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A protein knockdown strategy to study the function of β-catenin in tumorigenesis

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Abstract

Background: The Wnt signaling pathway plays critical roles in cell proliferation and cell fate determination at many stages of development. A critical downstream target of Wnt signaling is the cytosolic β-catenin, which is stabilized upon Wnt activation and promotes transcription of a variety of target genes including c-myc and cyclin D. Aberrant Wnt signaling, which results from mutations of either β-catenin or adenomatous polyposis coli (APC), renders β-catenin resistant to degradation, and has been associated with multiple types of human cancers.

Results: A protein knockdown strategy was designed to reduce the cytosolic β-catenin levels through accelerating its turnover rate. By engineering a chimeric protein with the β-catenin binding domain of E-cadherin fused to βTrCP ubiquitin-protein ligase, the stable β-catenin mutant was recruited to the cellular SCF (Skp1, Cullin 1, and F-box-containing substrate receptor) ubiquitination machinery for ubiquitination and degradation. The DLD1 colon cancer cells express wild type β-catenin at abnormally high levels due to loss of APC. Remarkably, conditional expression of βTrCP-E-cadherin under the control of a tetracycline-repressive promoter in DLD1 cells selectively knocked down the cytosolic, but not membrane-associated subpopulation of β-catenin. As a result, DLD1 cells were impaired in their growth and clonogenic ability *in vitro*, and lost their tumorigenic potential in nude mice.

Conclusion: We have designed a novel approach to induce degradation of stabilized/mutated β-catenin. Our results suggest that a high concentration of cytoplasmic β-catenin is critical for the growth of colorectal tumor cells. The protein knockdown strategy can be utilized not only as a novel method to dissect the role of oncoproteins in tumorigenesis, but also as a unique tool to delineate the function of a subpopulation of proteins localized to a specific subcellular compartment.

Background

Wnt signaling plays diverse roles at many stages of development by regulating the stability of β-catenin [1]. In cells that do not receive a Wnt signal, cytoplasmic β-catenin is bound to a multi-protein β-catenin destruction complex that contains several proteins including Axin, APC, and

glycogen synthase kinase-3 (GSK3), and it is constitutively phosphorylated at a cluster of Ser and Thr residues at its N-terminus by GSK3. Phosphorylated β-catenin is recognized by βTrCP, a component of the $SCF^{\beta TrCP}$ ubiquitin-protein ligase complex, and degraded by the ubiquitin-proteasome pathway. Wnt signaling disassembles the β-catenin destruction complex, which prevents the phosphorylation and subsequent ubiquitination of β-catenin, thus diverting β-catenin from the proteasome machinery. Accumulated β-catenin then enters the nucleus, binds to the LEF/TCF family transcription factors, and activates the expression of β-catenin target genes.

Deregulated Wnt signaling contributes to tumorigenesis. Wnt-1, the founding member of the Wnt family, was first identified as a gene activated by insertion of a mouse mammary tumor provirus, leading to the formation of mouse mammary tumors [2]. Aberrant activation of Wnt signaling, which results from activating mutations of β-catenin or inactivating mutations of APC or Axin, has been associated with a wide variety of human malignancies, such as colorectal, heptocellular, ovarian endometrial, desmoid, and pancreatic tumors [3]. Among these tumor types, Wnt signaling is most frequently deregulated in colorectal tumors. APC is mutated in the majority of colorectal cancers, and those tumors with wild-type APC often contain mutated β-catenin [4]. Thus, aberrant activation of Wnt signaling appears to be obligatory for the initiation or progression of colorectal tumors. Recent studies suggested that β-catenin promotes tumorigenesis through increasing the expression of oncogenes like *c-myc* and *cyclin D1* [5–7].

β-catenin is a "dual function" protein, which is determined by its membrane and nuclear localizations. Membrane-associated β-catenin plays an important role in cell-cell adhesion. It binds to the intracellular domain of E-cadherin, and links E-cadherin to α-catenin and thereby to the cortical actin cytoskeleton. E-cadherin-mediated cell adhesion plays an inhibitory role in tumor invasion [8], and loss of E-cadherin promotes tumor progression [9]. Nuclear β-catenin enhances transcription of Wnt-responsive genes through interacting with TCF/LEF transcription factors and recruiting different transcriptional co-activators to the TCF/LEF binding sites.

To study the function of β-catenin in tumorigenesis, one needs to develop a strategy to selectively block the nuclear activity of β-catenin while leaving the membrane activity of β-catenin intact. Such an approach would enhance our understanding of the oncogenic function of β-catenin, and might further serve as a strategy for targeted therapy for tumors derived from aberrant Wnt signaling. In this study, a protein knockdown method was designed to induce the degradation of unphosphorylated β-catenin, which resulted in the suppression of neoplastic growth of colorectal tumor cells.

Results

Ubiquitin-dependent proteolysis constitutes the major pathway for eukaryotic cells to degrade specific proteins. This pathway involves a cascade of enzymatic reactions catalyzed by the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin-protein ligase [10]. The substrate specificity of this system is determined by the E3 ligase. One such E3 ligase, designated SCF (Skp1, Cul-1, F-box-containing substrate receptor, and the Ring domain protein Rbx1/Roc1/Hrt1), is a multimeric protein complex that targets key regulators of cell cycle and signaling pathways for ubiquitination (reviewed in [11]). Among the SCF subunits, the F box proteins serve as receptors that recruit substrates through various protein-protein interaction domains and bring them to the core E3 (Skp1/Cul-1/Rbx1) through interaction between the F box and Skp1. βTrCP is such an F box protein that binds to its substrates, such as IκB and β-catenin, through its WD40 repeats. Specifically, serine phosphorylation of IκB and β-catenin is a prerequisite for their binding to βTrCP [12–15].

The SCF ubiquitination machinery can be harnessed to degrade a specific target protein by fusing an F box protein with a peptide that is able to bind to the target protein [16]. Here we investigated whether an F-box protein can be redesigned to target their usual substrates that have become resistant to degradation (eg, by mutations in the N-terminus of β-catenin). Recognition of β-catenin by βTrCP normally requires phosphorylation of serine residues within the N-terminal DSGxxS motif of β-catein [13,15]. To target unphosphorylated and thus stabilized β-catenin to the core SCF for ubiquitination and degradation, we fused the β-catenin binding domain of E-cadherin (amino acids 794–883, designated Ecad) to the C-terminus of βTrCP (Fig. 1A). A glycine-serine-rich sequence was inserted between βTrCP and Ecad to relieve the potential steric hindrance between these two protein structures. Ser37 is one major GSK3 phosphorylation site of β-catenin that is recognized by βTrCP [13]. Substitution of Ser37 with Ala abrogates the association between β-catenin and βTrCP. It has been shown that β-catenin S37A is about 9-fold more stable than the wild-type β-catenin [17]. The binding between β-catenin S37A and βTrCP-Ecad was assayed in 293 cells using a co-immunoprecipitation assay. HA-tagged β-catenin S37A and FLAG-tagged βTrCP-Ecad were coexpressed in 293 cells. Total cell lysates were immunoprecipitated with the anti-FLAG antibody, and immunoprecipitates were subjected to SDS PAGE and immunoblotting with the anti-HA antibody. As shown in Fig. 1B, β-catenin S37A strongly interacted with F-TrCP-Ecad, but not F-TrCP, indicating that the

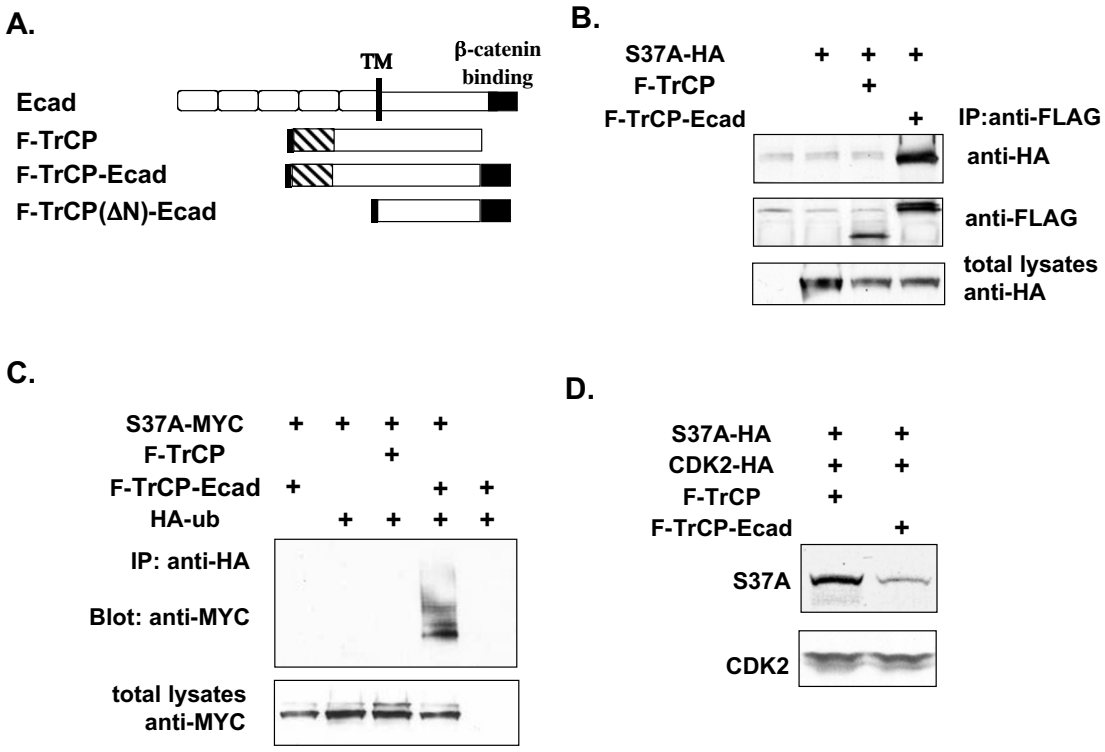

Figure 1

Engineering a βTrCP-E-cadherin chimera that targets oncogenic β-catenin for degradation. **A.** Schematic diagrams of F-TrCP-Ecad chimeras used in this study. The β-catenin binding domain (amino acids 794–883) of E-cadherin (Ecad) was fused to the C-terminus of FLAG-tagged βTrCP to form F-TrCP-Ecad. The N-terminus of βTrCP (amino acid 1–297) was deleted from F-TrCP-Ecad to make F-TrCP(ΔN)-Ecad. The transmembrane (TM) region and the β-catenin binding domain in E-cadherin are indicated. The F box of βTrCP is hatched. The FLAG epitope fused at the N-termini of βTrCP, βTrCP-Ecad, or βTrCP(ΔN)-Ecad is indicated by a solid rectangle. **B.** F-TrCP-Ecad binds to β-catenin S37A *in vivo*. HA-tagged β-catenin S37A and FLAG-tagged βTrCP derivatives were co-expressed in 293 cells. Cells were treated with the proteasome inhibitor MG132 for 4 hours before harvesting. Cell lysates were immunoprecipitated with the anti-FLAG antibody, precipitates were resolved by a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, and immunoblotted with the anti-HA antibody (top panel). Note that a nonspecific band that cross-reacted with the anti-HA antibody migrated slightly above β-catenin S37A in all lanes of this panel. The membrane was stripped and blotted with the anti-FLAG antibody (middle panel). The expression of β-catenin S37A in total cell lysates was examined by immunoblotting with the anti-HA antibody (bottom panel). **C.** F-TrCP-Ecad induces ubiquitination of β-catenin S37A *in vivo*. MYC-tagged β-catenin S37A, HA-tagged ubiquitin, and indicated βTrCP derivatives were co-expressed in 293 cells. Cells were treated with the proteasome inhibitor ALLN for 4 hours, and lysed in the denaturing buffer by boiling to disrupt non-covalent protein-protein interactions. Cell lysates were immunoprecipitated with the anti-HA antibody, and immunoprecipitates were resolved in SDS-PAGE and blotted with the anti-MYC antibody (top panel). The expression of β-catenin S37A in total cell lysates was determined by immunoblotting with the anti-MYC antibody (bottom panel). **D.** F-TrCP-Ecad reduces the steady state levels of β-catenin. HA-tagged β-catenin and CDK2 were co-expressed with indicated βTrCP derivatives in 293 cells. Total cell lysates were resolved by SDS-PAGE, and immunoblotted with the anti-HA antibody.

intracellular domain of E-cadherin binds to the armadillo repeats of β-catenin in a phosphorylation-independent manner.

Next, we tested whether binding between F-TrCP-Ecad and β-catenin S37A could induce ubiquitination of β-catenin S37A. 293 cells were cotransfected with plasmids encoding HA-tagged ubiquitin and MYC-tagged β-catenin S37A. Cell lysates were immunoprecipitated with the anti-HA antibody under conditions that denature proteins in cell lysates, and probed with an anti-MYC monoclonal antibody against MYC-tagged β-catenin. As seen in Fig. 1C, β-catenin S37A was ubiquitinated only in the presence of F-TrCP-Ecad, but not F-TrCP.

To examine whether ectopic expression of F-TrCP-Ecad affects degradation of β-catenin S37A, plasmids encoding F-TrCP-Ecad and HA-tagged β-catenin S37A were cotransfected into 293 cells. A plasmid encoding HA-tagged CDK2 was also included in the transfection as an internal control. Consistent with the association between F-TrCP-Ecad and β-catenin S37A, co-expression of F-TrCP-Ecad significantly reduced the steady state level of β-catenin S37A, but not that of the control CDK2 (Fig. 1D). Therefore, the engineered F-TrCP-Ecad can recapture the "non-degradable" β-catenin S37A, and promote its ubiquitination and degradation.

We further tested the effect of F-TrCP-Ecad on the transcriptional activity of β-catenin. β-catenin S37A was transiently expressed in 293 cells with TOP-FLASH, a luciferase reporter that contains multiple copies of TCF binding sites and serves as a readout for β-catenin signaling activity [18]. As shown in Fig. 2A, co-expression of F-TrCP-Ecad dramatically decreased the transcriptional response to β-catenin S37A from the TOP-FLASH reporter. Two other β-catenin targeting peptides, which constitute the N-terminal domains of human TCF4 or *Xenopus* TCF3, were also fused to βTrCP, and demonstrated similar inhibitory effects on β-catenin signaling as F-TrCP-Ecad (data not shown).

The F box is critical for the ubiquitin-protein ligase activity of βTrCP. To investigate whether βTrCP-mediated β-catenin degradation is required for the attenuation of β-catenin signaling, an N-terminal region of βTrCP, including the F box, was deleted from F-TrCP-Ecad to form F-TrCP(ΔN)-Ecad, and tested for its effect on the transcriptional activity of β-catenin. Deletion of the N-terminal region from βTrCP resulted in a complete loss of inhibition of β-catenin signaling (Figure 2A), although F-TrCP(ΔN)-Ecad and F-TrCP-Ecad bound to β-catenin to the similar extent (Figure 2B). This finding suggests that the inhibitory effect of F-TrCP-Ecad on β-catenin signaling is dependent on targeted degradation of β-catenin by the engineered F-TrCP-Ecad ubiquitin-protein ligase.

There are two subpopulations of β-catenin inside cells, the nuclear/cytosolic pool and the membrane pool. Only the nuclear/cytosolic pool of β-catenin is subjected to direct regulation by Wnt signaling. The nuclear β-catenin binds to TCF/LEF transcriptional factors and activates the expression of Wnt target genes. The membrane β-catenin bridges the interaction between α-catenin and E-cadherin, and links the E-cadherin complex to the actin cytoskeleton.

We tested the effect of F-TrCP-Ecad induction on the levels of the membrane and cytosolic β-catenin in the DLD1 colon cancer cells. β-catenin signaling is deregulated in DLD1 cells due to inactivation mutations of APC, resulting in the accumulation of unphosphorylated β-catenin resistant to βTrCP-mediated proteolysis. A stable DLD1-F-TrCP-Ecad cell line was constructed in which the engineered F-TrCP-Ecad was expressed under the tetracycline-repressible promoter [19]. The expression of F-TrCP-Ecad was induced when DLD1-F-TrCP-Ecad cells were cultured in the absence of doxycycline (Dox), a tetracycline analogue (Fig. 3C). To test whether induction of F-TrCP-Ecad affects the steady state levels of the membrane and cytosolic β-catenin, DLD1-F-TrCP-Ecad cells were grown in the presence or the absence of Dox, and fractionated into the cytosolic pool (S100) and the membrane pool (P100) by centrifugation. As seen in Fig. 3A, induction of F-TrCP-Ecad expression reduced the steady state level of cytosolic β-catenin, while it had no effect on the level of the membrane-associated β-catenin. In contrast, Dox treatment of the parental DLD1 cells had no effect on the steady state level of cytosolic β-catenin (data not shown). Therefore, F-TrCP-Ecad preferentially targets soluble nuclear/cytosolic β-catenin for degradation, sparing the membrane-associated fraction, which is tightly associated with endogenous membrane E-cadherin.

The signaling activity of β-catenin was further assessed in response to induced expression of F-TrCP-Ecad. DLD1-F-TrCP-Ecad cells were transiently transfected with TOP-FLASH and a control plasmid encoding *Renilla* luciferase, then cultured in the presence or the absence of Dox. As seen in Fig. 3B, induction of F-TrCP-Ecad reduced TOP-FLASH activity by 4-fold, indicating decreased β-catenin signaling activity.

c-myc has been previously identified as a β-catenin target gene in colon cancer cells [5]. We asked whether c-myc expression was affected by down-regulation of β-catenin signaling. Indeed, induction of F-TrCP-Ecad by Dox withdrawal decreased the concentration of c-myc oncoprotein (Fig. 3C).

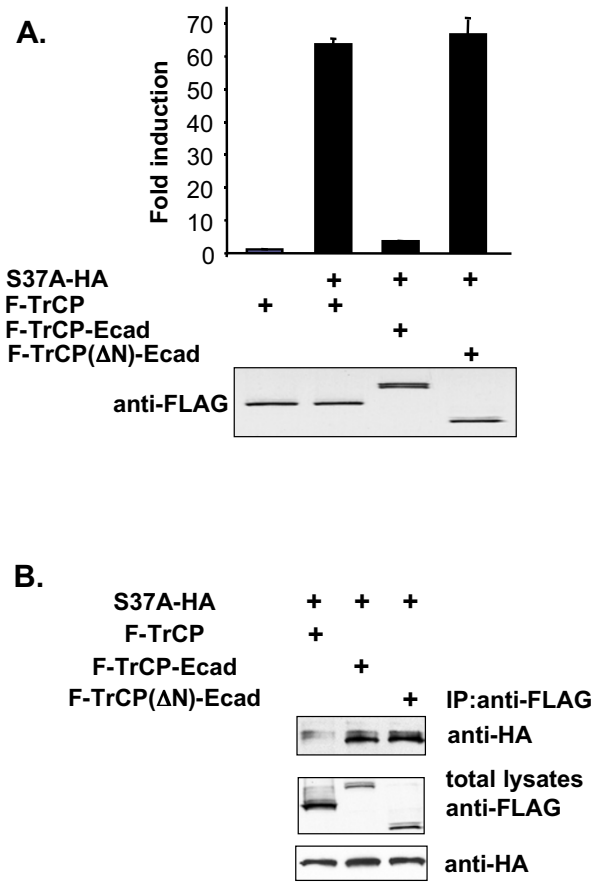

Figure 2
F-TrCP-Ecad inhibits the transcriptional activity of β-catenin S37A. **A.** 293 cells were co-transfected with a β-catenin S37A expression plasmid, indicated βTrCP expression plasmids, TOP-FLASH, and a CMV-*Renilla* reporter. The luciferase activities were measured and normalized against the control *Renilla* activities (top panel). The luciferase activity of cells transfected with βTrCP was arbitrarily set to 1. The expression of βTrCP derivatives was determined by immunoblotting with the anti-FLAG antibody (bottom panel). **B.** F-TrCP-Ecad and F-TrCP(ΔN)-Ecad bind to β-catenin to the similar extent. HA-tagged β-catenin S37A and FLAG-tagged βTrCP derivatives were co-expressed in 293 cells. Cells were treated with the proteasome inhibitor MG132 for 4 hours before harvesting. Cell lysates were immunoprecipitated with the anti-FLAG antibody, precipitates were resolved by SDS-PAGE and immunoblotted with the anti-HA antibody (top panel). The expression of FLAG-tagged βTrCP derivatives and β-catenin S37A in total cell lysates was examined by immunoblotting with the anti-FLAG and anti-HA antibodies (middle and bottom panels).

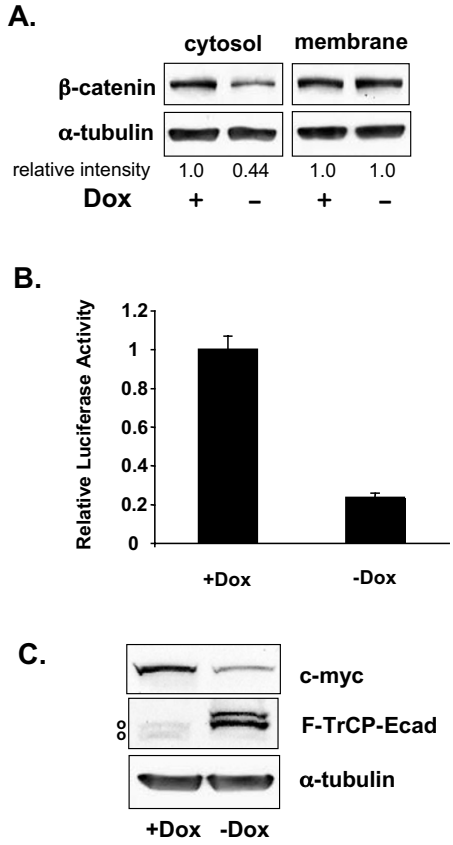

Figure 3
F-TrCP-Ecad promotes β-catenin degradation and inhibits Wnt signaling in DLD1 cells. **A.** Effects of F-TrCP-Ecad on the levels of cytosolic and membrane-associated β-catenin. A DLD1 inducible cell line was generated with the tetracycline (tet)-off system; these cells expressed F-TrCP-Ecad only in the absence of Dox. Cells were grown in the presence or absence of Dox for 5 days, and subjected to subcellular fractionation. Equal amounts of proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with the anti-β-catenin antibody. α-tubulin levels were determined by immunoblotting with the anti-α-tubulin antibody as an internal loading control. Quantified representation of immunoblot is shown at the bottom of each blot (relative intensity). **B.** F-TrCP-Ecad inhibits the transcriptional activity of β-catenin. DLD1-F-TrCP-Ecad cells were transfected with TOP-FLASH and a CMV-*Renilla* reporter, and cultured in the presence or absence of Dox. The luciferase activity of cells grown in the presence of Dox was arbitrarily set to 1. **C.** F-TrCP-Ecad induction inhibits c-MYC expression. DLD1-F-TrCP-Ecad cells were cultured in the presence or absence of Dox for four days. The expression level of c-myc and F-TrCP-Ecad were determined by immunoblotting with the anti-MYC (C-19) and the anti-FLAG antibodies (top and middle panels). Equal loadings were confirmed by immunoblotting with the anti-α-tubulin antibody (bottom panel). 'o' indicates two unknown species immuno-reactive with the anti-FLAG antibody, which migrated right below βTrCP-Ecad of the middle panel.

To study the effect of F-TrCP-Ecad induction on cell proliferation, we first measured the growth rate of DLD1-F-TrCP-Ecad cells in the presence or absence of Dox. A reduced rate of cell growth was observed when cells were cultured in the absence of Dox (Fig. 4A). This effect of F-TrCP-Ecad on cell proliferation was not a result of increased cell death, as determined by trypan blue staining (data not shown). A clonogenic assay was performed, in which DLD1-F-TrCP-Ecad cells were plated at a low density (2,000 cells/10 cm plate) and cultured in the presence or absence of Dox. Induction of F-TrCP-Ecad expression dramatically reduced the colony forming capability of DLD1 cells (Fig. 4B).

The effect of targeted-degradation of β-catenin on the tumorigenicity of DLD1 cells was examined by subcutaneous injection of DLD1-F-TrCP-Ecad cells into nude mice. Remarkably, nude mice fed with Dox-free food exhibited significant attenuation of tumor growth compared with those treated with Dox, indicating a requirement for maintenance of β-catenin levels for the tumorigenicity of DLD1 cells *in vivo* (Fig. 4C).

Collectively, these results provide strong evidence that induced expression of the engineered F-TrCP-Ecad ubiquitin-protein ligase downregulated β-catenin levels and significantly blocked Wnt signaling in and tumorigenicity of a colon cancer cell.

Discussion

The stability of β-catenin is tightly regulated in normal cells: β-catenin is constitutively phosphorylated by GSK3, which triggers its recognition and degradation by the $SCF^{\beta TrCP}$ ubiquitination machinery. In tumor cells with aberrant activation of the Wnt signaling pathway, β-catenin is no longer phosphorylated due to mutations in β-catenin itself or upstream elements that are critical for its phosphorylation, and thus escapes degradation. We have designed a protein knockdown strategy to recapture non-phosphorylated cytoplasmic β-catenin and to target it to the SCF machinery for degradation. This knockdown strategy can degrade both mutant β-catenin lacking N-terminal phosphorylation sites and wild-type β-catenin stabilized by APC mutations, and it preferentially targets cytoplasmic versus membrane β-catenin. Finally, we show that degradation of β-catenin results in inhibition of colorectal tumor cell growth both *in vitro* and *in vivo*.

β-catenin has both membrane and nuclear functions. To inhibit β-catenin signaling, one needs to specifically block the nuclear function of β-catenin while leaving the membrane function of β-catenin intact. Methods such as targeted gene knockout mutagenesis, RNA interference, or inhibition with antisense oligonucleotides will eliminate both the nuclear and the membrane functions of β-catenin. The nuclear function of β-catenin can be blocked by enhancing the degradation of cytosolic β-catenin with excess Axin [20,21] or APC [22,23]. However, neither Axin nor APC can induce the degradation of β-catenin mutants lacking N-terminal phosphorylation sites, and Axin has an apoptotic activity that appears to be independent of its effects on the Wnt pathway [24]. Another way to block the nuclear function of β-catenin specifically is to overexpress a dominant negative form of TCF; this approach has proved to be valuable for studying the role of the β-catenin in tumorigenesis [25]. However, there is evidence that at least some signaling functions of β-catenin might not be mediated by TCF. For example, activation of WISP-1 by Wnt and β-catenin appears to be TCF-independent [26]. Recently, it has also been shown that Pitx2 can recruit β-catenin to the promoter of Cyclin D2, and activate the transcription of Cyclin D2 [27].

The F-TrCP-Ecad chimera that we have engineered decreases the levels of cytosolic and presumably nuclear β-catenin, but has no obvious effect on the levels of membrane β-catenin. βTrCP has a short half-life [28](J.Z. and P.Z., unpublished results), so the steady state level of F-TrCP-Ecad in cells is likely low. It is possible that β-catenin, when in complex with the membrane E-cadherin, is protected from the action of F-TrCP-Ecad. However, prolonged high level expression of F-TrCP-Ecad eventually affects the abundance of membrane β-catenin through affecting the equilibrium between the two pools of β-catenin (J.Z. and P.Z., unpublished results).

In this study, we have found that down-regulation of β-catenin signaling in DLD1 cells reduces c-myc expression levels and impairs cell growth (Fig. 3 and 4) without causing obvious cell death (data not shown). Similarly, van de Wetering et al have shown that overexpression of a dominant-negative form of TCF4 in colon cancer cells causes growth arrest but not cell death [25]. Importantly, ectopic expression of *c-myc* can reverse the growth arrest induced by dominant-negative TCF4, demonstrating that c-myc is a crucial mediator of β-catenin oncogenic activity [25].

We have shown that the tumorigenicity of DLD1 cells appears to be dependent on the concentration of cytoplasmic β-catenin. However, this may not be true for all colon cancer cells with stabilized β-catenin. Using somatic cell gene targeting to disrupt either wild-type or mutant form of β-catenin, it has been shown that elevated β-catenin is not essential for the growth of HCT116 cells [29,30], but is required for the growth of DLD1 and SW48 cells [30]. Presumably, additional mutations in HCT116 cells override the need for an oncogenic signal mediated by β-catenin.

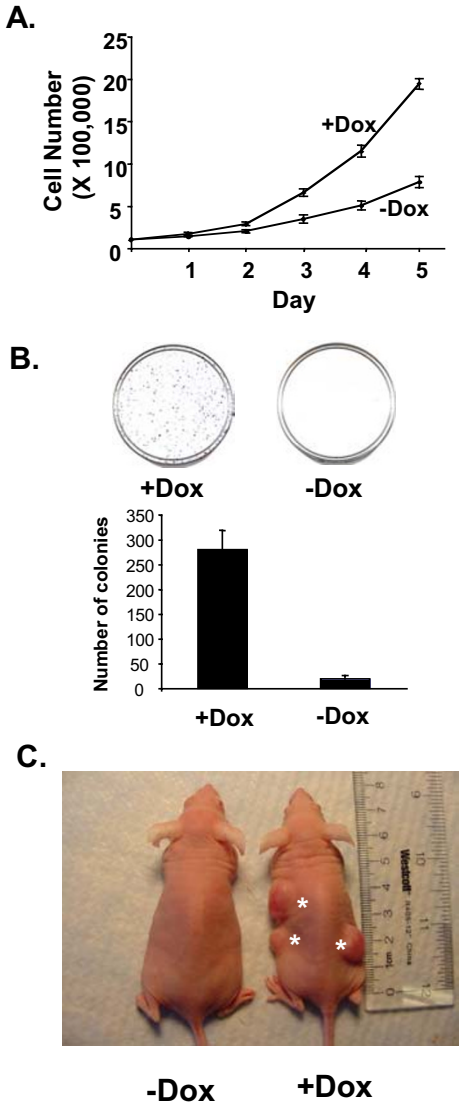

Figure 4
F-TrCP-Ecad inhibits clonogenic and tumorigenic potential of DLD1 cells. **A.** βTrCP-Ecad inhibits the growth rate of DLD1 cells. DLD1-F-TrCP-Ecad cells were grown in the presence or the absence of Dox for five days. The numbers of cells were counted daily in triplicates. **B.** Induction of F-TrCP-Ecad decreases the colony forming ability of DLD1 cells. DLD1-F-TrCP-Ecad cells were diluted, plated at a low density, and cultured in the presence or the absence of Dox for 14 days. Plates were photographed (top panel) and the number of colonies was counted (bottom panel). The experiment was performed in triplicates **C.** F-TrCP-Ecad induction inhibits tumorigenicity of DLD1 cells in nude mice. 2×10^6 DLD1-F-TrCP-Ecad cells were injected subcutaneously in nude mice. Each mouse received three injections. Nude mice were fed with either regular or Dox-containing food, and photographed three weeks after injection. In this observation period, six of six injections gave rise to tumors (indicated with asterisks) on mice fed with Dox-impregnated food, while none of the six injections generated tumor on mice fed with regular food.

Cancer stems from the step-wise accumulation of genetic changes. In simplified mouse models for cancer, continued activity of an initiating oncogene is usually required for tumor growth [31]. However, one cancer-causing gene important for tumor initiation might also become dispensable for tumor maintenance due to accumulation of additional mutations when the tumor progresses to an advanced stage [32]. Therefore, it is necessary to establish whether an oncoprotein in a late stage tumor is still a valid molecular target for the development of pharmacological inhibitors. The protein knockdown system provides a unique strategy for assessing the contribution of each oncoprotein to the tumorigenic phenotype of a late stage tumor as well as a novel approach for targeted tumor therapy.

Conclusions

We have developed a protein knockdown strategy to degrade unphosphorylated or mutated β-catenin, and demonstrated that suppression of β-catenin inhibits the neoplastic growth of colorectal tumor cells.

Methods

Cell culture

The 293 cell line was obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. A tetracycline (tet)-off system was used for inducible expression of F-TrCP-Ecad protein. In this system, the repression of gene expression was achieved by maintaining cells in the presence of a tetracycline analog, doxycycline (Dox) and the induction of gene expression was achieved by Dox withdrawal. The DLD1-tTA cell line was a generous gift of Bert Vogelstein [19], which expresses tetracycline-suppressible transactivator (tTA)-IRES-neo under control of the CMV promoter. The DLD1-tTA cells were maintained in McCoy's 5A, 10% fetal bovine serum, 400 μg/ml G418, and 20 ng/ml Dox (ICN). The F-TrCP-Ecad expression construct was generated by cloning F-TrCP-Ecad into pBI-MCS-EGFP [19]. The construct was linearized and cotransfected with pTK-hygro (Clontech) into DLD1-tTA cells. Single colonies were obtained by limiting dilution with medium containing 400 μg/ml G418, 250 μg/ml hygromycin B (Clontech), and 20 ng/ml of Dox for three weeks. Clones with homogenous GFP induction were subjected to immunoblot analysis for the expression of F-TrCP-Ecad with anti-FLAG antibodies (Sigma). 293 and DLD1 cells were transfected with a calcium phosphate transfection kit (Stratagene).

Luciferase Reporter assay

Cells were split into 12-well plates 24 hours before transfection. Each well received 0.2 μg TOP-FLASH, 0.02 μg CMV-*Renilla* expression plasmid, together with 0.5 μg indicated plasmids. Thirty-six hour after transfection, cells were lysed and luciferase assays were performed with the Dual Luciferase Assay kit (Promega) according to the manufacturer's instructions. The luciferase activity was normalized with the *Renilla* activity.

Co-immunoprecipitation and immunoblotting assay

For co-immunoprecipitation experiments cells were lysed in EBC buffer (50 mM Tris, PH 7.6, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM DTT, and protease inhibitors). Cell lysates were clarified by centrifugation at 13,000 rpm for 15 minutes at 4°C. For immunoprecipitation, the cell lysates were incubated with 1 μg of appropriate antibodies at 4°C for 1 to 2 hours. The immunocomplexes were collected with Protein A or Protein G agarose beads, and washed five times with lysis buffer. The bound proteins were eluted with Laemmli sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk for 1 hour at room temperature, and incubated with appropriate primary antibodies for 1 hour. Membranes were then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG secondary antibodies for 1 hour. Membranes were washed extensively with TBST and developed with an ECL kit (Amersham). Protein bands were semiquantitated by densitometry. Commercial antibodies used in this study include anti-FLAG (M2) and anti-α-tubulin monoclonal antibodies (Sigma), anti-HA (HA.11) monoclonal antibody (Covance), anti-β-catenin monoclonal antibody (Transduction Laboratories), anti-MYC (9E10) monoclonal antibody and anti-MYC (C-19) polyclonal antibody (Santa Cruz).

Cell fractionation assay

DLD1-F-TrCP-Ecad cells were grown in medium with or without Dox for 5 days before being harvested. Cells were washed and scraped on ice into TBS (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 2 mM DTT, protease inhibitors). Cells were homogenized with 30 strokes in a dounce homogenizer, and the nuclei were removed by low speed centrifugation. The post-nuclear supernatants were spun at 100,000 g for 90 min at 4°C to generate a supernatant, or cytosolic, fraction and membrane-rich pellet fraction. Samples normalized for protein content were analyzed by SDS-PAGE.

Cell growth and colony formation assay

For growth curve analysis, cells (10^5) were seeded into a well of 6-well plates in the presence or the absence of 20 ng/ml Dox. Each day of 5 days, cells were counted using a hemocytometer after trypsinization. For colony formation assay, cells were plated at 2,000 cells/10 cm plate. Cells

were grown in the presence or the absence of Dox for 14 days and stained with 0.05% crystal violet (Sigma).

In vivo *Ubiquitination assay*

293 cells were transfected with a HA-tagged ubiquitin expression construct together with indicated plasmids. Thirty-six hours after transfection, cells were treated with the proteosomal inhibitor N-acetyl-leucinyl-leucinyl-norleucinyl-H (ALLN) for four hours. Cells were then harvested and lysed in 1% SDS lysis buffer (50 mM Tris-HCl pH7.5, 0.5 mM EDTA, 1% SDS, 1 mM DTT, protease inhibitors). Cell lysates were sonicated, heated at 80°C for 30 minutes, and clarified by centrifugation. Supernatants were diluted 10 times in Triton X-100 lysis buffer (50 mM Tri-HCl pH8.0, 150 mM NaCl, 1% Triton X-100, protease inhibitors). Cell lysates were incubated with anti-HA affinity matrix (Roche) overnight at 4°C. Immunoprecipitates were washed with Triton X-100 lysis buffer 5 times. The bound proteins were eluted with Laemmli sample buffer. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblotting analysis.

Nude mouse assay

2×10^6 of DLD1-F-TrCP-Ecad cells were injected subcutaneously into 8-week-old athymic nu/nu (nude) mice (NCI). Animals were administered with regular or Dox-impregnated food pellets, and inspected for 3 weeks.

Authors' contributions

FC and PZ conceived the study and designed the experiments. FC, JZ, and WP performed the experiments. PZ and HV supervised the work. All authors read and approved the final manuscript.

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