human colon cancer cells. Cby knock-down cells become flat and show increased cell-cell contact whereas Cby overexpressing cells display rounded cell shape and appear to have decreased cell-cell adhesion. Additionally, in SW480 colon cancer cells, Cby overexpression mediates significantly increased viable floating cells at confluence. Given that β-catenin also resides at the plasma membrane and functions in cell adhesion, we examined if these phenotypes are linked with alteration of β-catenin localization at the plasma membrane and adherens junctions. Fluorescent staining shows that in Cby knock-down cells, β-catenin is largely enriched at the plasma membrane. Accordingly, cells overexpressing Cby show a more diffuse distribution of β-catenin throughout the cytoplasm in both human breast adenocarcinoma MCF7 cells and Canine Kidney MDCKII cells. These data suggest that Cby might also present at the plasma membrane, and Cby binding may cause dissociation of β-catenin from membrane-bound E-cadherin, thereby contributing to the cytosolic accumulation of β-catenin. Taken together, my research demonstrates a dual role of Cby in regulation of cell growth and mesencymal-to-epithelial transitions showing that Cby overexpression reduces growth in colon cancer cells by reducing β-catenin-mediated nuclear signaling, yet that loss of Cby leads to MET-like changes, marked by increased levels of E-cadherin and changes in cell behavior consistent with epithelialization, in two different cell lines.

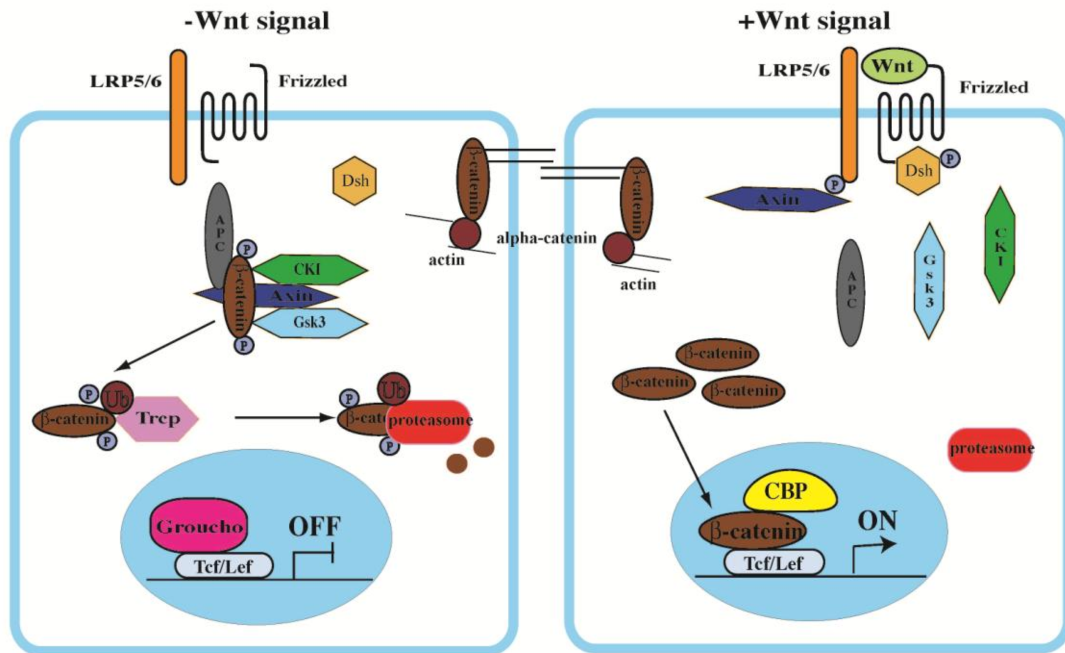


Figure 1-1 The canonical Wnt signaling pathway

A. In the absence of Wnt signaling (“-Wnt signal”), cytoplasmic β-catenin is continuously degraded subsequent to phosphorylation in the destruction complex.
B. Upon Wnt-ligand binding (“+Wnt signal”), β-catenin is stabilized, translocates to the nucleus and activates gene transcription. See text for more details. DSH: disheveled

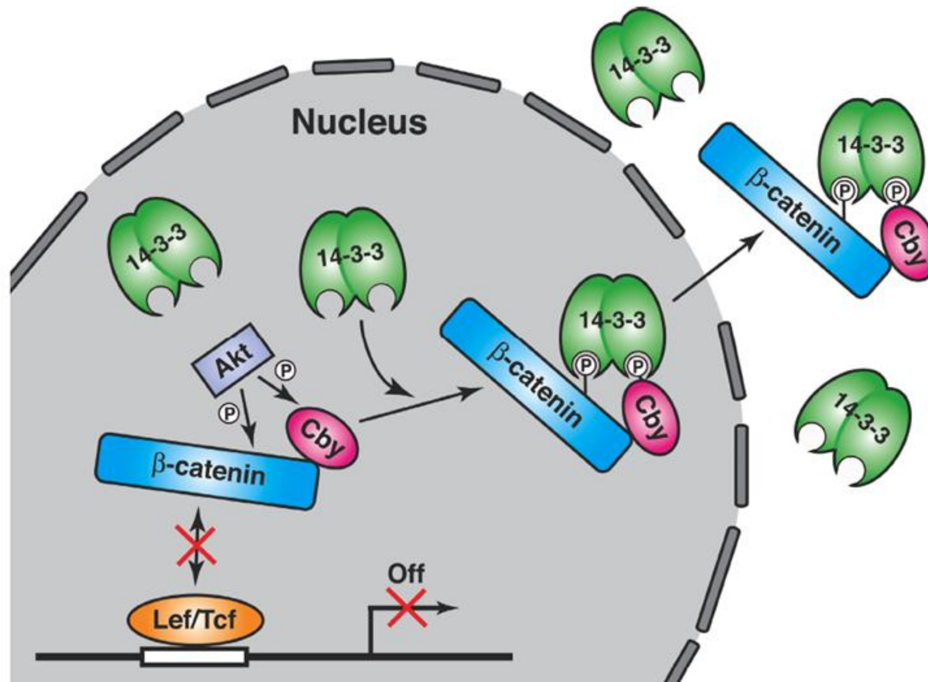


Figure 1-2 Dual mechanism model for inhibition of β-catenin signaling activity by Cby

Cby reduces nuclear β-catenin signaling by competing for β-catenin binding with Tcf/Lef transcription factors and by shuttling β-catenin out of the nucleus in a tripartite complex with 14-3-3 proteins. See text for details.

(Li, Mofunanya et al. 2008), with permission

Chapter 2 Chibby suppresses growth of human SW480 colon adenocarcinoma cells through inhibition of β-catenin signaling

This chapter has been accepted by the Journal of Molecular Signaling. The majority of the data were produced by Victoria Fischer. The stable cell lines and the first figure were generated by Dr. Feng-Qian Li. Co-author Dex-Anne Brown-Grant assisted with experiments for the revision.

Abstract

The canonical Wnt signaling pathway is crucial for embryonic development and adult tissue homeostasis. Activating mutations in the Wnt pathway are frequently associated with the pathogenesis of various types of cancer, particularly colon cancer. Upon Wnt stimulation, β-catenin plays a central role as a coactivator through direct interaction with Tcf/Lef transcription factors to stimulate target gene expression. We have previously shown that the evolutionarily conserved protein Chibby physically binds to β-catenin to repress β-catenin-dependent gene activation by 1) competing with Tcf/Lef factors for binding to β-catenin and 2) facilitating nuclear export of β-catenin via interaction with 14-3-3 proteins. In this study, we employed human colon adenocarcinoma SW480 cells with high levels of endogenous β-catenin to address a potential tumor suppressor role of Cby. In SW480 stable cells expressing wild-type Cby (CbyWT), but not 14-3-3-binding- defective Cby mutant CbyS20A, a significant fraction of endogenous β-catenin was detected in the cytoplasm. Consistent with this, CbyWT-expressing cells showed low levels of β-catenin signaling activity, leading to reduced growth. Our results suggest that Cby, in collaboration with 14-3-3 proteins, can counteract oncogenic β-catenin signaling in colon cancer cells.

Background

The canonical Wnt/β-catenin signaling pathway is highly conserved throughout evolution and plays diverse roles in embryonic development and adult homeostasis by regulating cell proliferation, cell fate decisions, as well as stem cell maintenance and self-renewal (Klaus and Birchmeier 2008; Cadigan and Peifer 2009; MacDonald, Tamai et al. 2009; van Amerongen and Nusse 2009). β-Catenin serves as a key transcriptional coactivator for transducing canonical Wnt signals from the cell surface to the nucleus. In the absence of Wnt ligands, cytoplasmic β-catenin becomes sequentially phosphorylated by casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3) in the so-called "destruction complex" containing the tumor suppressors Axin and APC, and then targeted for ubiquitin-mediated proteasomal degradation (Kimelman and Xu 2006; Cadigan and Peifer 2009; MacDonald, Tamai et al. 2009). Wnt binding to the Frizzled (Fz)

receptors and LRP5/6 co-receptors leads to inhibition of β-catenin phosphorylation, resulting in stabilization of β-catenin protein. Subsequently, β-catenin translocates into the nucleus where it forms a complex with the Tcf/Lef transcription factors to stimulate expression of direct target genes such as cyclin D1 (Willert and Jones 2006; Takemaru, Ohmitsu et al. 2008; Mosimann, Hausmann et al. 2009).

Sustained activation of Wnt/β-catenin signaling, due to loss-of-function mutations in APC or Axin or gain-of-function mutations in β-catenin, has been linked to various human malignancies including melanoma and colon and hepatocellular carcinomas (Polakis 2000; Pinto and Clevers 2005; Klaus and Birchmeier 2008; Takemaru, Ohmitsu et al. 2008). Notably, greater than 70% of colon cancers exhibit elevated Wnt/β-catenin signaling activity. Mutations in APC or Axin compromise their function within the β- catenin destruction complex, whereas oncogenic mutations in the N-terminal phosphorylation domain of β-catenin block its degradation via the ubiquitin-proteasome pathway. In all cases, the common outcome is the stabilization and nuclear accumulation of the central player β-catenin and subsequent activation of its target genes. Accordingly, the Wnt/β-catenin pathway has gained recognition as an enticing molecular target for therapeutics of human cancers (Moon, Kohn et al. 2004; Barker and Clevers 2006; Takemaru, Ohmitsu et al. 2008).

Chibby (Cby) was originally identified as a β-catenin binding partner using the C-terminal transactivation domain of β-catenin as bait in a yeast Ras recruitment system (Takemaru, Yamaguchi et al. 2003). Cby is a 14.5-kDa protein evolutionarily conserved from fly to human. We have previously reported that Cby acts as a Wnt/β-catenin antagonist through two distinct molecular mechanisms (Li, Mofunanya et al. 2008; Takemaru, Fischer et al. 2009). It competes with Tcf/Lef factors for β-catenin binding in the nucleus. Cby also interacts with 14-3-3 chaperones to export β-catenin out of the nucleus. 14-3-3 proteins specifically recognize serine 20 within the 14-3-3-binding motif of Cby. More recently, we have demonstrated that Cby harbors functional nuclear localization signal (NLS) and nuclear export signal (NES) motifs and constitutively shuttles between nucleus and cytoplasm (Li, Mofunanya et al. 2010). Direct interaction of 14-3-3 proteins with Cby facilitates Cby binding to the CRM1 export receptor, while inhibiting Cby binding to the nuclear import receptor importin-α, thereby promoting cytoplasmic compartmentalization of Cby and β-catenin.

It has been reported that Cby expression is significantly down-regulated in pediatric ependymomas (Karakoula, Suarez-Merino et al. 2008) and colon cancer cell lines (Schuierer, Graf et al. 2006). However, the tumor suppressive properties of Cby remain largely uncharacterized. In this study, we evaluated whether Cby suppresses β-catenin-dependent signaling activity and growth of the human colon adenocarcinoma cell line SW480 in which APC is mutated, causing stabilization and nuclear accumulation of β-catenin (Korinek, Barker et al. 1997; Morin, Sparks et al. 1997). We show that overexpression of Cby in SW480 cells causes a dramatic shift in distribution of β-catenin towards the cytoplasm, thereby repressing β-catenin-mediated gene activation. Furthermore, stable expression of Cby inhibited SW480 cell growth.

Notably, these effects are, at least in part, exerted through interaction with 14-3-3 proteins since stable expression of 14-3-3-binding- defective Cby mutant CbyS20A has no significant influence on β-catenin signaling and cell growth. Taken together, these findings support a role for Cby in the control of colon tumorigenesis.

Results

Ectopic expression of Cby represses endogenous β-catenin signaling in SW480 colon cancer cells

Previously, we reported that Cby serves as a β-catenin signaling antagonist by competing with Tcf/Lef factors for β-catenin binding and facilitating nuclear export of β-catenin in concert with 14-3-3 proteins (Li, Mofunanya et al. 2008; Takemaru, Fischer et al. 2009; Li, Mofunanya et al. 2010). To investigate whether Cby influences endogenous β-catenin signaling, we turned to the human colon adenocarcinoma SW480 cell line. It is well established that the canonical Wnt/β-catenin signaling pathway is aberrantly activated in most colon cancers. The SW480 cells carry truncating mutations in the *APC* gene and consequently accumulate nuclear β-catenin, leading to constitutive activation of β-catenin signaling (Korinek, Barker et al. 1997; Morin, Sparks et al. 1997). Interestingly, Cby expression in these cells is down-regulated due to hypermethylation of the *Cby* promoter region (Schuierer, Graf et al. 2006), providing a useful model system to study Cby function.

We first assessed if ectopic expression of Cby affects β-catenin subcellular distribution in SW480 cells. In agreement with prior reports (Henderson 2000; Rosin-Arbesfeld, Townsley et al. 2000), endogenous β-catenin was detected in the nucleus of the vast majority of cells (Figure 2-1). Transient expression of wild-type Cby (CbyWT) led to a significant shift in β-catenin localization towards the cytoplasm. Next, we examined the effect of a Cby mutant lacking the functional C- terminal NLS (CbyΔNLS) since this mutant is exclusively cytoplasmic and able to retain exogenous β-catenin in the cytoplasmic compartment (Li, Mofunanya et al. 2010). Ectopic expression of CbyΔNLS resulted in even more profound cytoplasmic enrichment of β-catenin where they colocalized (Figure 2-1A and B).

Western blot analysis using anti-Cby antibody revealed that as previously noted at mRNA levels (Schuierer, Graf et al. 2006), endogenous Cby protein was present at low levels in non-transfected SW480 cells (Figure 2-1C, lane 1). Upon transient transfection, expression levels of CbyΔNLS were lower compared to those of CbyWT. We therefore increased the amount of DNA for the mutant for the subsequent β-catenin-dependent luciferase reporter (TopFlash) assays. SW480 cells exhibit high levels of TopFlash activity resulting from constitutive activation of β-catenin signaling (Figure 2-1D). Ectopic expression of CbyWT repressed TopFlash activity in a dose-dependent manner. In accordance with our previous observations in HEK293T cells (Li, Mofunanya et al. 2010), CbyΔNLS exerted slightly more potent inhibitory

effects through efficient sequestration of β-catenin in the cytoplasm. Taken together, our results indicate that Cby negatively modulates endogenous β-catenin signaling in SW480 cells.

Stable expression of Cby suppresses colon cancer cell growth

It was shown that the proliferation of colon cancer cells largely depends on the continuous presence of β-catenin signaling (Tetsu and McCormick 1999; van de Wetering, Sancho et al. 2002). Having established that Cby interferes with β-catenin signaling activity in SW480 cells, we investigated whether Cby influences their growth. To this end, we generated stable cell lines by infecting SW480 cells with a retrovirus carrying CbyWT, 14-3-3-binding-defective CbyS20A, 14-3-3ζ or both Cby and 14-3-3ζ along with a control retrovirus. The efficient expression of the proteins was confirmed by immunoblotting using anti-Cby and anti-14-3-3 antibodies (Figure 2-2A). Worthy of note, stable expression of CbyWT substantially stabilized endogenous 14-3-3 proteins, reaching near exogenous expression levels (compare lanes 2 and 4). Similarly, stable expression of 14-3-3ζ resulted in an increase in endogenous Cby protein levels (compare lanes 1 and 4).

As shown in Figure 2-2B, stable expression of CbyWT significantly suppressed growth of SW480 cells. Coexpression with 14-3-3ζ showed no further influence on their growth. This is most likely explained by the fact that stable expression of CbyWT increases levels of endogenous 14-3-3 protein (Figure 2-2A). In contrast, CbyS20A displayed no obvious effect, suggesting that Cby – 14-3-3 interactions are crucial for suppression of SW480 cell growth. Stable expression of 14-3-3ζ alone showed a tendency to slow cell growth but it was not statistically significant, implying that efficient growth inhibition requires relatively high expression levels of both Cby and 14-3-3 proteins. Cell cycle analysis by flow cytometry showed that there is a marked increase in the G2/M population with a concomitant decrease in the G0/G1 population in CbyWT-overexpressing cells in comparison with vector-control cells (Figure 2-2C). Collectively, these observations suggest that Cby, in cooperation with 14-3-3 proteins, attenuates colon cancer cell growth by inducing a G2/M cell-cycle arrest.

Stable expression of Cby reduces nuclear β-catenin levels in SW480 cells

We previously reported that Cby is a nuclear-cytoplasmic shuttling factor and acts with 14-3-3 proteins to promote nuclear export of β-catenin (Li, Mofunanya et al. 2008; Li, Mofunanya et al. 2010). Thus, we examined whether the growth-suppressive effect of Cby on SW480 cells is mediated in part by changes in β-catenin subcellular localization using immunofluorescence microscopy. In control SW480 cells, β-catenin was predominantly detected in the nucleus (Figure 2-3A). However, in stable cells expressing CbyWT but not CbyS20A, there was a shift in β- catenin localization towards the cytoplasm (Figure 2-3A).

To confirm these results, we performed subcellular fractionation of SW480 cell lysates in order to assess relative β-catenin levels in nuclear and cytoplasmic compartments. Consistent

with our immunofluorescence staining data, stable expression of CbyWT led to a cytoplasmic enrichment of β-catenin with a concomitant reduction in its nuclear levels (Figure 3-3B, lanes 5 and 6). CbyS20A-expressing cells exhibited no major changes in β-catenin distribution (lanes 8 and 9). It is also noteworthy that CbyWT showed marked enrichment in the cytoplasmic fraction (lanes 5 and 6), whereas CbyS20A was more abundant in the nuclear fraction (lanes 8 and 9). This further confirms our prior data demonstrating that association of 14-3-3 with Cby at serine 20 is responsible for cytoplasmic sequestration of Cby (Li, Mofunanya et al. 2008; Li, Mofunanya et al. 2010). These findings suggest that Cby, in cooperation with 14-3-3 proteins, shuttles β-catenin out of the nucleus into the cytoplasm, leading to reduced cancer cell growth.

Stable expression of Cby inhibits β-catenin signaling in colon cancer cells

To investigate whether the compromised growth of CbyWT-expressing cells is accompanied by a reduction in the transcriptional output of β-catenin, we conducted TopFlash reporter assays. As expected, we observed that β-catenin signaling was markedly reduced in SW480 stable cells expressing CbyWT but not CbyS20A (Figure 2-4A). Cyclin D1 is a direct target for the β-catenin/Tcf complex, and plays a crucial role in proliferation of colon cancer cells (Arber, Doki et al. 1997; Tetsu and McCormick 1999). We found that expression of cyclin D1 was reduced in SW480 cells expressing CbyWT but not CbyS20A (Figure 2-4B and C). Taken together, our findings suggest that Cby, in cooperation with 14-3-3 proteins, suppresses colon cancer cell growth by modulating subcellular distribution and signaling activity of endogenous β-catenin.

Discussion

It has been established that the canonical Wnt/β-catenin signaling pathway is aberrantly activated in the vast majority of colon cancers and to a lesser extent in other tumor types. Most common mutations occur in the *APC* or *β-catenin* gene, which ultimately results in stabilization and nuclear accumulation of β-catenin in cancer cells. Thus, a deeper understanding of β-catenin regulation is important for the development of effective cancer therapies.

The evolutionarily conserved Cby protein binds to the C-terminal transactivation domain of the β-catenin oncoprotein and acts as a potent repressor, thereby attenuating canonical Wnt signaling (Takemaru, Yamaguchi et al. 2003; Li, Singh et al. 2007; Li, Mofunanya et al. 2008; Mofunanya, Li et al. 2009; Li, Mofunanya et al. 2010). Cby mRNA levels have been shown to be down-regulated in various colon cancer cell lines including SW480 cells due to promoter hypermethylation (Schuierer, Graf et al. 2006). However, a potential tumor suppressor function of Cby remains largely unknown. The goal of this research was to determine whether Cby, in conjunction with 14-3-3, regulates growth of SW480 colon cancer cells by controlling β-catenin localization and signaling activity. Consistent with our hypothesis, stable expression of Cby suppresses SW480 cell growth. In these cells, β-catenin is predominantly found in the cytoplasm

and its signaling activity is significantly reduced. This effect of Cby is most likely to be mediated by 14-3-3 proteins since CbyS20A defective in 14-3-3 binding has no significant effect on cell growth and β-catenin signaling. Manipulation of Cby function might therefore provide a novel means for the therapeutic intervention of Wnt/β- catenin-driven tumors.

Interestingly, stable expression of Cby causes a dramatic increase in G2/M phase cells (Figure 2-2C). In support of this, β-catenin has been implicated in the control of cell cycle at G2/M (Olmeda, Castel et al. 2003; Davidson, Shen et al. 2009), and small molecule inhibitors of β-catenin signaling (quercetin and NC043) have been shown to block the cell cycle of SW480 cells at the G2/M phase (Shan, Wang et al. 2009; Wang, Liu et al. 2011). These findings concur with the notion that reduced SW480 cell proliferation by Cby overexpression is primarily attributable to a G2/M cell-cycle arrest. At present, the exact fate of Cby-14-3-3-bound β-catenin is unknown. However, we speculate that the Cby-14-3-3-β-catenin complex might be protected from degradation pathways and remains stable in the cytoplasm. This may serve as a reservoir of signaling competent β-catenin that is readily available for release in response to upstream signals.

Our findings are in good agreement with the idea that Cby may function as a tumor suppressor. Interestingly, deletion and epigenetic silencing of the *Cby* gene was detected in over 60% of pediatric ependymonas (Karakoula, Suarez-Merino et al. 2008). However, it remains unclear whether Wnt/β- catenin signaling is up-regulated in ependymonas. On the other hand, no mutations or expression changes of the *Cby* gene have been reported in colon cancer (Gad, Teboul et al. 2004; Schuierer, Graf et al. 2006) and Wilms tumors (Zirn, Wittmann et al. 2005). Nonetheless, it is possible that alterations of Cby function might occur through changes in posttranslational modifications that affect protein stability and subcellular distribution. Clearly, further experiments are required to define the tumor suppressor role of Cby in human cancer.

Methods

Expression constructs

The expression plasmids for Flag-tagged wild-type Cby (CbyWT) and CbyΔNLS have been described previously (Li, Mofunanya et al. 2008; Li, Mofunanya et al. 2010). For generation of SW480 stable cell lines, Cby and 14-3-3ζ cDNAs were amplified by PCR and subcloned into the retroviral vector pQCXIP (puromycin resistant; Clontech). Additionally, a CbyWT cDNA was subcloned into pLXIN (neomycin resistant; Clontech) for establishment of the double-stable cell line with 14-3-3ζ. The sequence of all expression vectors was confirmed by DNA sequencing.

Cell culture, transfection and viral infection

SW480 cells were purchased from ATCC and propagated in DMEM with 10% FBS and 100 U/ml penicillin-streptomycin. For transient transfection, cells were seeded onto 6- or 12-well tissue culture dishes, cultured overnight, and then transfected using Lipofectamine 2000 (Invitrogen) or Expressfect (Denville Scientific, Inc.) according to the manufacturer's instructions. Empty vector was added to adjust the total amount of DNA to be the same in each transfection. For establishing stable cell lines, SW480 cells were infected with retroviruses bearing CbyWT, CbyS20A and 14-3-3ζ individually or CbyWT and 14-3-3ζ in combination, followed by selection of pools of cells with either 2.5 µg/ml of puromycin (Invitrogen) alone or both puromycin and 500 µg/ml of G418 (Invitrogen) as described previously (Li, Singh et al. 2007).

Immunofluorescence microscopy

Cells were seeded at $5x10^5$ cells/well onto coverslips in 12-well dishes and allowed to adhere and proliferate for 48 h. Cells were then fixed with methanol/acetone (1:1, v/v), incubated with anti-β-catenin antibody (1:1000; mouse monoclonal; BD Transduction Laboratories) and anti-Cby antibody (1:1000; rabbit polyclonal; (Takemaru, Yamaguchi et al. 2003)) in 1% BSA and 0.1% Triton X-100 in PBS, washed and incubated with DyLight 488-conjugated goat anti-mouse IgG and DyLight 549-conjugated goat anti-rabbit IgG secondary antibodies (1:500; Jackson ImmunoResearch Laboratories). Cells were counterstained with DAPI and mounted onto glass slides. Images were taken on a Zeiss LSM510 confocal microscope and processed with Adobe Photoshop for brightness and contrast. To quantify subcellular localization of β-catenin, independent transfections were performed at least three times and a minimum of 100 cells were counted for each transfection as described previously (Takemaru, Ohmitsu et al. 2008; Li, Mofunanya et al. 2010).

Subcellular fractionation and immunoblotting

Whole cell lysates were prepared from subconfluent cells and fractionated as described (Mihara and Moll 1993). Protein concentration was determined using the DC protein assay kit (Bio-Rad). Equal amounts of nuclear and cytoplasmic fractions were treated with concanavalin A- Sepharose (Sigma), using 7 µg concanavalin A/µg total protein, at 4°C for 3 h. The resultant supernatants were separated by SDS-PAGE, and subjected to western blotting. The primary antibodies used were as follows: rabbit anti-Cby (Takemaru, Yamaguchi et al. 2003); rabbit anti-pan-14-3-3 (Santa Cruz Biotechnology); mouse anti-GAPDH (BioDesign International); rabbit anti-cyclin D1 (Epitomics); and mouse anti-β-catenin and mouse anti-nucleoporin p62 (BD Transduction Laboratories). HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories.

TopFlash luciferase reporter assays

SW480 cells were seeded onto 12- or 24-well plates, and 24 h later, cells were transfected with the appropriate combinations of plasmids in triplicate. A Renilla luciferase (pRL- TK) was cotransfected to normalize transfection efficiency. Luciferase activity was measured 24 to 48 h post-transfection using the Dual-Luciferase Reporter Assay System (Promega) with a luminometer (Berthold Technologies) as described previously (Li, Mofunanya et al. 2008; Mofunanya, Li et al. 2009; Li, Mofunanya et al. 2010).

Cell growth and cell cycle assays

Cells were plated in triplicate at 10^5 cells/well in 24-well plates. On day 5, cells were trypsinized, diluted, stained with 0.4% trypan blue (Sigma), and live cells were counted using a hemocytometer. For cell cycle analysis, cells were seeded at $5x10^5$ cells/well in 12-well plates and allowed to proliferate for 3 days. The cells were then harvested by trypsinization, fixed in ice-cold 70% ethanol overnight, and stained with propidium iodide (Sigma). The DNA content was measured using a Guava EasyCyte Plus flow cytometer (Millipore).

Statistical analysis

Statistical significance was calculated by the unpaired Student's *t*-test using Microsoft Excel. Data are presented as means ± SE or SD as indicated, and *P* values of <0.05 were considered statistically significant.

Figure 2-1: Forced expression of Cby in SW480 colon cancer cells promotes translocation of nuclear β-catenin towards the cytoplasm, resulting in inhibition of endogenous β-catenin signaling.

A. SW480 cells were transiently transfected with a control empty vector or an expression plasmid for Flag-tagged CbyWT or CbyΔNLS, and doubly immunostained with anti-Cby (red) and anti-β-catenin (green) antibodies. Nuclei were stained with DAPI. A merged image of β-catenin and DAPI is also shown.

B. Quantitative analysis of the results in (A). The subcellular localization of endogenous β-catenin 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. For cells transfected with Cby plasmids, β-catenin localization was scored only in those expressing ectopic Cby.

C. Western blot analysis of Cby expression in SW480 cells using anti-Cby antibody. Note that, to compensate protein levels, higher amounts of DNA for CbyΔNLS were used for transfection. The anti-Cby antibody detected both exogenous and endogenous proteins. An asterisk indicates a non-specific band that overlaps with Flag-Cby. GAPDH was used to confirm equal loading.

D. The ability of Cby to repress endogenous β- catenin signaling was tested by TopFlash assays. SW480 cells were transfected with 100 ng of TopFlash luciferase reporter and the indicated amounts of a Flag-tagged Cby expression plasmid. Luciferase activity was measured 24 h post-transfection, and normalized to Renilla luciferase activity used as an internal control. All transfections were carried out in triplicates and the means ± SD are shown.

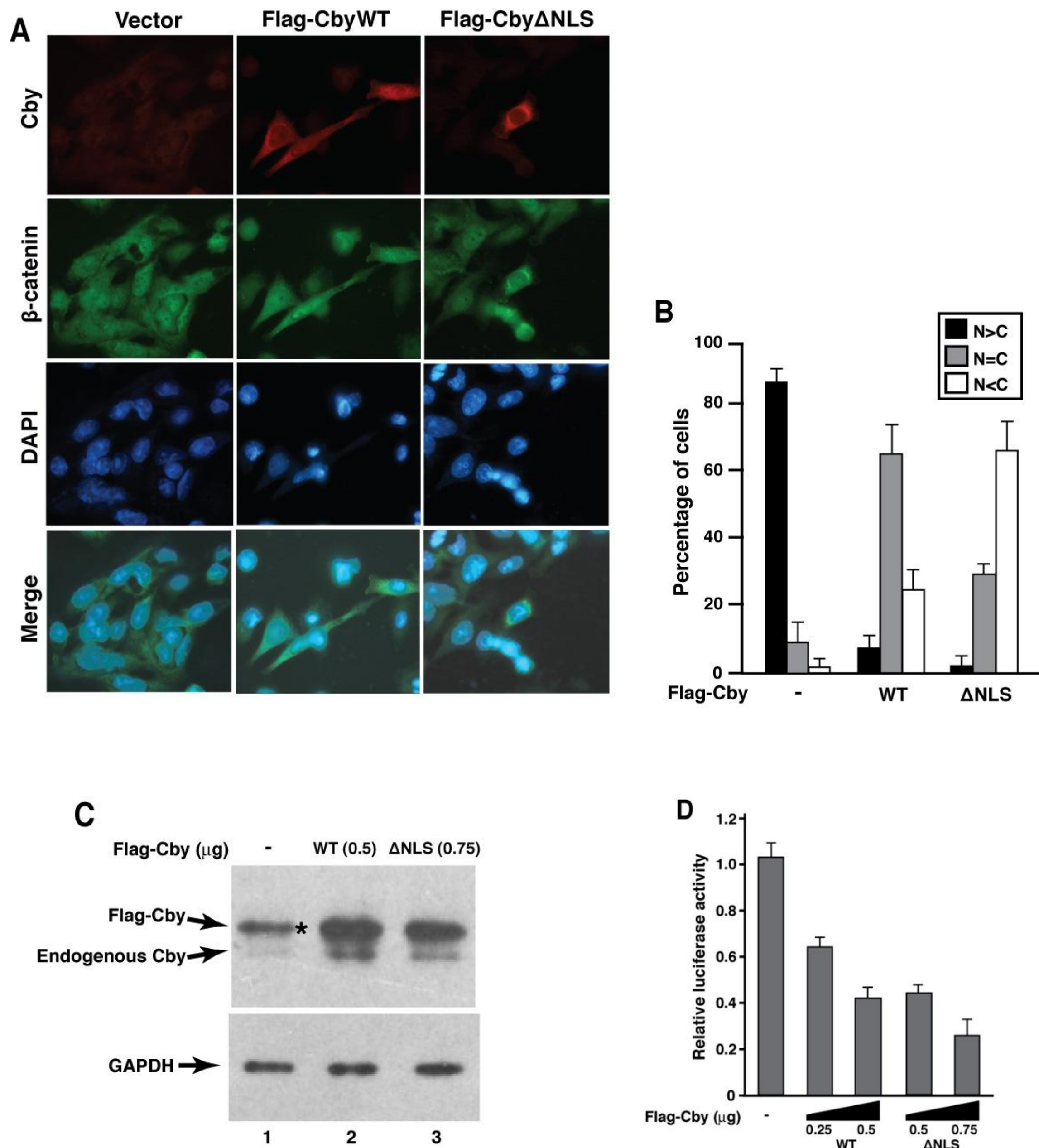


Figure 2-1: Forced expression of Cby in SW480 colon cancer cells promotes translocation of nuclear β-catenin towards the cytoplasm, resulting in inhibition of endogenous β-catenin signaling.

Figure 2-2: Stable expression of Cby reduces SW480 cell growth.
A. Stable SW480 cell lines expressing CbyWT, CbyS20A, 14-3-3ζ or both Cby and 14-3-3ζ, and vector-control cells were established, and the cell lysates were analyzed by western blotting using antibodies against Cby, 14-3-3ζ, β-catenin and GAPDH.
B. The stable SW480 cell lines were seeded on 24-well plates (10^5 cells/well), and total cell numbers were counted 5 days later. The data are the means ± SE of triplicate samples and representative of three independent experiments. Student's t-test; *P < 0.05, **P < 0.01, NS = not significant when compared with vector-control cells.
C. Cell cycle analysis of vector-control and CbyWT-expressing cells. Following propidium iodide staining, the DNA content of individual cells was measured by flow cytometry. The data shown are from one representative experiment out of three.

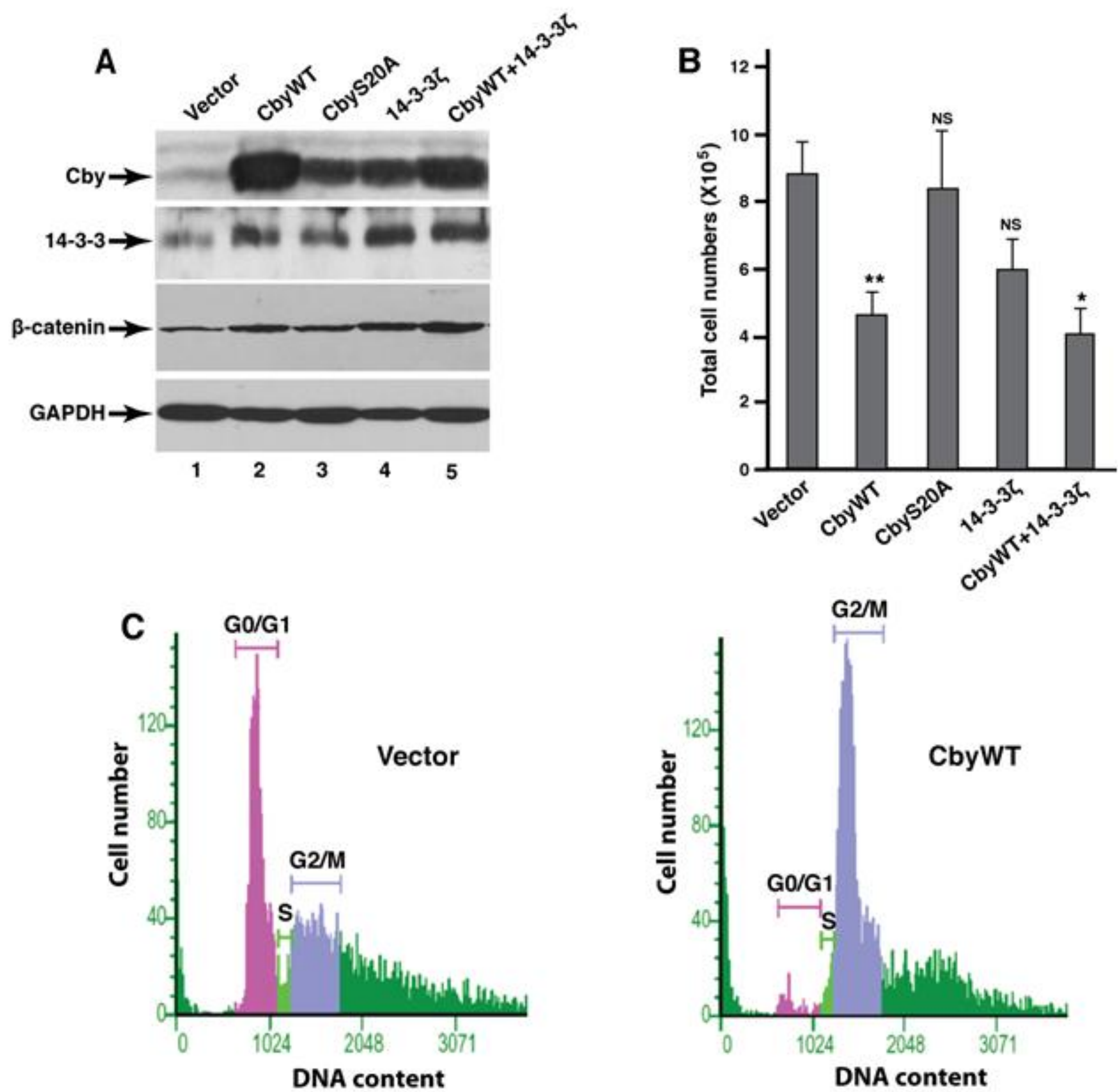


Figure 2-2 Stable expression of Cby reduces SW480 cell growth.

Figure 2-3: Cby facilitates nuclear export of endogenous β-catenin.
A. SW480 cells stably expressing CbyWT or CbyS20A and control cells were fixed and subjected to immunofluorescence staining of endogenous β-catenin (green). Nuclei were visualized with DAPI.
B. Whole-cell (W), nuclear (N) and cytoplamic (C) extracts were prepared from the indicated SW480 stable cells, and β-catenin levels were analyzed by western blotting. The relative purity of nuclear and cytoplasmic fractions was evaluated by probing for nucleoporin p62 (NUP62) and GAPDH, respectively.

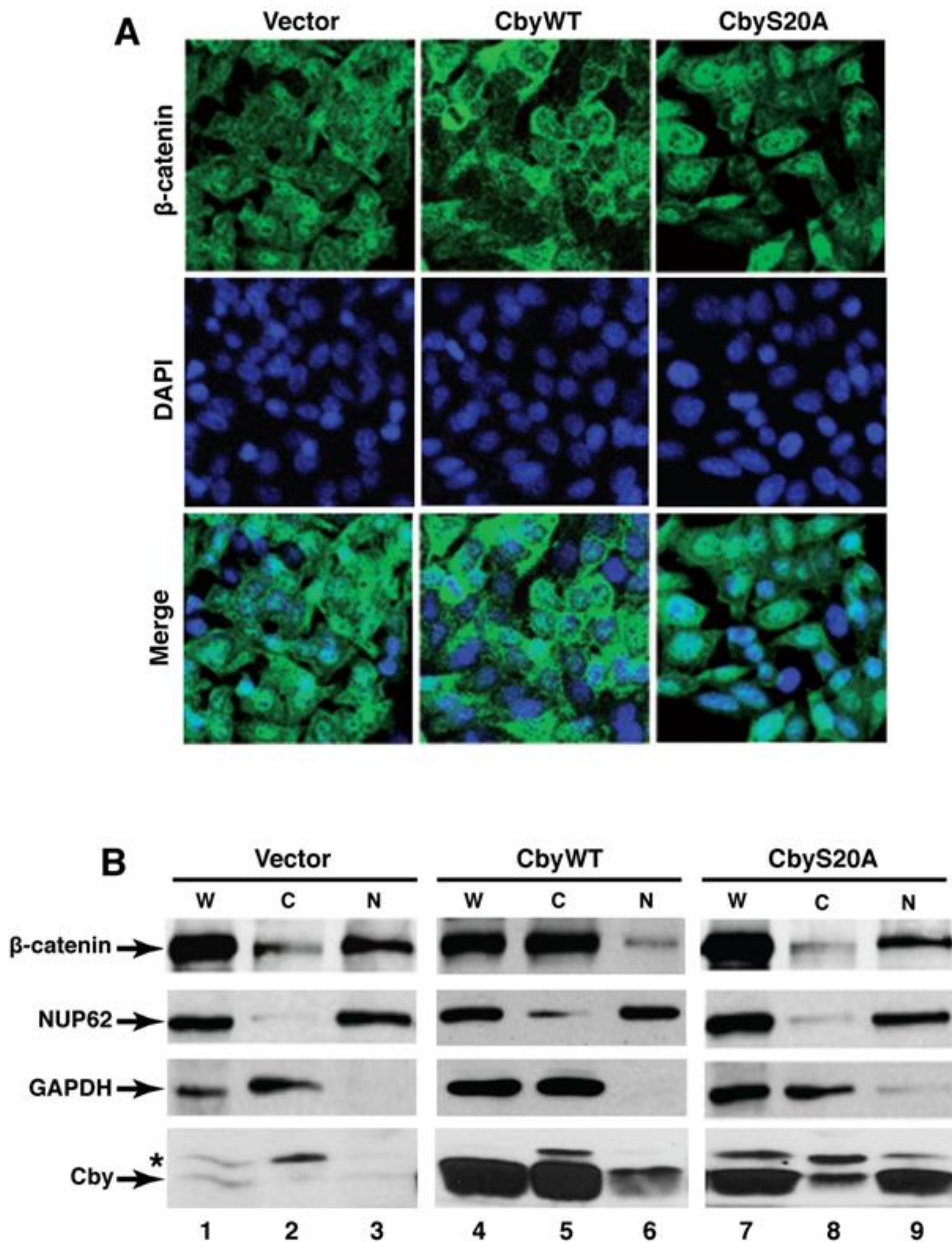


Figure 2-3 Cby facilitates nuclear export of endogenous β-catenin.

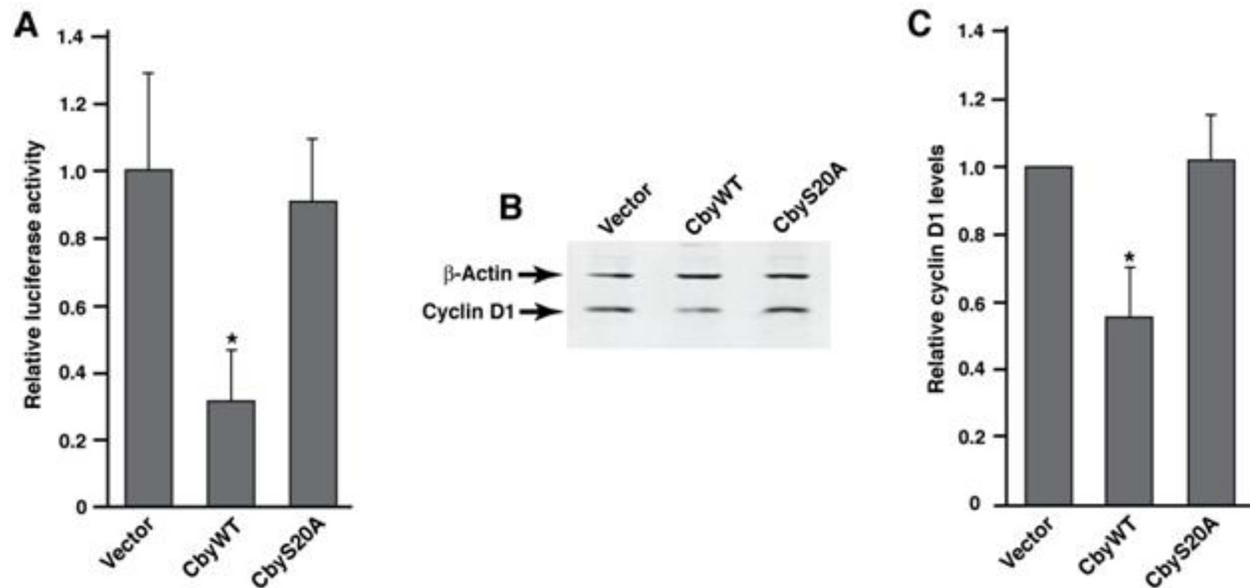


Figure 2-4 β-Catenin signaling activity is attenuated in SW480 cells stably expressing Cby.

A. CbyWT, but not CbyS20A, represses TopFlash activation. SW480 stable cells were transfected in 24-well plates with 25 ng of TopFlash or mutant FopFlash reporter as well as a Renilla luciferase plasmid to normalize transfection efficiency. Luciferase activities were measured 48 h post-transfection, normalized and corrected for background by subtraction of FopFlash values from corresponding TopFlash values. Each transfection was performed in triplicate and repeated at least three times. Shown are the means ± SD from one representative experiment. Student's t-test; *P<0.05 when compared with vector-control cells.
B. CbyWT, but not CbyS20A, inhibits cyclin D1 expression. Whole-cell lysates from the indicated SW480 stable cells were subjected to western blot analysis for cyclin D1 and nucleoporin p62 (NUP62) as a loading control.
C. Quantification of cyclin D1 expression levels. The intensity of cyclin D1 bands from western blots was quantified using the NIH Image software and normalized to that of NUP62 bands. Data are the mean band intensities ± SD from at least three independent experiments. Student's *t*-test; *P < 0.05 when compared with vector-control cells.

Chapter 3 Cby knock-down promotes Mesenchymal-to-epithelial reversion-like changes

The data for this chapter was produced by Victoria Fischer. The stable HEK293 cell lines were generated by Dr. Feng-Qian Li.

Background

Cancer is currently the second leading cause of death in the US (Minino, Xu et al. 2010), with colon cancer ranking among the top four cancer types (Eheman, Henley et al. 2012). Among colon cancers, 90% of sporadic cases are ascribed to aberrant Wnt/β-catenin signaling (Herbst and Kolligs 2007). In healthy cells, in the absence of Wnt signaling, β-catenin is continuously degraded unless associated with cadherins at the plasma membrane (Moon, Kohn et al. 2004). Upon Wnt-signaling, β-catenin is stabilized, localizes to the nucleus and activates gene transcription in conjunction with transcription factors of the T-cell factor/lymphoid enhancer factor-family. β-Catenin's target genes function in the regulation of many cellular processes relevant in development and wound healing, but also in tumorigenesis, including cell proliferation, cell cycle progression, apoptosis, differentiation, tissue invasion, and angiogenesis (Orford, Orford et al. 1999; Herbst and Kolligs 2007). Pathogenically increased levels of β-catenin can additionally cause aggressive fibromatosis and pulmonary fibrosis (Moon, Kohn et al. 2004; Thiery, Acloque et al. 2009), while other alterations in Wnt-ligands cause a variety of diseases (Moon, Kohn et al. 2004).

One process that can be induced by Wnt-signaling is Epithelial-to-Mesenchymal Transition (EMT). This process includes a loss of adherens junctions, adoption of spindle-shaped morphology with cytoskeletal reorganization, and increased cell motility, along with loss of epithelial and gain of mesenchymal markers (Xie, Law et al. 2003; Aroeira, Aguilera et al. 2007). While essential in development and for some processes in adult life (Palacios, Tushir et al. 2005; Thiery, Acloque et al. 2009), in tumorigenesis, EMT is believed to promote metastasis, allowing cells to become invasive and to enter blood vessels (Moustakas and Heldin 2007; Kalluri 2009). Meanwhile at the new host sites for metastatic cells, the opposite process, Mesenchymal-to-Ephithelial Reversion (MET), allows cells to form metastatic tumors (Moustakas and Heldin 2007; Blick, Widodo et al. 2008). Tight control of the Wnt/β-catenin signaling pathway is therefore critical throughout life.

β-Catenin plays an opposing role at the plasma membrane, however, where it binds to the adherens junction protein E-cadherin and is required for E-cadherin's functions in polarization and regulation of cellular behavior. Formation of E-cadherin-mediated adherens junctions

between cells contributes to regulation of cell shape, migration, and differentiation (Theard, Raspe et al. 2008), and effectuates coordinated behavior of cells as a cohesive unit (Christiansen and Rajasekaran 2006). Loss of E-cadherin expression and formation of single migratory cells has been described as a result of EMT at the invasive front in colon carcinoma (Brabletz, Jung et al. 2001), and such loss of E-cadherin expression has been observed in most carcinomas including colorectal carcinomas (Peinado, Portillo et al. 2004; Natalwala, Spychal et al. 2008). In fact, transcriptional repression, alteration of stability and delocalization of cadherins as well as β-catenin from the plasma membrane form part of EMT (Peinado, Portillo et al. 2004; Hugo, Ackland et al. 2007). In accordance with this observation, E-cadherin re-expression has been shown to induce MET in tumor cells (Peinado, Portillo et al. 2004), and the protein is accordingly considered an invasion suppressor gene (Peinado, Portillo et al. 2004) and a metastasis suppressor (Reynolds and Carnahan 2004). However, the effect of E-cadherin on suppression of invasion depends on its binding to β-catenin (Wong and Gumbiner 2003).

Several studies have researched whether the opposing effects of β-catenin – EMT on one side and epithelialization on the other – are exerted by the same pool of protein, or whether post-translational modification defines the function. Gottardi et al. demonstrate that these pools are, indeed, different when cells are stimulated by Wnt signal (Gottardi and Gumbiner 2004), but the same in SW480 colon cancer cells, where β-catenin is stabilized due to a truncating mutation in APC (Gottardi, Wong et al. 2001). The truncated APC in SW480 cells still allows interaction between β-catenin and APC but prevent β-catenin's destruction (Su, Fu et al. 2008), leading to stabilization of β-catenin and subsequent transcriptional activation of its target genes. Stabilized, signaling active β-catenin can then trigger cell proliferation and EMT (Orford, Orford et al. 1999).

The evolutionarily conserved protein Chibby (Cby) was discovered as a β-catenin binding partner in a Ras recruitment assay (Takemaru, Yamaguchi et al. 2003). We have recently shown that its overexpression leads to reduced nuclear levels and signaling of β-catenin, both by competition with Tcf/Lef for β-catenin binding (Takemaru, Yamaguchi et al. 2003) and by shuttling β-catenin out of the nucleus in a tripartite complex with 14-3-3ζ protein (Li, Mofunanya et al. 2008), and effectuates reduced proliferation in SW480 human colon cancer cells (see chapter 2). These results would suggest a role as tumor suppressor in colorectal carcinogenesis. However, Cby was found to be overexpressed in some but repressed in other colon carcinomas (Gad, Teboul et al. 2004). Additionally, Cby mRNA was found downregulated in seven standard colon cancer cell lines compared to primary colon epithelial cells, including SW480 cells used for the present study. In these cells, demethylation restored Cby mRNA levels to that of control cells (Schuierer, Graf et al. 2006).

We show here that Cby regulates function of β-catenin at the membrane. We found that Cby knock-down leads to MET-like changes, including increased total protein levels of E-cadherin; increased membrane-bound E-cadherin and β-catenin; increased protein levels and plasma membrane localization of the tight junction protein zonula occludens-1 (ZO-1); and

decreased levels of vimentin. Importantly, migratory pattern upon injury modeled by wound scratch assays changes toward that of epithelial cells, and anchorage-independent growth as indicator of metastatic capacity is dramatically reduced in Cby knock-down cells. We find that nuclear β-catenin signaling is reduced, while levels of E-cadherin mRNA are increased. These findings point to the possibility that levels of Cby protein in tumor cells are decisive at specific time points.

Results

Cby knock-down changes the growth pattern of HEK293 and SW480 cells

Cby has been shown to function as Wnt/β-catenin antagonist. We have shown that Cby shuttles β-catenin out of the nucleus and represses nuclear β-catenin signaling when overexpressed (Li, Mofunanya et al. 2008; Li, Mofunanya et al. 2010). In order to further investigate the functions of Cby, we generated stable monoclonal lines expressing a short-hairpin RNA for Cby in human embryonic kidney (HEK) 293 cells. These cells harbor relatively high levels of both Cby and β-catenin protein, thus are well suited to study effects of Cby protein levels on β-catenin function. We infected these cells with a retrovirus expressing Cby shRNA or a control virus, and the infected cells were selected with puromycin. Cby protein was substantially depleted by Cby shRNA, with a 56% reduction, compared to those infected with a control virus (Fig. 3-1A, Western Blot and graph).The growth pattern of the Cby knock-down cells was immediately recognizable as remarkably different from the vector control line (Fig. 3-1A, phase-contrast microscopy images). These cells grow as distinct clones with apparent cell-cell contacts typical for epithelial cells while control cells grow as mostly single cells in a mesenchymal, spindle-shaped pattern and rapidly cover the entire plate even when seeded at low density.

In order to investigate whether the effects caused by Cby knock-down is a cell-type dependent phenotype, we next examined this observation using a different cell line. Based on our previous results that Cby shuttles β-catenin out of the nucleus and reduces nuclear signaling of β-catenin (Li, Mofunanya et al. 2008; Li, Mofunanya et al. 2010) even in cells with stabilized β-catenin (see chapter 2), we used SW480 human colon adenocarcinoma cells. These cells express a truncated APC protein (Korinek, Barker et al. 1997; Brabletz, Jung et al. 2001). The mutant protein still binds to β-catenin but can no longer shield it from dephosphorylation by PP2A (Su, Fu et al. 2008), leading to accumulation of β-catenin and high levels of nuclear signaling (Henderson 2000; Rosin-Arbesfeld, Townsley et al. 2000). SW480 cells express Cby protein in their wild-type form, but at a lower level than the HEK293 cells, likely due to a methylated promoter region of the *Cby* gene (Schuierer, Graf et al. 2006). Our knock-down reduced Cby protein levels by about 45% (Fig. 3-1B Western Blot and graph). This moderate change in Cby protein levels led to a notable change in cellular phenotype. The Cby knock-down cells appeared to grow in more densely packed clones, with tighter connections between the cells and less

spindle shaped cells (Fig. 3-1B, phase contrast images), thus adopting a less mesenchymal, more epithelial phenotype, though the change was not as dramatic as in HEK293 cells.

Cby knock-down increases levels of beta-catenin and E-cadherin at the plasma membrane

Cell-cell contacts are based on a variety of cell-cell junctions. It is thought that the formation of junctions is initiated by E-cadherin-mediated adherens junctions. The establishment of such junctions then triggers other connections to form, including tight junctions (Wong and Gumbiner 2003; Cavey and Lecuit 2009). E-cadherin can bind β-catenin at adherens junctions, and this binding is required for some functions of E-cadherin, including the suppression of invasiveness (Wong and Gumbiner 2003). Since Cby is known for, and was initially discovered by, its interaction with β-catenin (Takemaru, Yamaguchi et al. 2003), we tested whether the increased apparent cell-cell connections were based on adherens junctions, thus contained E-cadherin and β-catenin.

Indeed, in the HEK293 cells, β-catenin appears to localize solely to the plasma membrane in the Cby sh cells, while it is diffusely distributed in control lines (Fig. 3-2A, left panel). Even more dramatically, E-cadherin is barely detectable at all in control lines, while it stains distinctly and strongly to cell-cell contacts in Cby sh cells (Fig. 3-2B, left panel). In agreement with the differences in growth pattern, the effect of Cby knock-down is less dramatic but still very clear in the SW480 cells. While SW480 vector control cells show a characteristic β-catenin localization, with high levels of β-catenin throughout the cell, yet distinct nuclear accumulation, Cby knock-down cells show β-catenin localized to cell-cell contacts (Fig. 3-2A, B right panels). The change in growth pattern observed by phase-contrast microscopy (Fig. 3-1) becomes more evident in the β-catenin or E-cadherin-stained cells. Cby knock-down cells grow in a monolayer with clear cell-cell contacts, whereas the vector control cells at the same density tend to lie on top of each other, with little β-catenin at cell-cell contacts and very low levels of E-cadherin. This localization is evident throughout the height of the cells, as the galleries of images from a representative z-stack show for E-cadherin in HEK293 cells (Fig. 3-3).

It has been shown previously that growth to confluence leads to formation of adherens junctions with β-catenin and E-cadherin at the plasma membrane of both SW480 and HEK293 cells (Davies, Roberts et al. 2004; Maher, Flozak et al. 2009). We maintained our cell lines at a maximum of 80% confluence, to avoid this effect, and only allowed them to grow to confluence in a specific assay when needed. The staining assays were performed both at subconfluence and at day 1 of confluence, and images were taken of fields of comparable nuclear density by DAPI stain. Comparison of Cby sh and vector cells stained in this way show increased localization of β-catenin and E-cadherin to the plasma membrane both in confluent and subconfluent areas, suggesting vastly increased formation of adherens junctions in Cby sh cells.

Cby knock-down leads to changes in protein levels and localization towards an epithelial phenotype

Carcinomas stem from epithelial cells. However, in histological examinations of colon carcinomas, it was found that particularly at the invasive front of the tumor, single migratory cells, displaying a mesenchymal phenotype, can be observed that do not express E-cadherin (Brabletz, Jung et al. 2001). However, tumor metastases adopt the epithelial-like phenotype of the primary tumor, with increased E-cadherin expression (Chao, Shephard et al. 2010). In *in vitro* experiments, reexpression of E-cadherin in tumor cells has been shown to reverse the EMT changes, leading to epithelialization of the mesenchymal tumor cells in numerous systems (Nawrocki-Raby, Gilles et al. 2003; Moustakas and Heldin 2007).

Given the dramatic relocalization and apparent increased levels of E-cadherin in the Cby knock-down cells, we first confirmed that protein levels had changed, by Western Blot analysis (Fig. 3-4C). In parallel, we assessed levels of the mesenchymal filament protein vimentin that is typically higher in mesenchymal than epithelial cells (Lee, Dedhar et al. 2006; Thiery and Sleeman 2006; Hugo, Ackland et al. 2007; Polette, Mestdagt et al. 2007), in HEK293 cells (Fig. 3-4C). Both E-cadherin and vimentin levels change as expected for epithelialization, with E-cadherin levels increased five-fold, and vimentin levels decreased by about half.

Formation of E-cadherin-mediated adherens junctions is thought to initialize the establishment of cell-cell contacts, triggering subsequent formation of other cell-cell junctions (Wong and Gumbiner 2003; Cavey and Lecuit 2009). We therefore tested if tight junctions were also present in the Cby sh cells by staining for ZO-1, one of the proteins involved in this junction (Reichert, Müller et al. 2000) and frequently used as marker for epithelial cells (Zavadil, Bitzer et al. 2001; Rastaldi, Ferrario et al. 2002; Yang, Camp et al. 2006; Polette, Mestdagt et al. 2007; Blick, Widodo et al. 2008; Kalluri 2009; Hicks, O'Neil et al. 2010; Pohl, Radacz et al. 2010). The protein clearly localizes to cell-cell contacts in both cell lines. SW480 cells only show this difference when plated at subconfluent levels though. We furthermore confirmed increased protein levels in the HEK293 cells (Fig. 3-4B). Levels of ZO-1 in control cells were very low, making meaningful densitometry measurements very difficult, but underlining the dramatic difference in ZO-1 protein levels. The low band intensity in the vector cells led to large variation in the calculated difference in protein levels, so that statistically, the difference has to be denoted as not significant, despite the obvious large difference in the blots.

In addition to changes in cell-cell junctions and intermediate filaments, epithelialization includes changes in the actin cytoskeletal arrangement, from stress-fibers toward a cortical actin ring (Zavadil, Bitzer et al. 2001; Das, Becker et al. 2009). Despite the otherwise less dramatic change in phenotype in the SW480 cells, both cell lines show the according changes very clearly (Fig. 3-6; Fig. 3-7 for z-stacks). Parallel to the membranous expression of E-cadherin, β-catenin and ZO-1, the changes in actin cytoskeletal organization are present in subconfluent cultures. For HEK293, they persist in confluent cultures, whereas SW480 cells generally adopt a more epithelial formation in confluent cells (Davies, Roberts et al. 2004) but show clear differences in

subconfluent cells and early in confluence (day 1). These changes support a change in overall phenotype of the cells, far beyond changes in the levels of single proteins.

Cby knock-down affects migratory pattern and reduces the capacity for anchorage-independent growth

Cancer survival rates decrease dramatically when distant metastases are present (Stage IV) compared to even regionally spread disease (American Cancer Society 2008) . Various *in vitro* assays are used to predict the ability of cells to metastasize. The capacities required for this complex process are multiple, and include the ability to migrate and to grow independent of adhesion to substrate or other cells (Hanahan and Weinberg 2011). HEK293 cells are not considered tumorigenic (Ha, Kim et al. 2010), but are able to migrate (Ma, Zhang et al. 2009). SW480 cells are tumorigenic and able to migrate (Faux, Ross et al. 2004; Bowen, Doan et al. 2009; Pohl, Radacz et al. 2010; McInroy and Määttä 2011), and anchorage-independent survival of SW480 cell has been shown previously (Arber, Doki et al. 1997). We therefore first assessed if migratory capacity is affected by Cby knock-down, in wound scratch assays. To this end, cells were seeded to wells at equal density. We allowed at least 72 hours for the cells to reach confluence because E-cadherin expression after replating requires three days to reach maximum levels in both cell lines (data not shown). A scratch was then applied to the cells with a pipette tip, washed to remove and debris and to reduce the number of loosely attached cells in SW480 lines, and allowed to grow in various growth conditions. In order to assess differences in the speed of wound closure, we imaged the wound scratch at various time points in the same locations, marked on the bottom of the wells. We applied various growth conditions, including serum starvation prior to wound scratch and low serum media (2% FBS), as well as growth in complete media, but found no difference in speed of wound closure when measuring the distance between the cells closed to the edge in the same spot over time (data not shown).

However, while the Cby knock-down cells do not show slower wound closure, the pattern of movement is different. Cells can move, and do in tumors, either as single cells or as cellular sheets (Roerth 2009). Cby knock-down cells moved into the scratch area at the same speed, but as a sheet rather than the single cell movement observed in control cells. In the HEK293 cells, this difference was easily observed by phase contrast microscopy (Fig. 3-8A top panel). In SW480 cells, as described previously (Faux, Ross et al. 2004), two distinct populations exist. While most cells adhere tightly to the substrate, a refractile, less adherent population can be observed. These less adherent cells can detach and resettle in the wound area (Faux, Ross et al. 2004), generating the impression of single-cell migration. We had assessed that the number of these loosely attached cells is not statistically significant between vector and Cby sh cells in proliferation assays (data not shown). In order to exclude this population from the wound closure analysis as far as possible, we thoroughly washed the cells before and after application of the scratch, and rinsed the cells again in 24hr intervals. Measuring the distance between the wound edges as criterion to further exclude an impact of cells floating into the wound rather than

migrating, we did not observe a difference in speed of wound closure between vector-control and Cby sh cells (Fig. 3-8B, quantification not shown).

Since the SW480 cells appear to adopt a more epithelial phenotype at confluence, we proceeded to stain cells at the wound edge with phalloidin, to allow better determination of the moving cells' phenotype (McInroy and Määttä 2011). This technique revealed that the vector cells at the wound edge showed detachment of single cells from the wound edge that more often displayed spindle-shaped morphology (Fig. 3-8C). While the wound edge of Cby sh cells did show single cells, at approximately one third of the number in control cells, these cells consistently were cuboidal rather than spindle-shaped, thus the Cby sh cells maintained an epithelial organization of the actin cytoskeleton.

To quantify the findings, random images along the wound scratch were evaluated for cells disconnected from the confluent sheet. These single cells were scored for phenotype by cytoskeletal arrangement as spindle shaped when the cell was no wider than the nucleus, but were much longer. Otherwise, cells were scored as cuboidal. An equal number of visual fields was evaluated for each line. Since Cby sh cells were observed to be slightly more dense at the wound edge, though not statistically significant ($p=0.18$), the numbers obtained for single cells at the wound edge were normalized to the number of cells immediately at the edge of the wound but attached to the confluent sheet. We then compared the ratio of cuboidal, spindle shaped and total single cells near the wound edge between the lines (Fig. 3-8D). The numbers indicate clearly that there are significantly fewer single cells near the wound edge in Cby sh cells ($p=0.002$), and that the cells that are in the wound are more cuboidal (the ratio of spindle-shaped to cuboidal cells is 25 times lower than that in vector cells, $p=0.001$). Thus while the number of cells with a cuboidal cytoskeletal shape is similar in both lines, there are very few spindle-shaped cells near the wound edge in Cby sh cells ($p=0.001$). While the cells at the wound edge of Cby sh cells clearly extend structures into the wound, suggesting that they migrate, these cells remain firmly attached to adjacent cells. In contrast, vector cells are more likely to show single cells close to the wound edge, and in concordance with the general phenotype of this cell line, display a high ratio of spindle-shaped cell in the wound. This observation confirms that vector cells near the wound edge tend to show mesenchymal phenotype, despite their high density, whereas Cby knock-down cells maintain their epithelial actin cytoskeletal ring, even 48hrs after application of the scratch, and have less tendency to migrate as single cells. This supports that Cby sh cells have indeed undergone MET, and don't revert to mesenchymal phenotype even upon injury modeled by wound scratch (Klarlund and Block 2011).

It was thought that single cell migration is required at least for distant metastasis, the progression step that dramatically reduces patient survival (American Cancer Society 2008). This notion is questioned at this time since groups of cells have been found to invade the lymphatic system (Roerth 2009), and it is thought that cells enter the blood stream not as a result of extravasation, but rather rupture capillary walls as a result of proliferation (Christiansen and Rajasekaran 2006). To better assess potential changes in metastatic capacity we next used soft

agar assays, considered highly predictive of this characteristic *in vivo* (Roh, Green et al. 2001; Mani, Guo et al. 2008). As predicted from the changes in levels and localization of E-cadherin and ZO-1, Cby knock-down cells showed a dramatic reduction in colony formation in soft agar assays, throughout different seeding densities, reducing colony number by 50% in SW480 cells (Fig. 3-9A, B). Additionally we observed that colony size was smaller in the Cby sh cells. Consistent with the more dramatic phenotype in the HEK293 cells with respect to cell-cell junctions and levels of vimentin, Cby sh cells of that cell line show reduced capacity to grow anchorage-independently by nearly two orders of magnitude, to 3% of the number of colonies observed in control cells, at two different seeding densities (Fig. 3-9C). To ascertain that this difference was not caused by a lower survival rate after replating, we plated an aliquot of each line into a regular dish when seeding a soft agar assay, and counted surviving cells after 24hrs by trypan blue. The cells did not show a difference in plating efficiency in this test.

Taken together, these results show that Cby knock-down leads to a mesenchymal-to-epithelial reversion in HEK293 and SW480 cells, and reduces capacity for single-cell migration and metastasis in SW480 colon cancer cells. These findings immediately raise the question as to the mechanism by which these changes are effectuated.

Cby knock-down leads to increased amounts of E-cadherin mRNA in HEK293 cells

β-Catenin is known to directly transcriptionally activate EMT-associated target genes, including the E-cadherin repressors *snail*, *slug* and *twist* (Hlubek, Spaderna et al. 2007; Prosperi and Goss 2010). Since β-catenin localizes increasingly to the plasma membrane in the Cby knock-down cells while β-catenin total protein levels do not change, we next tested if nuclear β-catenin signaling was reduced. To this end, we performed a Topflash assay. We used the SW480 cells for the assay although these cells show the milder phenotype, compared to the HEK293 cells, because they are more easily transfected. Indeed, we found a 50% reduction in Topflash activation in the Cby knock-down cells (Fig. 3-10A). This finding suggests that the observed changes may be due, at least in part, to a reduction in nuclear β-catenin signaling. We did, however, not observe reduced levels of nuclear β-catenin in fractionation assays (data not shown). This may be due to the binding preferences of E-cadherin, that in SW480 cells binds the same fraction of β-catenin LEF-1 binds to (Gottardi, Wong et al. 2001). Since the majority of β-catenin present in SW480 cells appears to have little signaling activity (Wong and Gumbiner 2003), the change in concentration of signaling active β-catenin may not become visible by fractionation or staining for total β-catenin.

However, reduced nuclear β-catenin signaling could reduce repression of E-cadherin gene transcription, and the observed MET-like changes in the HEK293 and SW480 cells could be ascribed to the increased levels of E-cadherin (Fig. 3-4C), as demonstrated by Gottardi et al. (Gottardi, Wong et al. 2001). The gene coding for E-cadherin, *cdh1*, is controlled by multiple factors (Peinado, Portillo et al. 2004). The E-cadherin gene repressor slug is under direct transcriptional control of β-catenin (Hlubek, Spaderna et al. 2007), thus reduced nuclear

signaling of β-catenin may be responsible for the reduced E-cadherin gene repression, allowing higher levels of transcription. We therefore next investigated by RT-qPCR if mRNA levels of E-cadherin were present. Our results indicate that E-cadherin mRNA is increased by about 2.4-fold, across all three monoclonal lines of the HEK293 cells, and 1.3-fold in the SW480 cells. Levels of mRNA are regulated not only at the transcriptional level but also via their stability. However, the observed increase in mRNA levels explains, at least in part, the observed increase in protein levels, suggesting that Cby knock-down induces MET-like changes in SW480 and HEK293 cells by allowing for derepression of *cdh1*.

Discussion

Aberrant Wnt-β-catenin signaling is involved in tumorigenesis of various cancers, most notably colon cancer, where it is thought to underlie as many as 90% of spontaneous tumors (Herbst and Kolligs 2007). In these tumors, β-catenin is stabilized, in the majority by mutations of the APC tumor suppressor, but mutations of Axin and β-catenin have also been reported (Liu, Dong et al. 2000). The stabilization of β-catenin leads to increased transcriptional activation of its target genes, which can result in EMT. A switch in phenotype from epithelial to mesenchymal is associated with numerous changes, including loss of adherens and tight junctions, changes in cytoskeletal arrangement and morphology and changes in the levels of various proteins, like decreased E-cadherin and increased vimentin (Christiansen and Rajasekaran 2006).

We show here that Cby knock-down in cells of mesenchymal phenotype leads to increased presence of β-catenin and E-cadherin at the plasma membrane (Fig. 3-2), along with dramatically increased levels of E-cadherin (Fig. 3-3C) and reduced nuclear β-catenin signaling (Fig. 3-10A), suggesting that Cby knock-down induces MET. This hypothesis is further supported by decreased levels of vimentin and increased levels of ZO-1 in HEK293 cells, with ZO-1 localizing to the plasma membrane (Fig. 3-3), and a characteristic change in the actin cytoskeleton from stress fibers to a cortical actin ring (Fig. 3-6), associated with an epithelial phenotype (Christiansen and Rajasekaran 2006). Perhaps more relevant to tumorigenesis, however, is the change in cell behavior. Both HEK293 and SW480 Cby sh cells maintain cell cohesion when injured in a wound scratch assay, in contrast to the single cell migration observed in the control lines (Fig. 3-8). Most remarkably, Cby sh cells lose the ability to form colonies in anchorage-independently (Fig. 3-9). This ability has been shown to correlate with formation of metastases from xenografts in mice (Cifone and Fidler 1980), and is widely used to predict this property (Arber, Doki et al. 1997; Mani, Guo et al. 2008; Ha, Kim et al. 2010). The changes observed in the Cby sh cells therefore clearly point to a relevant change in behavior in both cell lines consistent with the concept of MET.

Cby is known to interact with β-catenin, and an effect of this interaction was shown in Cby's function to shuttle it out of the nucleus in conjunction with 14-3-3 proteins (Li, Mofunanya et al. 2008; Li, Mofunanya et al. 2010). 14-3-3 proteins have been reported to sequester proteins in the cytoplasm (Kumagai and Dunphy 1999; Yang, Winkler et al. 1999;

Brunet, Kanai et al. 2002). No function of Cby is known as of yet at the plasma membrane. However, our findings presented here make an at least indirect effect of Cby on β-catenin-E-cadherin complexes likely. Since release of β-catenin from E-cadherin leads to loss of E-cadherin from the plasma membrane (Nelson and Nusse 2004), the adherens junction protein E-cadherin is a likely mediator in the chain of events that leads to the observed MET in Cby sh cells. Loss of E-cadherin is considered a major factor in tumor progression (Peinado, Portillo et al. 2004), and is associated with poor prognosis (Reichert, Müller et al. 2000). Conversely, re-expression of E-cadherin in tumor cell lines has been shown to ablate invasive growth (Nawrocki-Raby, Gilles et al. 2003), but also to occur in metastases (Peinado, Portillo et al. 2004). Any protein affecting E-cadherin protein levels in invasive tumors may therefore act as a double-edged sword, preventing further invasion of the primary tumor but promoting successful formation of metastases from cells that have reached new host tissues. We did not detect a direct interaction of Cby with E-cadherin, nor a competition of Cby with E-cadherin for β-catenin (data not shown), however, the mechanism that leads to increased localization of β-catenin to the plasma membrane upon loss of Cby may be indirect and involve nuclear β-catenin signaling.

The *cdh1* gene coding for E-cadherin is controlled by a complex interplay of factors repressing or promoting transcription (Peinado, Portillo et al. 2004), and β-catenin is part of this control, repressing E-cadherin gene transcription (Novak, Hsu et al. 1998; Masszi, Fan et al. 2004), both directly (Novak, Hsu et al. 1998) and by induction of the E-cadherin gene repressor snail1 (Yook, Li et al. 2006). We show that by Topflash assay, nuclear β-catenin-mediated signaling is reduced in the Cby sh lines. It has been shown that β-catenin signaling contributes to EMT in MDCK Madin-Darby canine kidney cells (Reichert, Müller et al. 2000), and that β-catenin inhibition reduces anchorage-independent growth and invasiveness in SW480 cells (Roh, Green et al. 2001). Furthermore, Scholer-Dahirel et al. show that that continued β-catenin signaling is required for maintenance of proliferation and dedifferentiated state in APC-mutant colorectal tumor xenografts in mice, such that β-catenin inhibition in established tumors leads to reduced growth and to differentiation (Scholer-Dahirel, Schlabach et al. 2011). The finding that Cby knock-down reduces β-catenin signaling therefore reveals one mechanism by which the observed MET can be effectuated.

In accordance with this notion, E-cadherin re-expression alone is sufficient to reduce nuclear β-catenin signaling, in SW480 cells (Gottardi, Wong et al. 2001), but also in BZR transformed human bronchial epithelial cell line carrying the SW40 large T-antigen and a v-Ha-ras oncogene (Nawrocki-Raby, Gilles et al. 2003). The observed changes can therefore result from the higher levels of E-cadherin.

Interestingly, Perrais et al. show that E-cadherin-mediated growth inhibition is independent of β-catenin/TCF transcriptional activity while it does depend on E-cadherin binding to β-catenin (Perrais, Chen et al. 2007). The fact that we do not observe significant reduction of growth in the Cby sh cells, but do find changes in β-catenin-mediated characteristics like anchorage-independent growth (Orford, Orford et al. 1999) increases the probability that the

MET-like changes observed depend more on reduced nuclear β-catenin/TCF signaling than on E-cadherin/β-catenin complex-mediated signaling. This notion is further supported by the finding that re-expression of E-cadherin does not cause significant changes in MDA-MB 231 breast cancer and TSU-Pr1 prostate cancer cells that do not have activation of Wnt-signaling (Wong and Gumbiner 2003).

The question remains why β-catenin signaling is reduced in Cby knock-down cells. We have previously shown that Cby functions to shuttle β-catenin out of the nucleus and is required for 14-3-3 mediated sequestration of β-catenin in the cytoplasm (Li, Mofunanya et al. 2008). Several other proteins are known to export β-catenin from the nucleus, including Axin (Cong and Varmus 2004) and APC (Neufeld, Zhang et al. 2000), and the truncated APC in SW480 cells still binds β-catenin (Henderson 2000). It is not clear, at this point, if the signaling-active form of β-catenin is preferentially bound by any of the shuttling proteins, so that it is possible that the theoretically reduced cytoplasmic sequestration of possibly preferentially signaling inactive β-catenin due to the reduced levels of Cby leads to sufficient competition between signaling-active and signaling-inactive β-catenin in the nucleus to tip the balance of the complex control of *cdh1* transcription (Peinado, Portillo et al. 2004). This would lead to de-repression of the gene. Increased levels of E-cadherin protein would then bind more signaling-active β-catenin, further reducing repression of the *cdh1* gene, until other control mechanisms that normally operate in epithelial cells limit E-cadherin protein levels.

In summary, we show here that Cby knock-down results in MET-like changes both in HEK293 human embryonic kidney cells and in SW480 human adenocarcinoma cells, with dramatically increased levels of E-cadherin and drastically reduced capacity for anchorage-independent growth. These parameters suggest reduced metastatic capacity of the cells. However, they also suggest that if Cby is inhibited in cells that have reached a new host site as mesenchymal cells, formation of new secondary tumors might be promoted. Our findings therefore underline the inherent difficulties in preventing metastasis by inhibiting EMT that result from MET, the reversal of EMT at new host sites.

Materials and Methods

Cell lines and cell culture

HEK293 and SW480 cells were obtained from ATCC and maintained in DMEM with 10% Fetal bovine serum (Denville Scientific Inc.) with 100U/ml Penicillin-Streptomycin (Gibco), at 37°C with 5% CO_2. To generate stable Cby knock-down lines, cells were transfected with Sure Silencing^{TM} pGeneClip^{TM} plasmid, containing non-coding sequence (vector control) or short hairpin RNA for Cby (NM_015373) (GGCTGAAAGTGGACATCTTAT) under control of the U1 promoter, with a puromycin resistance gene. For transfection, Expressfect (Denville Scientific Inc.) was used according to manufacturer's instructions. Transfected cells were selected with 2.5μg/ml Puromycin (Gemini BioProducts) and maintained in medium

supplemented with 1.25µg/ml Puromycin. Continued knock-down of Cby was tested periodically. For HEK293 cells, cells were seeded at clonal density and single clones were isolated to generate three monoclonal lines for each vector and Cby sh cells.

Western blot analyses

Cells were lysed in RIPA (radioimmunoprecipitation assay) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 µg/ml Aprotinin, 5 µg/ml Leupeptin, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS) with Complete protease inhibitor cocktail (Roche) and sonicated for 10 seconds at 18% amplitude using a Branson Digital Sonifier, or lysed in immunoprecipitation buffer (IPB) (20mM Tris.HCl pH 8.0, 135mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 1% Triton X-100, 10% Glycerol) with Complete protease inhibitor cocktail (Roche) and rotated at 4°C for 15minutes. Lysates were cleared by centrifugation at 15,000x *g* for 10 minutes at 4°C. For Western blot analysis, protein concentration of the lysates was adjusted with lysis buffer to achieve equal protein concentration of all samples, after assaying for protein concentration using the BioRad Dc protein assay kit (BioRad). 5x loading dye (250mM Tris-HCl pH 6.8, 10% SDS, 500mM β-mercaptoethanol, 0.02% Bromophenol Blue, 50% glycerol) was added to a final concentration of 20% of sample volume, and lysates were boiled for 5 minutes at 97.5°C. Proteins were separated by SDS-PAGE and transferred to a 0.45µm pore size nitrocellulose membrane (BioRad) in transfer buffer (20mM Tris acetate pH 8.3, 0.1% SDS, 20% isopropanol). Transfer was checked using Ponceau S stain (0.1% Ponceau S (Sigma) w/v, 0.5% acetic acid v/v). Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST; 20mM Tris base, 137mM NaCl, 0.1% Tween-20), incubated with primary antibody in blocking milk, washed in TBST, incubated in secondary antibody in blocking milk and developed depending on the secondary antibody used. For HRP-conjugated secondary antibodies (Jackson Laboratories), HyGlo Quick Spray (Denville Scientific Inc.) was used for chemiluminescent detection. For detection of fluorescent secondary antibodies, LI-COR Odyssey Infrared Imager was used. Densitometry was performed using ImageJ for blots developed with chemiluminescence, and Odyssey software (version 3.0) for blots read with the LI-COR Odyssey Imager.

Immunofluorescence and phase-contrast microscopy

For immunofluorescent staining, cells were seeded to glass coverslips (SW480 cells), or glass coverslips coated with collagen VI (HEK293 cells) at equal density. Cells were cultured for 48 to 72 hrs prior to treatment. Cells were then washed with PBS (phosphate-buffered saline) fixed with fresh 4% formaldehyde in PBS for 20minutes at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes at room temperature, or fixed with methanol acetone (1:1, v/v) for 5 minutes at room temperature, as indicated. Cells were blocked with 1% BSA (bovine serum albumin, Sigma) in 0.1% Triton X-100 in PBS for 1hr at room temperature, incubated with primary antibody as indicated, washed, and incubated with fluorophore-

conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and coverslips were mounted onto glass slides with Fluoromount-G (Southern Biotech). Cells were analyzed using a Zeiss LSM 510 META confocal laser scanning microscope with Zeiss LSM 510 software.

For phase-contrast microscopy, cells were plated into culture dishes, treated as described, and imaged using a DFC300 FX camera and Leica Application Suite software version 3.0 with a Leica DM IL inverted contrasting microscope.

Wound scratch assays

Cells were grown to confluence in 35mm wells, in triplicate wells. For HEK293 cells, wells were coated with collagen VI prior to plating, to increase adhesion of the cells. Confluent cells were washed carefully with PBS, and a scratch was applied with a plastic pipette tip. The scratch was marked on the bottom of the dish, and the wound area was imaged immediately (0hrs) and at 24hrs intervals at the same locations, guided by the markings. SW480 cells were washed with PBS prior to imaging, to eliminate refractile cells with low adhesion from images and to reduce their impact on wound closure. To evaluate speed of wound closure, the largest distance between cells at a point defined by the marking was measured using ImageJ (NIH). For SW480 cells, this was the distance between the wound edges. For HEK293 cells the distance measured the distance between the farthest advanced cells.

Soft agar assays

SW480 cells were plated in complete medium with 0.3% agarose at $2.5x10^3$, 10^4 and $5x10^4$ cells/well onto 0.6% agarose in complete medium into 35mm wells, in triplicate. For HEK293 cells, 10^4 and $5x10^4$ cells/well were used. The agar was left at room temperature for 45minutes to solidify before incubation at 37°C with 5% CO_2, and complete medium was added 4 hours later. Cells were fed three times weekly (Faux, Ross et al. 2004). 4 weeks after plating, cells were stained with 0.005% crystal violet (Sigma) in PBS for 1hr at room temperature, and thoroughly washed and destained with PBS for several hours. Wells were photographed using a FluoChem^{TM}8900 Imaging System with AlphaEaseF software. Colonies were counted under a dissection microscope, counting all visible colonies. For wells containing more than 1000 colonies, 5 fields marked by graph paper were counted and total colony number was calculated based on this count.

TopFlash assays

Cells were seeded at 10^5 cells/well in triplicate and allowed to adhere for 24hrs before transfection with 25ng TopFlash or FopFlash luciferase plasmid (Korinek, Barker et al. 1997), in combination with 25ng Renilla luciferase plasmid (pRL-TK), using Expressfect (Denville

Scientific Inc.) according to manufacturer's directions. 48hrs post infection, cells were lysed in the well with lysis buffer S, and the luciferase assay was performed using a dual luciferase reporter assay system (Promega) in a luminometer (Berthold Technologies) with MicroWin 2000 software (Mikrotek Laborsysteme, Germany). 10µl lysate was used for the luciferase assay, and each well was assayed in duplicate. Luminescence was measured for 10 seconds after a 2 second delay post injection of reagent, for each luciferase. Transfection efficiency was corrected for by calculation the ratio of firefly to renilla luciferase activity after correction for blank measurements. The baseline activation indicated by activation of the mutant FopFlash was subtracted, and activation was then normalized to vector.

RT-qPCR assays

For quantitative analysis of mRNA, RNA was isolated using the RNEasy Mini kit (Qiagen), following manufacturer's instructions. Potentially contaminating DNA was digested in the process using RNase-free DNase (Qiagen). RNA quality was measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Only RNA with a A260/280 >1.8 was used, to exclude DNA contamination (Taylor, Wakem et al. 2010). Subsequently cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), using the following thermal cycler times: 25°C for 10min, 37°C for 120min, 85°C for 5min, 4°C thereafter.

For the qPCR reaction, Fast SYBR Green master Mix (Applied Biosystems) was mixed with 200nM of forward and reverse primer, and 20ng cDNA, for a total reaction volume of 20µl. Each sample was assayed in triplicate for each primer pair. The reaction was carried out and analyzed using the StepOne Plus Real Time PCR system (Applied Biosystems). The cycler settings were as follows: 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. The melt curve followed immediately with 95°C for 15 seconds, 60°C for 1 minute, followed by 0.3°C increments to 95°C for 15 seconds (Fig. 3-11C). Primers used were E-cadherin (#1018 rtprimerdb.org, forward primer: AGTGCCAACTGGACCATTCA, reverse primer: TCTTTGACCACCGCTCTCCT, amplicon size 314bp) (Ueda, Yamashita et al. 2002; Pattyn, Robbrecht et al. 2006) and GAPDH (#813 rtprimerdb.org, forward primer: TGCCAAATATGATGACATCAAGAA, reverse primer: GGAGTGGGTGTCGCTGTTG, amplicon size 121 bp) (Nielsen, Birkenkamp-Demtroeder et al. 2003; Pattyn, Robbrecht et al. 2006). Primer-Blast (NIH) searches for the primers revealed one potential unintended target for the E-cadherin primer pair at a product size of 145bp, however, this target would have 5 and 6 mismatches with the forward and reverse primers, respectively. No amplicon of that size was seen on the agarose gels routinely run after the qPCR reaction. All other unintended targets for the primers used had amplicon sizes >1000bp. Primers were procured from the DNA Sequencing Facility, Stony Brook, NY.

Standard curves were established for the primers (Fig. 3-11A), showing borderline-high reaction efficiency for the E-cadherin primers, however, agarose gel analysis revealed no

amplicon at other than the expected size. Melt-curve analysis was performed for all assays, and the no-template-control (NTC) samples were run on an agarose gel, showing that the amplification shown at high cycle numbers (Fig. 3-11B) resulted from non-specific amplification at amplicon sizes smaller than the expected size visible in template-positive samples (Fig. 3-11D), and this amplification in the NTC was observed mainly in the GAPDH controls. This phenomenon has been described especially for SYBR Green I (Bustin, Benes et al. 2009; Taylor, Wakem et al. 2010), and was not considered an impediment to the assay since quantification cycle (C_q) numbers were at least 9 cycles lower than the C_q for the no-template controls in all assays.

Statistical analysis

Data was analyzed using Microsoft Excel. Statistical significance was determined by Student's *t*-test, and a $p<0.05$ was considered statistically significant. Graphs show data ±SD or SEM, as indicated.

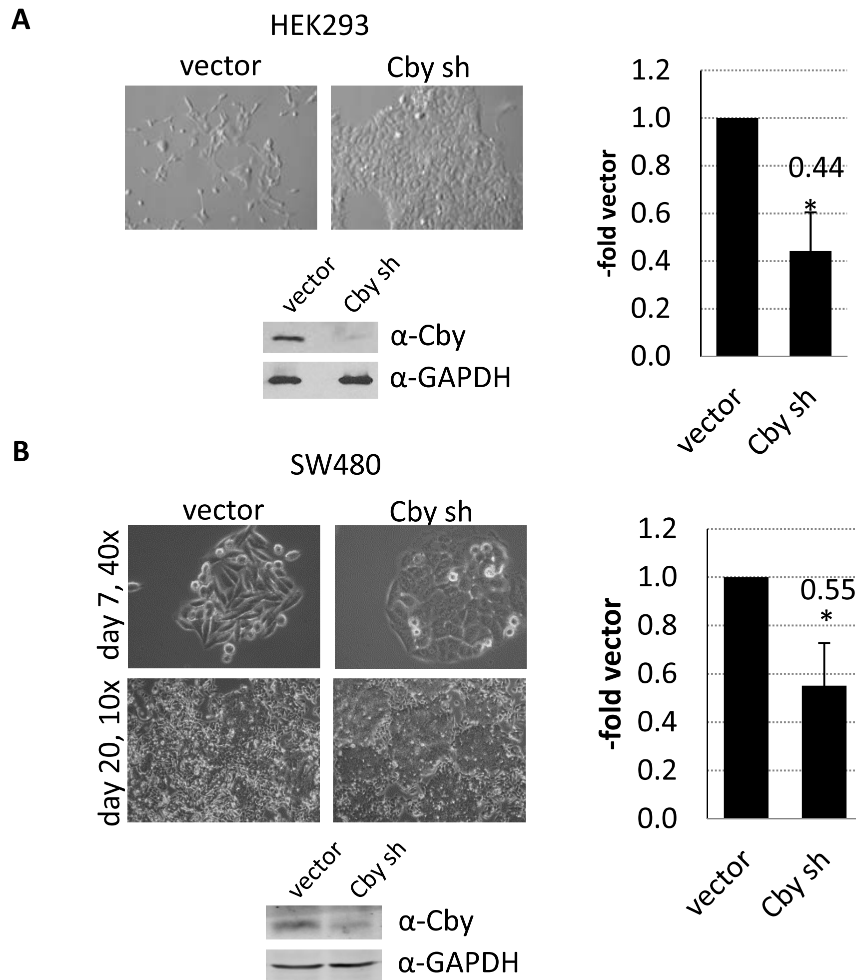


Figure 3-1 Cby knock-down changes cell morphology to an epithelial phenotype in HEK293 and SW480 cells.

A. HEK293 cells seeded at equal density were imaged by phase-contrast microscopy at 20x magnification. Western blot analysis of protein levels are shown below the images. Cells were lysed in RIPA buffer, and analyzed by Western blot with α-Cby (Takemaru, Yamaguchi et al. 2003) and α-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (Biodesign Int.) as loading control, with HRP-conjugated secondary antibodies. Densitometry was performed with ImageJ (NIH) to indicate relative remaining levels of Cby protein. Graph shows average knock-down over all three monoclonal lines from at least three independent experiments ±SD. *$p<0.05$

B. SW480 cells were seeded at 10^4cells/well to 6-well wells and photographed after 7 and 20 days as in A, at the magnification indicated. Western blot analysis was performed in A. Graph as in A.

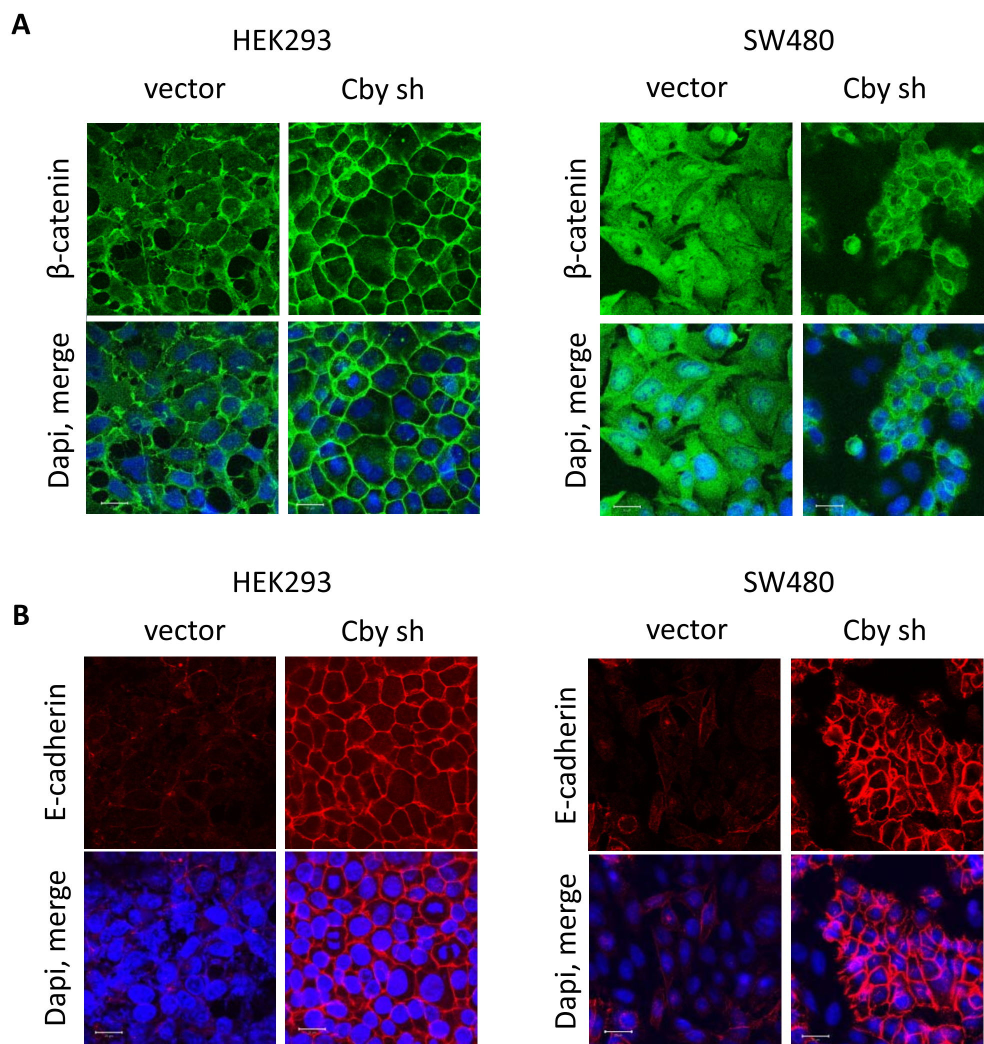
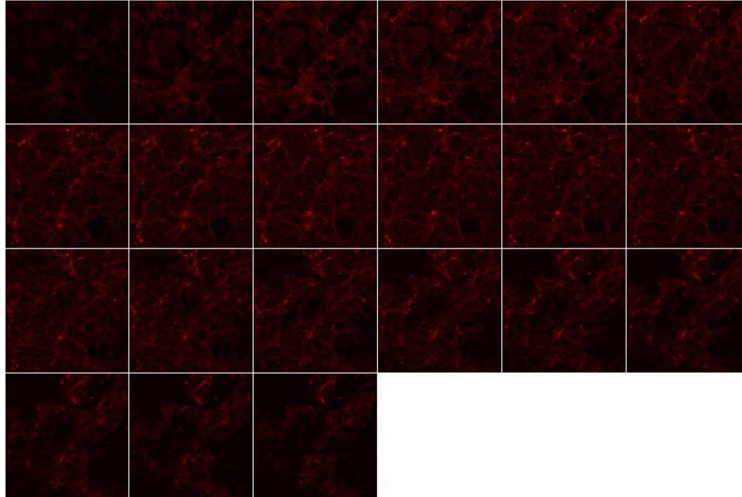


Figure 3-2 Cby knock-down increases levels of β-catenin and E-cadherin at the plasma membrane.

A. Cells were seeded at equal density and fixed after 48hrs with methanol/acetone (1:1, v/v). Cells were immunostained with α-β-catenin (BD Biosciences), followed by DL488-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and counterstained with Dapi. Cells were imaged on confocal microscope at 63x magnification. Scale bars are 20µm.
B. Cells were treated as in A. but immunostained with α-E-cadherin (BD Biosciences), followed by DL549-conjugated secondary antibody, and imaged as in A.

HEK293 vector, α-E-cadherin



HEK293 Cby sh, α-E-cadherin

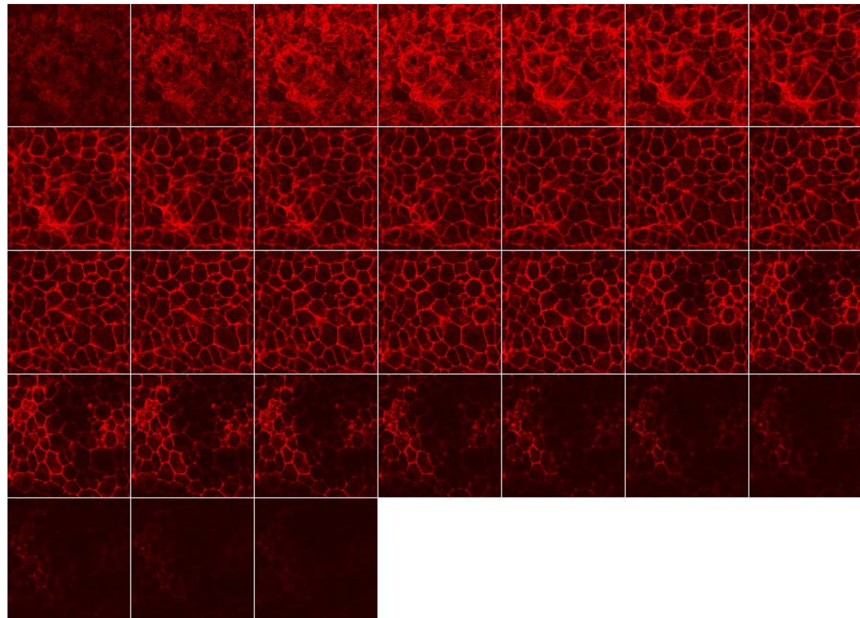


Figure 3-3 Supplemental images to Figure 3-2.
Cells were treated as described for Figure 3-2 B. z-stack images were taken at 0.3 µm slice thickness. Dapi stain not shown to allow better visibility of the E-cadherin distribution.

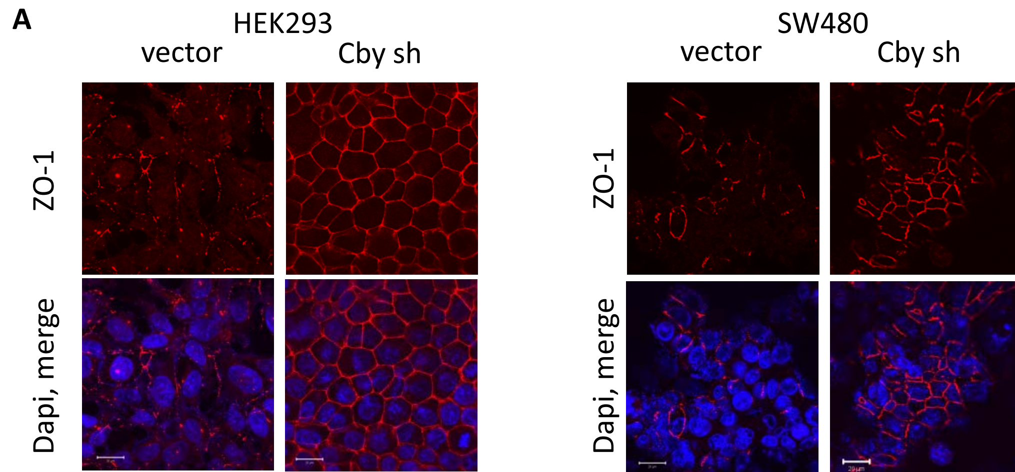
Figure 3-4: Cby knock-down leads to changes in protein levels and localization towards an epithelial phenotype.

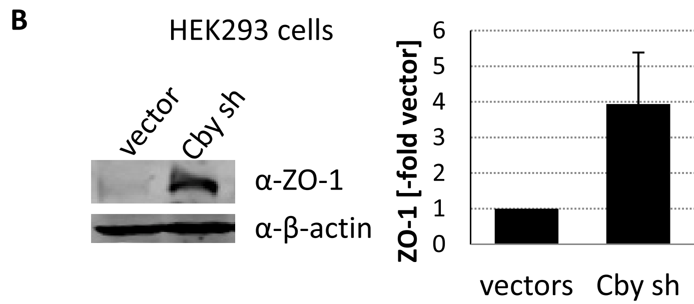
A. Localization of ZO-1 protein. Cells were seeded at equal density and fixed after 48hrs with methanol/acetone (1:1, v/v). Cells were immunostained with α-ZO-1 (BD Biosciences), followed by DL549-conjugated secondary antibody, and Dapi. Confocal images were taken at 63x magnification. Scale bars show 20μm.

B. Levels of ZO-1 protein in HEK293 cells. Cells were lysed with RIPA buffer. Western blot analysis was performed using α-ZO-1 and α-β-actin (Genscript) antibodies, with fluorescent secondary antibodies. Densitometry was performed using LI-COR Odyssey software, and relative protein levels averaged for two independent experiments are shown in the graph. Error bars indicate SD.

C. Protein levels of E-cadherin and vimentin by Western blot analysis. Cells were lysed with IPB with 20μM N-ethylmaleimide. Western blot analysis was performed using the primary antibodies indicated (α-vimentin: NeoMarkers), followed by HRP-conjugated secondary antibodies. GAPDH is used as loading control. Band intensity was measured with ImageJ (NIH), and set in ratio to the loading control. Values for vector and Cby sh lines were averaged and normalized to vector, using three independent experiments. Error bars indicate SEM. *$p<0.05$

D. Western blot analysis of E-cadherin protein levels in SW480 cells. Cells were grown to subconfluence, lysed with RIPA buffer and immunoblotted with primary antibodies as indicated. Fluorescent secondary antibodies were used, and densitometry was performed using LI-COR Odyssey software. The graph shows E-cadherin protein levels normalized to vector from a representative experiment of at least three independent repeats.





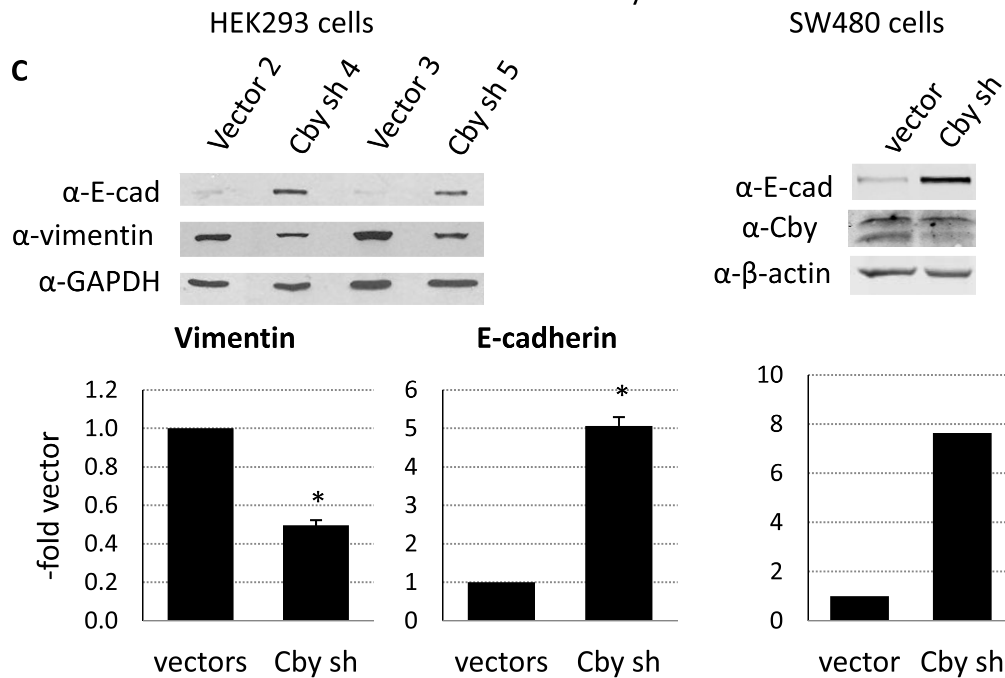
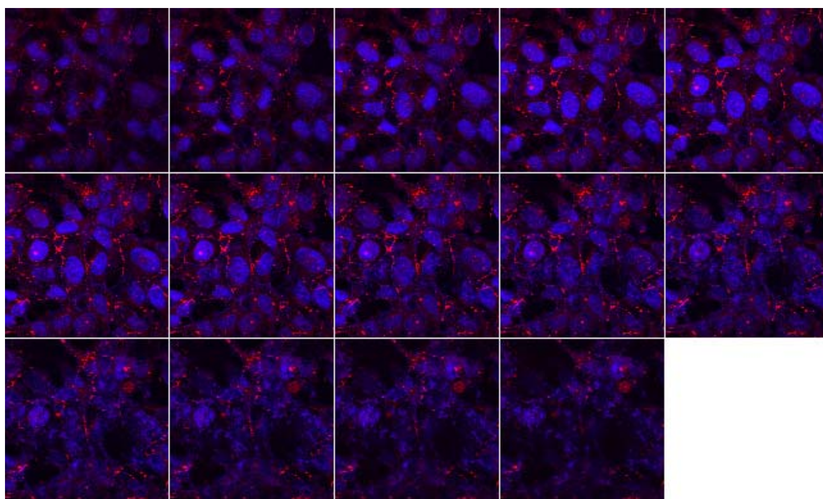


Figure 3-4 Cby knock-down leads to changes in protein levels and localization towards an epithelial phenotype.

HEK293 vector, α-ZO-1



HEK293 Cby sh, α-ZO-1

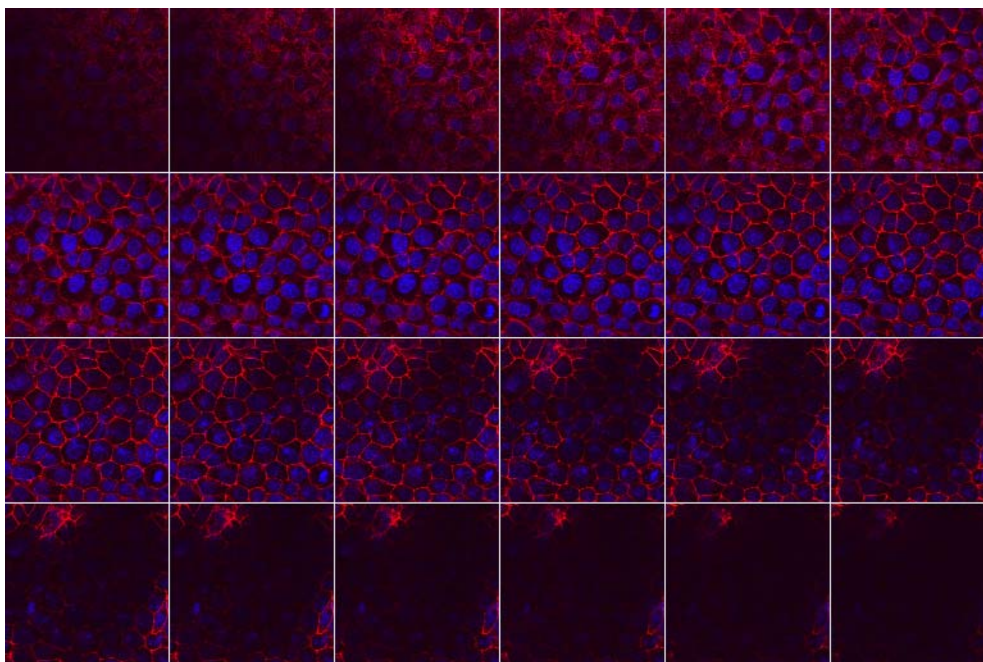


Figure 3-5 Supplemental images to Figure 3-4.
Cells were treated as described for Figure 3-4 A. z-stack images were taken at 0.3 μm slice thickness.

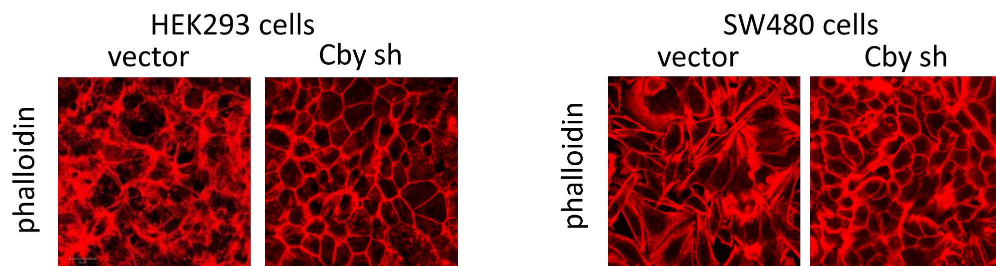
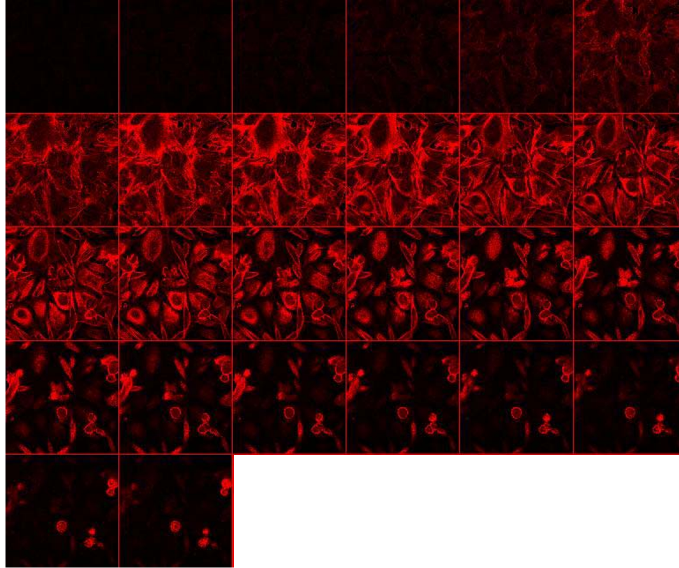


Figure 3-6 The actin cytoskeleton changes toward a cortical actin ring in Cby knock-down cells.

Cells were seeded at equal density and allowed to adhere for 48hrs before fixing with 4% formaldehyde in PBS, permeabilizing, and staining with Tritc-conjugated phalloidin. Counterstain with Dapi not shown. Images were taken by confocal laser-scanning microscopy at 63x magnification.

SW480 vector, Tritc-phalloidin



SW480 Cby sh, Tritc-phalloidin

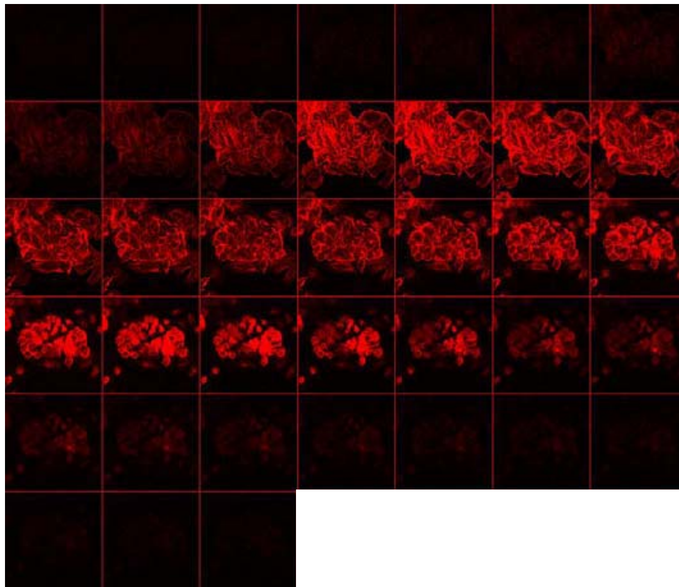


Figure 3-7 Supplemental images to Figure 3-6.
z-stack images at 0.3µm slice thickness of cells treated as described for figure 3-6. Dapi stain not shown so that F-actin distribution can be better evaluated.

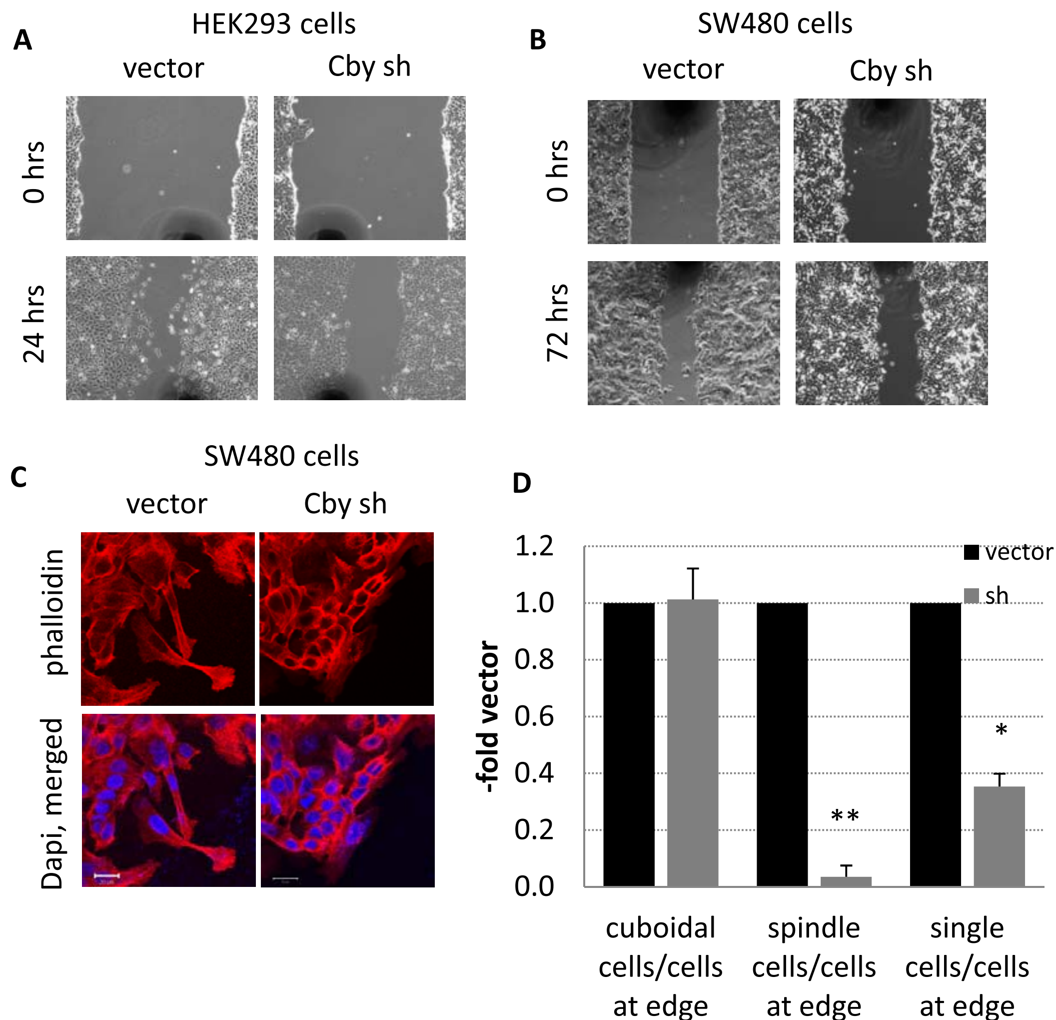
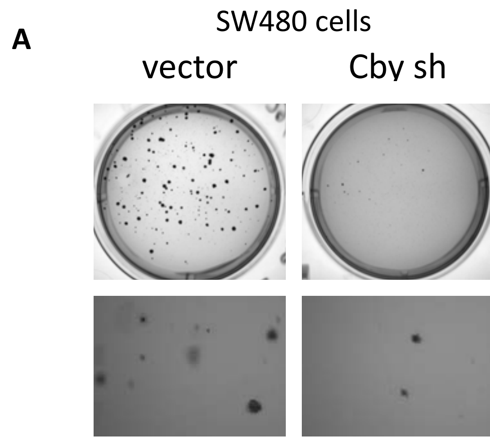
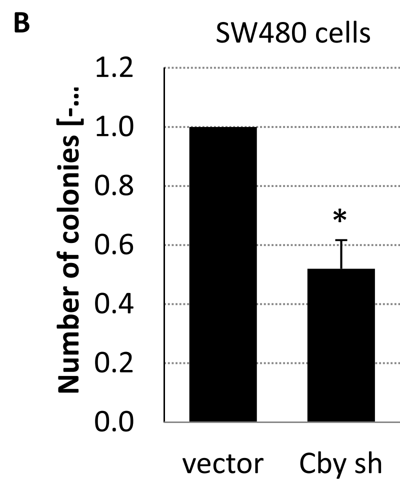


Figure 3-8 Cby knock-down leads to changes in migratory behavior.
A. HEK293 cells were seeded at equal density and allowed to grow to confluence for at least 72hrs. A scratch was applied and marked on the bottom of the plate to allow repeated imaging of the same area. Phase-contrast images were taken after the time indicated.
B. SW480 cells were treated as described in A.
C. SW480 cells were seeded to coverslips. 48hrs after application of a scratch, cells were fixed and stained with Tritc-phalloidin, counterstained with DAPI. Confocal images were taken along the scratch.
D. Quantification of the migration pattern in C from random images. Cells were scored as follows: Cells immediately facing the wound edge were counted as "cells at edge". Cells in the wound were scored according to their shape by phalloidin stain, as spindle shaped or cuboidal. *p<0.05 (0.002), ** p<0.001

Figure 3-9 Cby knock-down reduces anchorage-independent growth.
A. SW480 cells were trypsinized to single cells and seeded in 0.3% agar in DMEM on top of a layer of 0.6% agar in DMEM. Cells were fed with complete medium. After 4 weeks, colonies were stained with 0.005% crystal violet in PBS and counted under a dissection microscope. Representative images of colonies in the plate (top) and under phase-contrast microscopy at 10x magnification (bottom) are shown.
B. The graph depicts the number of colonies normalized to vector averaged for three independent experiments in triplicates ±SEM. $*p<0.002$
C. Graphic representation of the relative number of colonies in HEK293 cell lines, averaged for 2 monoclonal lines each in 3 independent experiments. Error bars show SEM, $*p<0.05$





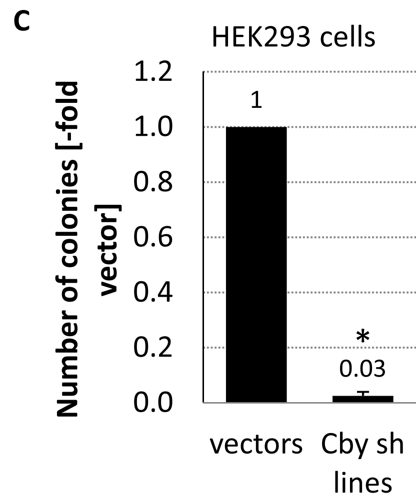


Figure 3-9 Cby knock-down reduces anchorage-independent growth.

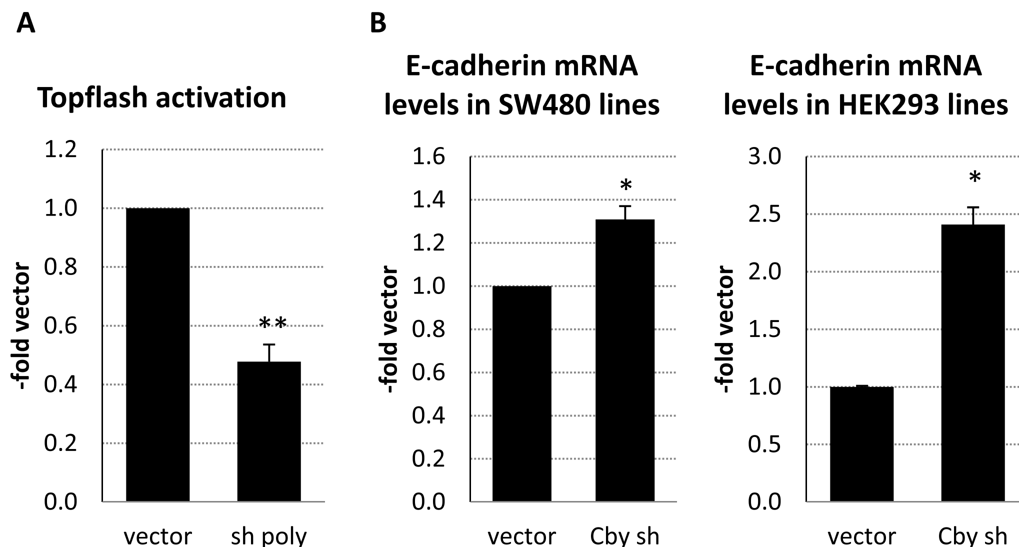


Figure 3-10 E-cadherin transcription is reduced, likely as a result of decreased nuclear activity of β-catenin.
A. TopFlash activation in SW480 cells. Topflash assays were performed as described in Materials and Methods. FopFlash activation was subtracted as background. TopFlash activation is shown normalized to vector, averaged over five independent experiments. Error bars represent SEM. **$p<0.001$.
B. E-cadherin mRNA levels by RT-qPCR. Assays were performed as described in Materials and Methods. Graphs show E-cadherin mRNA levels normalized to vector in SW480 (left) and HEK293 (right) cells.

Figure 3-11: Supplemental information to Figure 3-10: RT-qPCR validation
A. Standard curves for E-cadherin and GAPDH primer pairs. The legend provides the details on slope, efficiency and R^2 value. Standard curve was determined once in duplicate. Cq: quantification cycle, sometimes referred to as threshold cycle
B. Representative amplification plot. The green curves show the melt curves of the template-containing samples. The red curves show the no-template controls.
C. Melt curve for the same experiment as in B. Note the different peak in the no-template controls.
D. 2% Agarose gel showing no-template controls, with Cby sh samples as positive control for expected size of the respective amplicons.

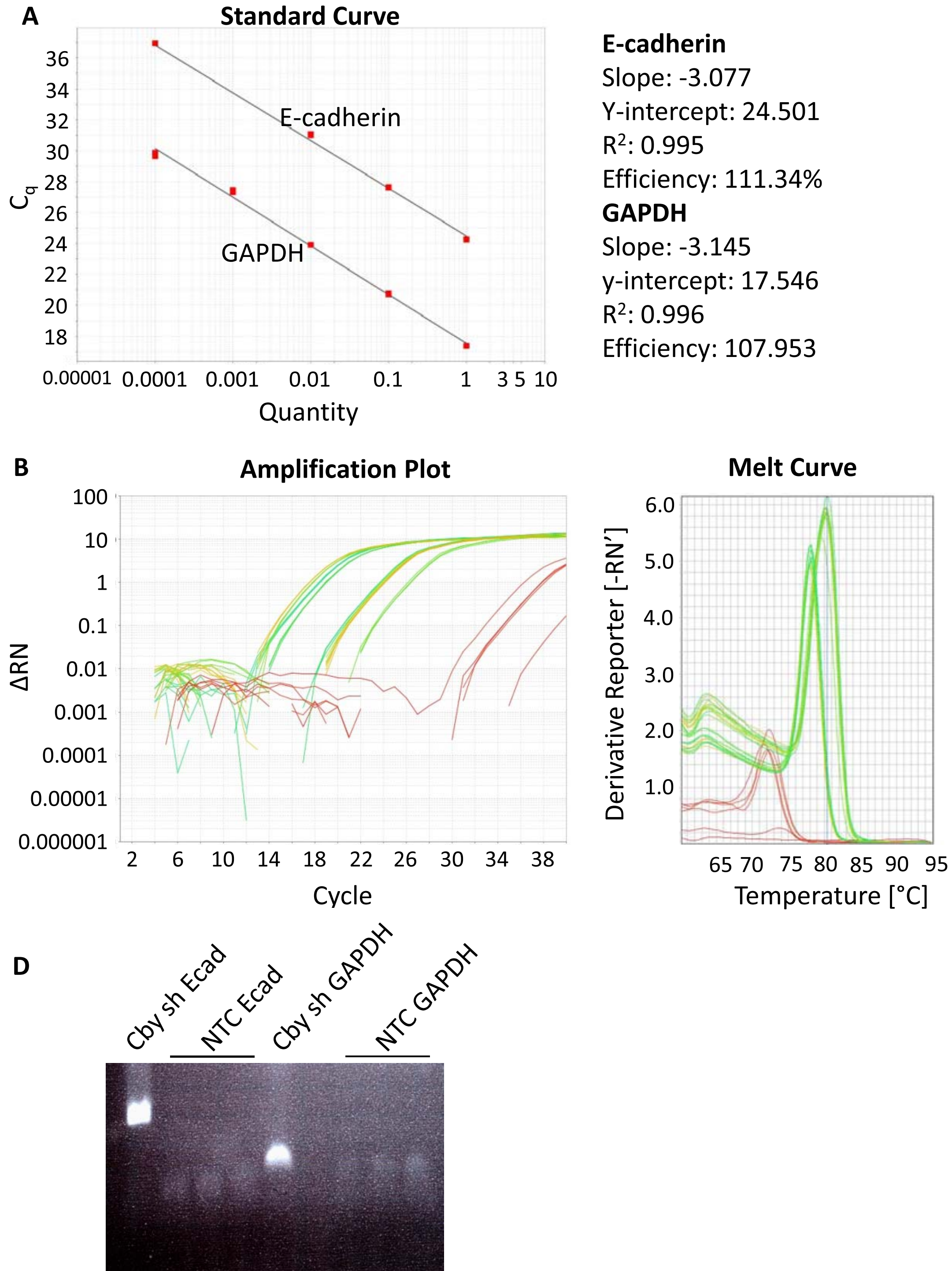


Figure 3-11 Supplemental information to Figure 3-10: RT-qPCR validation

Chapter 4 Discussion and Future Directions

The evolutionarily conserved protein Cby has been functionally characterized as Wnt/β-catenin antagonist, interacting directly with β-catenin (Takemaru, Yamaguchi et al. 2003). β-catenin functions downstream of Wnt-signaling in the nucleus as transcriptional co-activator for Tcf/Lef transcription factors, promoting gene transcription towards proliferation, migration, and anchorage-independent growth, and can prevent differentiation of cells including that of mouse ES cells into cardiomyocytes and differentiation of adipocyte precursors into adipocyte specify. These functions of β-catenin are associated with pathogenic processes including pulmonary fibrosis, cardiovascular disease, but most notably cancer, when dysregulated (Moon, Kohn et al. 2004). This study shows that Cby can counteract nuclear signaling of endogenous β-catenin even when dysregulated. I show that in SW480 cells that bear high levels of non-mutated β-catenin, Cby overexpression leads to relocalization of β-catenin from a predominantly nuclear to a more cytoplasmic distribution. Concomitantly, nuclear signaling is reduced and growth of confluent cells slows down. These findings are consistent with previous findings that Cby exports β-catenin from the nucleus in cooperation with 14-3-3 proteins (Li, Mofunanya et al. 2008; Li, Mofunanya et al. 2010). Relevance of this mechanism is further supported by my finding that serine-to-alanine mutation of the phosphorylation site required for Cby-14-3-3 interaction ablates Cby's effect toward β-catenin in the SW480 cells.

When analyzing causes for the growth reduction, I found significantly reduced levels of cyclin D1. Since this cell cycle regulator is a target gene of β-catenin, this result would be expected from reduced nuclear signaling. I additionally analyzed the cell cycle in Cby overexpressing cells and found a disproportionately high fraction of cells in G2/M phase (Fig. 2-2C). Wnt/β-catenin signaling has been suggested to be involved in induction of mitosis, so that reduction of available β-catenin would lead to cell cycle arrest in G2 (Davidson, Shen et al. 2009).

These findings confirm that Cby functions as β-catenin antagonist by promoting nuclear export of β-catenin, not only in cells transiently transfected with Cby, β-catenin and 14-3-3ζ, but also in cells with dysregulated β-catenin stably transfected with Cby, which may have implications for the development of cancer therapies. However, the levels of Cby achieved in the SW480 cells were very high. While it has been shown that the *Cby* gene promoter is hypermethylated in SW480 cells (Schuierer, Graf et al. 2006), downregulating Cby protein levels, endogenous levels of Cby are unlikely to achieve such high levels.

In order to confirm Cby's function toward β-catenin in a more physiologically relevant setting, we generated stable Cby knock-down lines using short hairpin RNA. In HEK293 cells transiently transfected with stabilized β-cateninS33Y-Flag, such reduction of Cby protein levels led to the expected nuclear accumulation of β-catenin (Li, Mofunanya et al. 2010). Surprisingly, in stable cell lines with endogenous β-catenin only, a dramatic change in phenotype from mesenchymal to epithelial occurred. Changes between mesenchymal and epithelial phenotypes have been described during essential developmental processes like neural crest development and

gastrulation, but also in wound healing and tissue homeostasis. They occur in pathogenic processes as well, including fibrotic diseases (Thiery, Acloque et al. 2009).

In tumors, transition from epithelial to mesenchymal phenotype has been observed and was thought to take place during and enable formation of distant metastases (Moustakas and Heldin 2007; Chao, Shephard et al. 2010) though it has now been shown that tumor cells can migrate as sheets and can enter the bloodstream and lymphatic system at sites of capillaries weakened subsequent to proliferation of the tumor cells (Christiansen and Rajasekaran 2006; Roerth 2009). EMT-like changes have been demonstrated in cells at the tumor front, with loss of E-cadherin and nuclear accumulation of β-catenin (Brabletz, Jung et al. 2001). Additionally, in vitro capability for anchorage-independent growth, a hallmark of mesenchymal cells (Christiansen and Rajasekaran 2006) correlates well with metastasis formation by cells injected into mice (Cifone and Fidler 1980; Roh, Green et al. 2001), supporting that EMT facilitates metastasis. The opposite process of MET takes place during formation of metastases at new host sites, with reexpression of E-cadherin and plasma membrane localization of β-catenin. I observed such MET-like changes in the Cby knock-down cells, in both HEK293 and SW480 cells. HEK293 cells are transformed with human adenovirus type 5 (Graham, Smiley et al. 1977) and have been shown to migrate and invade (Ma, Zhang et al. 2009; Ha, Kim et al. 2010). SW480 cells are colon carcinoma cells with the ability to migrate (Faux, Ross et al. 2004; Bowen, Doan et al. 2009) and invade (McInroy and Määttä 2007) in *in vitro* assays. With Cby knock-down however, the cells' ability to form colonies in soft agar, a test for capability of anchorage-independent growth, decreases by two orders of magnitude in HEK293 cells, and by 50% in SW480 cells, the latter showing about 70% reduction in Cby protein levels.

Additionally, in both cell lines, Cby knock-down leads to localization of β-catenin to the plasma membrane. Protein levels of E-cadherin increase several-fold, and the adherens junction protein localizes to cell-cell boundaries. The cells show characteristic changes in the actin cytoskeleton, forming a cortical actin ring, and no longer migrate as single cells in wound scratch assays. These changes were unexpected because our previous data had mainly supported a role for Cby in nuclear export of β-catenin, so that loss-of-function experiments were expected to show nuclear accumulation and increased signaling of β-catenin. Since we observed these changes in two cell lines of very different provenance, controlled with non-coding vector, and the stable lines were generated independently by two lab members (Feng-Qian Li and myself), it is unlikely that the procedure of generating the stable lines caused the switch in phenotype, although a switch at least in β-catenin and E-cadherin localization upon confluence has been reported (Davies, Roberts et al. 2004).

Several explanations are possible for the observed phenomena: Cby exports β-catenin from the nucleus in cooperation with 14-3-3 proteins. These proteins have been shown to stabilize cargo in the cytoplasm (Kumagai and Dunphy 1999; Yang, Winkler et al. 1999; Brunet, Kanai et al. 2002), or the nucleus, depending on the cargo. It is therefore possible that the tripartite complex persists in the cytoplasm, preventing β-catenin from localizing not only to the

nucleus but also to the plasma membrane. It has been described that β-catenin and E-cadherin form a complex early on during biosynthesis (Nakamura, Hayashi et al. 2008) and that E-cadherin is targeted for degradation upon loss of β-catenin (Miyashita and Ozawa 2007). However, it is not likely that this is the only point in time where complex formation can occur, especially since E-cadherin can also form a complex with γ-catenin (plakoglobin). Thus sequestration of β-catenin in the cytoplasm could reduce E-cadherin/β-catenin-mediated adherens junction. Consequently, the loss of cytoplasmic stabilization of the 14-3-3 – Cby – β-catenin complex upon loss of Cby far below endogenous levels could facilitate complex formation with E-cadherin and localization of β-catenin to the plasma membrane, possibly not only in complex with E-cadherin but also to the axin-phosphodestruction complex (Maher, Flozak et al. 2009).

The mechanism by which loss of Cby mediates MET-like changes could alternatively parallel Cby's function to export β-catenin from the nucleus. It is possible that Cby exerts a similar function at the plasma membrane, promoting removal of β-catenin from the membrane. While I have not been able to demonstrate competition of Cby with E-cadherin for β-catenin binding in competition Co-IP's (data not shown), a small effect restricted to β-catenin phosphorylated at a particular site, e.g. tyrosine 654, may be sufficient to tip the balance toward higher stability of the β-catenin-E-cadherin complex at the plasma membrane upon loss of Cby that would entrail formation of mature adherens junctions with the consequence of, ultimately, MET. Such difference has been shown previously, in form of E-cadherin binding increased amounts or β-catenin at the plasma membrane without depleting cytoplasmic or nuclear levels. In that case, the difference was ascribed to the phenomenon that E-cadherin binds the same fraction of β-catenin as Tcf/Lef transcription factors (Gottardi, Wong et al. 2001). The tyrosine 654 site of β-catenin is known to affect binding affinity between β-catenin and E-cadherin, where phosphorylation of β-catenin reduced affinity for E-cadherin (Castano, Raurell et al. 2002). Such differences in E-cadherin stability at the plasma membrane could be assessed, e.g., by pulse-chase assays, in combination with cell surface biotinylation assays. The former would allow assessment of protein turnover, while the cell surface biotinylation assays can be used to assess residence time at the plasma membrane. It would then be interesting to test if Cby interacts with this form of β-catenin, e.g. by Co-IP with phosphorylation mimic β-catenin S564D, and to test if expression of phosphorylation mutant β-catenin S564A would have an effect on β-catenin turnover at the plasma membrane depending on Cby levels.

The inverse change of less plasma membrane bound E-cadherin due to Cby overexpression would not become apparent in SW480 or HEK293 cells since E-cadherin levels in control cells are already at the lower end of the detection limit. Additionally, the change in E-cadherin and β-catenin to the plasma membrane was not observed either in SW480 cells overexpressing the 14-3-3-binding mutant Cby S20A that could be thought to outcompete endogenous Cby. However, the mutant did not act as a dominant negative in experiments. While it did not promote nuclear export (Li, Mofunanya et al. 2008), it did reduce nuclear β-catenin signaling, albeit not as much as Cby wt did, and it did not lead to further nuclear accumulation of

β-catenin in the stable SW480 cells. Since CbyS20A does not interact with 14-3-3 protein (Li, Mofunanya et al. 2008), it is thought that the reduced nuclear β-catenin signaling by Topflash assay is due to the second mechanism by which Cby reduces nuclear activity of β-catenin, competition with Tcf/Lef transcription factors for β-catenin binding. Since it does not promote β-catenin's relocalization to the cytoplasm, no effect on E-cadherin compared to vector control cells would be expected.

If this suggestion is true and Cby is involved in removal of β-catenin not only from the nucleus (Li, Mofunanya et al. 2008; Li, Mofunanya et al. 2010)(Chapter 2) but also from the E-cadherin complex, the contradiction remains that both Cby overexpression and Cby knock-down indisputedly lead to reduced nuclear β-catenin signaling activity (Fig. 2-4A and Fig. 3-10A), and that Cby knock-down but not overexpression leads to MET-like changes.To resolve this contradiction, additional details merit attention.

β-Catenin levels in the nucleus are reduced in Cby overexpressing cells (Fig. 2-2), but not in Cby knock-down cells (data not shown). Since nuclear signaling is reduced in the SW480 Cby sh cells (Fig. 3-10A), a change in the balance of β-catenin carrying specific posttranslational modifications is possible. β-catenin can be phosphorylated at several residues, and is exported from the nucleus not only by Cby but also by other proteins, including Axin, APC and Ran-BP. Loss of Cby-mediated export could tip the balance of signaling active to inactive β-catenin sufficiently to allow for derepression of the *cdh1* gene, ultimately increasing E-cadherin levels sufficiently to trigger E-cadherin-mediated signaling towards MET. E-cadherin reexpression in SW480 cells is sufficient to induce MET-like changes (Gottardi, Wong et al. 2001). Expression of *cdh1* is repressed by β-catenin, directly and via repressors snail and slug, snail being a gene target of β-catenin (Yook, Li et al. 2006). I demonstrated increased mRNA levels for E-cadherin in both HEK293 and SW480 cells (Fig. 3-10 B), supporting that higher levels of E-cadherin are due, at least in part, to increased mRNA levels.

Alternatively, the E-cadherin promoter region could be altered to allow higher levels of transcription. Cby's structure allowing for interaction with multiple partners in principle (Mokhtarzada, Yu et al. 2011), and known to compete with Tcf/Lef as well as to interact with TC-1, makes it thinkable that it affects more than β-catenin-mediated transcriptional coactivation, and analysis of the modifications of the E-cadherin promoter could show, e.g. by methylation-specific PCR, if changes occur depending on cellular levels of Cby. The mRNA levels, as well as translation into protein, are subject to additional steps of regulation. No data is available at this point regarding involvement of Cby in such regulation however, so that this aspect of regulation of E-cadherin is less likely to be affected by Cby protein levels.

Another possible mechanism by which Cby knock-down leads to increased presence of β-catenin and E-cadherin at the plasma membrane has to be taken into account however: Cby has been described to interact with the golgi marker protein GM130, and with polycycstin-1. The experiments showing these interactions used an N-terminally tagged form of Cby, and it is likely that such tagged Cby adopt a different conformation, or maybe one of several possible

conformations, compared to CbyWT, since one recently developed antibody for Cby recognizes N-terminally tagged, but not untagged Cby (Cyge, Fischer et al. 2011). Currently available antibodies to Cby have not shown Cby at the golgi, to the best of my knowledge, in staining assays, however, they rarely show localization to the nucleus, although Cby does localize to the nucleus both in overexpression experiments (Li, Mofunanya et al. 2008), and by cell fractionation (Fig. 2-2). A function of Cby at the golgi must therefore be regarded a serious possibility at this time (Hoffmeister, Babinger et al. 2011). Loss of Cby at the golgi could affect protein sorting and thereby direct more β-catenin-E-cadherin complex to the plasma membrane.

An alternative explanation for the observed dual role of Cby deserves further exploration: Recent publications provide increasing evidence for a role of Cby in ciliogenesis (Voronina, Takemaru et al. 2009; Enjolras, Thomas et al. 2012). Loss of Cby leads to defects in motile cilia in the nasal epithelia of Cby knock-out mice and to defects in formation of basal bodies in *Drosophila melanogaster*. Since cilia are critical in signaling for, e.g., the Hedgehog signaling pathway, but also for Wnt signaling (Hoffmeister, Babinger et al. 2011), it is possible that reduced levels of Cby impair regulation of cellular processes via its role at the basal body of cilia.

Irrespective of the initial shift in regulation however, be it by reduced β-catenin-mediated gene repression or altered sorting, once adherens junctions form, E-cadherin's signaling effects could induce the changes toward an epithelial phenotype, as it is shown to do in overexpression experiments in SW480 (Gottardi, Wong et al. 2001) and other tumor cells (Nawrocki-Raby, Gilles et al. 2003). Whether the MET-like changes upon Cby knock-down are E-cadherin-dependent is difficult to test. Knock-down of E-cadherin appears not meaningful because of the central role of E-cadherin in epithelial cells. Inducible short hairpin RNA for Cby however may allow to dissect the steps leading to the observed epithelialization, shedding further light on Cby's functions.

In summary, this study reveals a dual role for Cby, demonstrating that it reduces signaling of endogenous β-catenin in tumor cells with stabilized β-catenin on the one hand, and that Cby knock-down, on the other hand, promotes MET-like changes in two different cell lines, one with intact β-catenin signaling, and one with stabilized β-catenin. The discovery of this dual role raises interesting new questions regarding the interplay of membrane-bound versus nuclear signaling-active β-catenin that may reveal a new angle to target dysregulated Wnt-signaling in cancer thereapy but also for treatment of other Wnt-signaling-dependent diseases.

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