

## **The EDD E3 ubiquitin ligase ubiquitinates and up-regulates $\beta$ -catenin**

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Abbreviations: EDD, E3 identified by differential display; GSK-3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; CRC, colorectal cancer; APC, Adenomatous polyposis coli; CK1, casein kinase I; TCF, T-cell factor; LEF, lymphoid enhancer factor; siRNA, small interfering RNA

### **Abstract**

Wnt/ $\beta$ -catenin signaling plays a central role in development and is also involved in a diverse array of diseases.  $\beta$ -catenin activity is tightly regulated via a multi-protein complex that includes the kinase glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ). GSK-3 $\beta$  phosphorylates  $\beta$ -catenin, marking it for ubiquitination and degradation via the proteasome. Thus, in regulation of the Wnt pathway, the ubiquitin system is mostly known to be involved in mediating the turnover of  $\beta$ -catenin, resulting in reduced Wnt signaling levels. Here we report that an arm of the ubiquitin system increases  $\beta$ -catenin protein levels. We show that GSK-3 $\beta$  directly interacts with the E3 ubiquitin ligase EDD that also binds  $\beta$ -catenin. Expression of EDD leads to enhanced nuclear accumulation of both GSK-3 $\beta$  and  $\beta$ -catenin and results in up-regulation of  $\beta$ -catenin expression levels and activity. Importantly, EDD ubiquitinates  $\beta$ -catenin through Lys29 or Lys11 linked-ubiquitin chains, leading to enhanced stability of  $\beta$ -catenin. Our results demonstrate a role for the ubiquitin system in up-regulation of the Wnt signaling pathway, suggesting that EDD could function as a colorectal oncogene.

Key words: E3 identified by differential display (EDD);  $\beta$ -catenin; GSK-3 $\beta$ ; Wnt signaling pathway; ubiquitination.

## Introduction

The Wnt signaling pathway regulates numerous processes during normal animal development and controls adult stem cell self-renewal<sup>1 2</sup>. Hyperactivation of the canonical Wnt pathway is a hallmark of many human cancers, including the majority of sporadic and hereditary colorectal tumors<sup>3 4</sup>. As  $\beta$ -catenin is a major key component of the canonical Wnt pathway, its protein levels and activity are tightly regulated<sup>1;5</sup>. In the absence of Wnt stimulation, cytoplasmic  $\beta$ -catenin is targeted to a multi-protein complex, termed the " $\beta$ -catenin degradation complex" that includes axin, Adenomatous polyposis coli (APC), glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) and casein kinase I (CK1). GSK-3 $\beta$  and CK1 phosphorylate  $\beta$ -catenin at several serine/threonine residues<sup>6</sup> and the phosphorylated protein is then recognized and ubiquitinated by E3 ligases. The most well-studied is SCF <sup>$\beta$ -TrCP</sup>, a multi-protein complex that marks  $\beta$ -catenin for proteasomal degradation<sup>7</sup> and is regulated itself by the canonical Wnt pathway<sup>8 9</sup>. Wnt signaling is initiated by the binding of Wnt family members to a receptor complex consisting of the Frizzled transmembrane receptors, together with the co-receptors low-density lipoprotein receptor related proteins 5/6 (LRP5/6). The receptor activation leads to disassembly of the  $\beta$ -catenin degradation complex and, as a consequence, unphosphorylated  $\beta$ -catenin is no longer degraded but accumulates and translocates into the nucleus. In the nucleus,  $\beta$ -catenin functions as a transcription co-factor for members of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) families to activate Wnt target genes. Aberrant stabilization of  $\beta$ -catenin triggers up-regulation of Wnt target genes such as c-myc and cyclin D1<sup>1 10 11</sup>, and is believed to be the basis for tumorigenesis. In addition to the  $\beta$ -catenin degradation complex, several other mechanisms have been shown to repress the transcriptional activity of  $\beta$ -catenin<sup>12; 13; 14; 15; 16 17 18</sup>; however, the regulation of nuclear  $\beta$ -catenin remains poorly understood.

The ubiquitin pathway is initiated by the conjugation of a 76-residue ubiquitin moiety to lysine residues of a target protein. Ubiquitination occurs through a combined set of reactions involving activating (E1), conjugating (E2) and ligating (E3) enzymes. E3 enzymes physically interact with their substrates and are thus critical components in determining ubiquitination specificity. The ubiquitin system is involved in the regulation of various cellular processes, among them is the important role of targeting proteins for degradation. Nevertheless, ubiquitination also plays a significant role in DNA damage repair, cellular signaling, intracellular trafficking and ribosomal biogenesis<sup>19</sup>. In addition, ubiquitination is beginning to emerge as an important controlling factor in transcriptional switches<sup>20</sup>. In the Wnt signaling pathway, ubiquitination is known to mark  $\beta$ -catenin for degradation, thus reducing signaling levels. However, in this report we provide evidence indicating that the E3 ubiquitin ligase EDD serves as a positive regulator of  $\beta$ -catenin-mediated signaling. We show that EDD interacts with both GSK-3 $\beta$  and  $\beta$ -catenin, leading to  $\beta$ -catenin ubiquitination. Expression of EDD results in up-regulation of  $\beta$ -catenin protein levels and activity, suggesting a positive role for ubiquitination by EDD in the Wnt signaling pathway and cancer development.

## Discussion

The canonical Wnt/ $\beta$ -catenin signaling pathway regulates cell fate and proliferation in embryonic development and regeneration and tissue homeostasis in adults<sup>30 2</sup>. Up-regulation of this pathway is linked to a number of different diseases, including

cancer, Alzheimer's disease and bone density syndromes<sup>4</sup>. Activation of  $\beta$ -catenin, a key factor of the canonical Wnt pathway, is a critical step in the pathogenesis of many human cancers.  $\beta$ -catenin expression is known to be regulated primarily by proteasomal degradation, mediated by CK-1 and GSK-3 $\beta$  phosphorylation.

Using immunoprecipitation experiments we identified three potential GSK-3 $\beta$  binding proteins: aryl hydrocarbon receptor-associated 3 (ARA3), EDD1 E3 ubiquitin ligase, and APC. While APC is a known component of the canonical Wnt pathway and resides in the " $\beta$ -catenin degradation complex" together with GSK-3 $\beta$ ; the roles of ARA3 and EDD in the Wnt pathway are still unclear. The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor with important roles in metabolic adaptation, dioxin toxicology, and vascular development. ARA3 is a modifier of the AHR signaling pathway that regulates the concentration of functional AHR in mammalian cells<sup>31</sup>. Although no direct connection between the Wnt pathway and ARA3 has been demonstrated so far, it has been shown that AHR signaling mediates degradation of  $\beta$ -catenin in mice intestinal tumors<sup>32</sup> and may also down regulate the Wnt pathway during development<sup>33</sup>.

The E3 ubiquitin ligase EDD is the mammalian ortholog of the *Drosophila melanogaster* hyperplastic discs gene (*hyd*)<sup>34</sup>; a loss of function mutation of *hyd* causes imaginal disc overgrowth in *Drosophila*<sup>35</sup>. Reduced expression of EDD has been observed in several human cancers<sup>36 37</sup>, indicating that EDD may function as a tumor suppressor gene. However, in breast and ovarian cancers, EDD is over-expressed<sup>38 22</sup>, suggesting that it can potentially function as an oncogene. Therefore, though the mechanism is unclear, EDD plays important roles in the development of certain cancers.

The results presented in this study demonstrate a functional link between the canonical Wnt signaling pathway and the EDD protein. Here we show that EDD directly binds GSK-3 $\beta$  and forms a cellular complex with  $\beta$ -catenin. Moreover, EDD ubiquitinates  $\beta$ -catenin in a GSK-3 $\beta$ -dependent manner and up-regulates the expression levels and activity of  $\beta$ -catenin, possibly through increasing its stability. We demonstrate that endogenous EDD co-localizes with nuclear active  $\beta$ -catenin, and when over-expressed, EDD leads to increased nuclear accumulation of both  $\beta$ -catenin and GSK-3 $\beta$ . It is possible that in cells expressing high levels of EDD, such as ovarian cancer cells, EDD may interact with and alter the subcellular localization of GSK-3 $\beta$ , thus recruiting it into the nucleus. Nuclear phosphorylation by GSK-3 $\beta$  has been shown to regulate nuclear export, DNA binding, and transcriptional activity of different substrates<sup>39 40 41 42 43</sup>. By modulating GSK-3 $\beta$ 's nuclear localization, EDD may affect GSK-3 $\beta$ -dependent phosphorylation of different substrates, thus influencing a wide range of cellular processes, including  $\beta$ -catenin-mediated transcription.

Our data indicates that EDD ubiquitinates  $\beta$ -catenin, leading to enhanced  $\beta$ -catenin protein levels and activity. The regulation of Wnt signaling by ubiquitination is generally related to  $\beta$ -catenin degradation. Nevertheless, a number of recent studies have shown that the ubiquitin system is involved in activation of Wnt target genes. For example, Travid and Cezanne, both deubiquitylating (DUB) enzymes, antagonize the ubiquitination of APC, resulting in activation of  $\beta$ -catenin/TCF-mediated transcription<sup>44</sup>. In addition, Sierra *et al.* has demonstrated that *in-vitro* transcriptional activation by recombinant  $\beta$ -catenin/LEF-1 complexes requires ubiquitin<sup>16</sup>.

Our results indicate that the ubiquitin mutants K48R and K63R do not have much effect on EDD-mediated  $\beta$ -catenin ubiquitination, or protein levels and activity. The ubiquitin mutants K29R and K11R mutants, on the other hand, decreased EDD-

mediated ubiquitination and its effects on  $\beta$ -catenin. TRABID and Cezanne were shown to cleave K29 and K11-linked ubiquitin chains, respectively<sup>45 46</sup>. Although TRABID was first reported to activate the Wnt pathway by cleavage of the K63-linked chains<sup>44</sup>, it was recently demonstrated to be 40-fold more active towards the K29-linked chains<sup>45</sup>. These results should provide a drive to study the role of K29 and K11 linkages in the Wnt signaling biological pathway.

Rad6B is an E2-conjugating enzyme, reported to mediate  $\beta$ -catenin poly-ubiquitination and stabilization, thus activating the Wnt signaling pathway<sup>47</sup>. As both EDD and Rad6B are involved in DNA damage response<sup>23; 27; 48; 49</sup>, it is possible that Rad6B may function as an E2-ubiquitin conjugating enzyme (UBC) in combination with EDD to ubiquitinate  $\beta$ -catenin. We are currently testing this hypothesis and examining whether EDD/Rad6B-mediated ubiquitination of  $\beta$ -catenin can result in cancerous phenotypes.

Our data demonstrates that the constitutively active form of  $\beta$ -catenin (the S33A mutant which is GSK-3 $\beta$  insensitive) was only moderately ubiquitinated by EDD compared to the wild-type form. Furthermore, in COLO320 and SW480 cells, where GSK-3 $\beta$  cannot phosphorylate  $\beta$ -catenin, silencing of EDD had no effect on  $\beta$ -catenin/TCF-mediated transcription. Although GSK-3 $\beta$  is a negative regulator of the Wnt pathway<sup>6</sup> our results show that GSK-3 $\beta$  is involved in EDD-mediated activation of  $\beta$ -catenin and that phosphorylation of  $\beta$ -catenin by GSK-3 $\beta$  is required for its EDD-induced ubiquitination. Together, these results imply that GSK-3 $\beta$  may have a dual role in regulation of the Wnt pathway; on one hand as part of the destruction complex leading to degradation of  $\beta$ -catenin and on the other hand, as a binding partner of the EDD protein that functions to stabilize  $\beta$ -catenin.

Overall, our results reveal a correlation between EDD and up-regulation of  $\beta$ -catenin both in the expression levels and transcriptional activity. In a recent study EDD was shown to co-localize with APC in the cytoplasm<sup>50</sup> which somewhat conflict with our findings and other reports showing that EDD is a nuclear protein<sup>23 22</sup>. This paper also demonstrated that EDD up-regulates APC and down regulates  $\beta$ -catenin. Our data, however clearly show that over-expression of EDD up-regulates  $\beta$ -catenin and moreover, that EDD depletion resulted in decreased levels of  $\beta$ -catenin. The apparent discrepancy between the results of Ohshima *et al.* and our findings may result from the different cell lines and experimental conditions used in the EDD RNAi experiments.

The data presented in this work suggests that the ubiquitin system, mediated through EDD E3 ligase, is involved in stabilization of  $\beta$ -catenin, leading to up-regulated expression of Wnt target genes. The ubiquitin system, therefore, may be targeted for therapeutic intervention in CRC and other types of disorders that result from high levels of Wnt signaling.

## **Material and Methods**

### Cell culture and transfection

HEK293T, CHO and the human colorectal cancer cell lines COLO320, HCT116 and SW480 were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were transfected either by CaPO<sub>4</sub> precipitation or using the DNA transfection reagent jetPEI (Polyplus Transfection) following manufacturer's protocols.

### Plasmids and reagents

The FLAG-EDD expression vector was a generous gift from Dr. Michael J. Weber (University of Virginia Health Sciences Center). GFP- $\beta$ -catenin was constructed by

inserting the  $\beta$ -catenin cDNA into pEGFP-C1 (Clontech) using BamHI and XbaI restriction sites. This expression vector was used as a template for mutagenesis of  $\beta$ -catenin by the QuikChange site-directed mutagenesis kit (Stratagene) to replace serine<sup>33</sup> to alanine to generate GFP-S33A- $\beta$ -catenin.

The HA-GSK expression vector was kindly provided by Dr. T. C. Dale (Developmental Biology, Chester Beatty Laboratories, Institute of Cancer Research, London, UK). FLAG-GSK-3 $\beta$  was kindly provided by Dr. Hagit Eldar-Finkelman (Tel-Aviv University, Israel) and HA- $\beta$ -catenin was kindly provided by Dr. Arnona Gazit (Tel-Aviv University, Israel). GFP-GSK-3 $\beta$  was constructed by inserting the GSK-3 $\beta$  cDNA into pEGFP-C1 (Clontech). The Wnt-responsive TCF-dependent luciferase constructs pTOPFLASH (pTOPFLASH contains multimerized TCF-binding sites linked to a luciferase reporter) and its mutated version pFOPFLASH were kindly provided by Dr. H. Clevers (Center for Biomedical Genetics, Hubrecht, The Netherlands) and were described previously<sup>21</sup>. pSV- $\beta$ -Galactosidase Control Vector and pCMV-Renilla were purchased from promega. HA-ubiquitin was a generous gift from Dr. Dirk Bohmann (University of Rochester Medical Center). Ubiquitin mutants K48R, K63R, K29R and K11R expression vectors were kindly provided by Dr. Yosef Yarden (Weizmann institute of science, Israel). Control vectors were pEGFP-C1, mCherry (Clontech), pCMV-Flag (Sigma) and pCDNA3-HA (Invitrogen). Cycloheximide and LiCl (Sigma-Aldrich) were used at concentrations as indicated.

#### Antibodies

The following antibodies were used: mouse anti- $\beta$ -catenin (IB: 1:5000; IF: 1:300; BD Transduction Laboratories), rabbit anti- $\beta$ -catenin (IP:1:100; Santa Cruz Biotechnology), mouse anti-active  $\beta$ -catenin (ABC) (IB: 1:1000; IF: 1:150; Millipore) mouse anti-GSK-3 $\beta$  (IB: 1:1000; IF: 1:300; BD Transduction Laboratories), mouse anti-actin (1:10,000; ImmunO), rabbit anti-GFP (IB: 1:5000; IP: 1:100: Santa Cruz Biotechnology), rat anti-HA (IB: 1:5000; IF: 1:300; Roche), goat anti-EDD M-19 (1:500; Santa Cruz Biotechnology), goat anti-EDD ab4376 (1:500, Abcam), rabbit anti-EDD (IP: 1:100; Bethyl Laboratories. Inc.), mouse anti-FLAG (1:5000; Sigma), rabbit anti-FLAG (IB: 1:500; IP: 1:100; Sigma). Anti-rat and anti-goat horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Anti-mouse and anti-rabbit secondary antibodies were obtained from Jackson Immuno Research. For IF Alexa red and green (1:500; Molecular Probes) were used. IB: immunoblot; IP: immunoprecipitation; IF: immunofluorescence

#### Luciferase reporter assays

To assay TCF-mediated transcription cells were seeded at  $1 \times 10^5$  cells per well in a 24-well plate 24 hours prior to transfection. Cells were transfected with the indicated vectors, along with pTOPFLASH/pFOPFLASH and  $\beta$ -gal or pCMV-Renilla plasmids. Forty eight hours post transfection, the cell were harvested and subjected to luciferase assay according to manufacturer's instructions. Activity specificity was confirmed by the pFOPFLASH plasmid.

#### Western blot analysis and immunoprecipitation

Forty-eight hours following transfection, cells were washed with phosphate-buffered saline (PBS) and solubilized in lysis buffer (100 mM NaCl, 50 mM Tris, pH 7.5, 1% Triton X-100, 2 mM EDTA) or RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease inhibitor cocktail (Sigma). Extracts were clarified by centrifugation at  $12,000 \times g$  for 15 min at 4°C. Following SDS polyacrylamide gel electrophoresis (SDS-PAGE) separation, proteins were transferred to nitrocellulose membranes and, after blocking

with 5% low fat milk, filters were incubated with the specific primary antibody. Membranes were washed in PBS containing 0.001% Tween-20 and incubated for 1 hour with a secondary antibody. After washing in PBS/Tween, membranes were subjected to enhanced chemiluminescence detection analysis using horseradish peroxidase-conjugated secondary antibodies. Intensity of the protein bands was quantified by a computer-assisted densitometer (TINA 2.0c; Fuji BAS, Tokyo, Japan). For immunoprecipitation analysis, cells were solubilized in lysis buffer (see above). Cell lysates were incubated with anti-FLAG M2-agarose affinity gel (Sigma), with rotation for 18 hours at 4°C. Alternatively, cell lysates were incubated with the specific antibody for 1 hour at 4°C prior to 18-hour rotated incubation with protein A/G agarose (Santa Cruz Biotechnology) at 4°C. Beads were collected by slow centrifugation, washed four times in lysis or RIPA buffer and either used for ubiquitination assays or analyzed by SDS-PAGE followed by detection with specific antibody.

#### Pull-down assays

Pull-down experiments were performed using purified GST, GST- $\beta$ -catenin or 6XHis GSK-3 $\beta$  (kindly provided by Dr. Hagit Eldar-Finkelman). The EDD protein was produced in rabbit reticulocyte TnT system (Promega) using [ $S^{35}$ ]methionin or in the presence of non labeled amino acids mixture. Glutathione (Sigma) or Ni-NTA (Qiagen) beads were used to precipitate the GST-fusion proteins or the 6XHis GSK-3 $\beta$  respectively. For each experiment 10 $\mu$ g of recombinant proteins were incubated with 25 $\mu$ l of reticulocyte lysates and the appropriate beads for 3 hours at 4°C. Beads were then washed four times and analyzed by SDS-PAGE. The gel was stained with coomassie blue, dried and exposed to x-ray film or blotted and incubated with anti-EDD antibodies. For control, either GST protein or Ni-NTA beads were used respectively.

#### Nuclear extraction

Cells were harvested in low salt buffer (10 mM HEPES PH7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) containing protease inhibitor cocktail (Sigma). To extract cytosolic fraction, cells were centrifuged for 10 min at 7,000 x g. Pellets were then resuspended in high salt buffer (20 mM HEPES PH7.9, 0.42 mM NaCl, 1 mM EDTA, 1 mM EGTA). Nuclear fraction was collected following centrifugation of 5 min at 12,000 x g.

#### Immunofluorescence

HEK293T, CHO and SW480 cells grown on coverslips were fixed for 20 min in PBS containing 4% paraformaldehyde, washed three times with PBS, permeabilized with 0.1% Triton X-100 for 1 hour and blocked with bovine serum albumin for 1 hour. Subsequently, cells were incubated at room temperature with primary and secondary antibodies for 60 and 30 min, respectively. 4-6' diamidino-2 phenylindole (DAPI, Sigma) was used to stain cell nuclei. GFP and mCherry were detected without staining. Cells were visualized by Concofal Microscopy.

#### In vivo ubiquitination assay

HEK293T cells were transfected with HA-ubiquitin constructs together with the indicated plasmids. Five hours before harvesting, 25  $\mu$ M proteosomal inhibitor MG132 (CalBiochem) was added. Forty-eight hours after transfection cells were harvested and lysed in RIPA buffer. Cell lysates were immunoprecipitated with anti-GFP or anti- $\beta$ -catenin antibody as described. The bound proteins were eluted with Laemmli sample buffer. Western blot analysis was performed as described using an anti-HA antibody. The membrane was then stripped and reprobed with anti- $\beta$ -catenin

or anti-GFP antibodies. Lysates were also subjected to western blotting using anti-FLAG or anti-EDD antibodies.

#### SiRNA assay

The siRNA target sequence of EDD reported previously<sup>22</sup> was cloned into the BglII–HindIII site of pSUPER. EDD-RNAi, 5'-gcagtgttctctgccttctt-3'. The sequence of the control scrambled EDD-RNAi was 5'-ggtcctgtctctctctat-3'. Transfection of the colorectal cell lines SW480 and COLO320 using jetPEI was carried out as described. For EDD siRNA experiments in HEK293T and CHO cells, RNA oligonucleotides were purchased from Thermo Scientific Dharmacon (siGENOME SMART pool M-007189-02-0005, Human UBR5) as well as transfection reagent Dharmafect-1. Transfection was performed according to manufacturer's protocol. All experiments in HEK293T and CHO were performed using 100 nM siRNA oligonucleotides. siGSK was also purchased from Thermo Scientific Dharmacon (siGENOME SMART pool M-003010-03-0005 hGSK3b NM-002093). HEK293T were transfected with 10nM siRNA oligonucleotides using Interferin (Polyplus Transfection) according to manufacturer's order. Non targeting RNA oligonucleotides (Thermo Scientific Dharmacon) were used for both scEDD and scGSK controls.

## **Results**

### EDD forms cellular complexes with GSK-3 $\beta$ and $\beta$ -catenin

In our studies of the Wnt signaling pathway, we have tried to identify potential functional partner(s) of GSK-3 $\beta$ , a core component of this oncogenic pathway. To do so, lysates from HEK293T cells transfected with FLAG-GSK-3 $\beta$  or an empty FLAG vector were incubated with anti-FLAG M2 antibodies conjugated to agarose beads. After the captured proteins were resolved by SDS-PAGE, colloidal coomassie blue staining revealed several proteins that co-precipitated with FLAG-GSK-3 $\beta$  and not with the control vector. We excised these polypeptides and analyzed them by tandem mass spectrometry. Through this analysis, we identified three potential GSK-3 $\beta$  binding proteins: aryl hydrocarbon receptor-associated 3, EDD1 E3 ubiquitin ligase, and APC.

As several studies indicate that aberrations in EDD gene expression or function are a feature of many cancer types, we decided to further investigate the connection between EDD and the Wnt signaling pathway. First, we performed immunoprecipitation assay in order to verify the cellular interaction between EDD and GSK-3 $\beta$ . HEK293T cells were co-transfected with constructs encoding FLAG-EDD in the presence or absence of hemagglutinin (HA)-tagged GSK-3 $\beta$ . Both HA-GSK-3 $\beta$  (Fig. 1A) and endogenous GSK-3 $\beta$  (Fig. 1B) co-immunoprecipitated with FLAG-EDD, confirming the interaction between the two proteins. Next, we examined whether endogenous EDD can form a complex with endogenous GSK-3 $\beta$ . Indeed, EDD-GSK-3 $\beta$  complex was observed in both HEK293T cells (upper panel) and the colorectal cell line HCT116 where  $\beta$ -catenin is constitutively active (lower panel). As  $\beta$ -catenin is a core component of the canonical Wnt pathway, we tested whether  $\beta$ -catenin can interact with EDD as well. EDD is known to be a nuclear protein<sup>23</sup>, thus, we used the nuclear fraction of HEK293T cells to try and detect such an interaction. Our results show that endogenous nuclear  $\beta$ -catenin immunoprecipitated with endogenous EDD (Fig. 1D, upper panel). A similar result was obtained when using the colorectal cell line SW480, which expresses high levels of nuclear  $\beta$ -catenin due to a truncated APC protein (Fig. 1D, lower panel). To examine whether the interaction between EDD and GSK-3 $\beta$  or  $\beta$ -catenin is direct, *in-vitro* pull-down experiments were next performed. GSK-3 $\beta$  or  $\beta$ -catenin proteins were bacterially produced,

purified and tested for their ability to bind EDD translated in the TnT reticulocyte system. As shown in Fig. 1E (right panel), GSK-3 $\beta$  bound EDD, suggesting that the interaction between EDD and GSK-3 $\beta$  is direct.  $\beta$ -catenin, on the other hand, did not directly interact with EDD. Coomassie staining of bacterially produced proteins is shown in the left panel.

#### Over-expression of EDD enhances GSK-3 $\beta$ and $\beta$ -catenin's nuclear accumulation

Immunofluorescence was used to determine the subcellular localization of endogenous EDD, GSK-3 $\beta$  and  $\beta$ -catenin in both HEK293T and SW480 cells (Fig. 2A). In these experiments, we used anti-total and anti-active  $\beta$ -catenin antibodies. The anti-active  $\beta$ -catenin antibody (anti-ABC) specifically detects dephosphorylated nuclear  $\beta$ -catenin which is transcriptionally active in mediating the Wnt pathway, as opposed to  $\beta$ -catenin that functions in cell-cell adhesion<sup>24</sup>. As seen in HEK293T cells (upper panel), endogenous EDD shows nuclear staining and co-localizes with the active nuclear form of  $\beta$ -catenin. However, EDD does not co-localize with GSK-3 $\beta$  that is mostly cytoplasmic or with the membranal pool of  $\beta$ -catenin. Unlike HEK293T, SW480 cells express mainly nuclear  $\beta$ -catenin, which is transcriptionally active. In these cells (lower panel), endogenous EDD co-localizes with the nuclear  $\beta$ -catenin. To test whether over-expression of EDD affects the subcellular localization of GSK-3 $\beta$  or  $\beta$ -catenin, immunofluorescence assay was performed in HEK293T cells transfected with FLAG-EDD and HA-GSK-3 $\beta$  or HA- $\beta$ -catenin (Fig. 2B, upper panel). As can be seen, FLAG-EDD is nuclear, whereas HA-GSK-3 $\beta$  and HA- $\beta$ -catenin are mostly cytoplasmic. However, when co-expressed with FLAG-EDD, the subcellular distribution of both HA-GSK-3 $\beta$  or HA- $\beta$ -catenin was altered and staining was observed in the cell's nucleus as well as the cytoplasm. These results indicate that EDD enhances the nuclear localization of both GSK-3 $\beta$  and  $\beta$ -catenin. To quantify the nuclear accumulation of GSK-3 $\beta$  or  $\beta$ -catenin induced by EDD over-expression, co-transfected cells were counted (n=200) and the percentage of nuclear staining with or without FLAG-EDD was determined (Fig. 2B, lower panel). Expression of FLAG-EDD resulted in more than a 3 fold increase in the levels of nuclear GSK-3 $\beta$  or nuclear  $\beta$ -catenin. Taken together, these results indicate that EDD alters the subcellular distribution of both GSK-3 $\beta$  and  $\beta$ -catenin, leading to their nuclear accumulation.

#### EDD up-regulates the active form of $\beta$ -catenin and enhances Wnt signaling

Next, the effect of EDD over-expression on  $\beta$ -catenin and GSK-3 $\beta$  levels was examined (Fig. 3A). HEK293T cells were co-transfected with FLAG-EDD or the FLAG empty vector, along with HA-GSK-3 $\beta$ , GFP- $\beta$ -catenin or GFP-S33A- $\beta$ -catenin; a constitutively active  $\beta$ -catenin mutant that is insensitive to GSK-3 $\beta$ -mediated phosphorylation and proteasomal degradation<sup>25</sup>. Over-expression of EDD led to a significant increase in the levels of the active  $\beta$ -catenin fraction as compared to the levels of the total  $\beta$ -catenin protein pool. Although GSK-3 $\beta$  levels were unchanged by EDD over-expression, GSK-3 $\beta$  phosphorylation of  $\beta$ -catenin seemed to be important for EDD's function, as the GFP-S33A- $\beta$ -catenin protein was hardly affected by EDD (Fig. 3A). As HEK293T cells exhibit low levels of Wnt signaling due to the activity of the  $\beta$ -catenin degradation complex, GFP- $\beta$ -catenin was expressed in these cells to induce activation of signaling. The cells were co-transfected with GFP- $\beta$ -catenin and with increasing amounts of FLAG-EDD. Results show that over-expression of EDD led to increased levels of  $\beta$ -catenin in a dose-dependent manner (Fig. 3B, upper panel). Accordingly, EDD over-expression resulted

in enhanced  $\beta$ -catenin activity that was measured by the TCF/ $\beta$ -catenin dependent transcription system (pTOPFLASH/pFOPFLASH) (Fig. 3B, lower panel). Next, immunofluorescence analysis was performed in CHO (Fig. 3C, left panel) and HEK293T (Fig. 3C, right panel) cells, both exhibiting low levels of  $\beta$ -catenin and Wnt signaling activity. The data presented support our western blot observation as over-expression of EDD resulted in increased levels of the active  $\beta$ -catenin protein. In order to better understand the mechanism by which EDD affects  $\beta$ -catenin, we next examined  $\beta$ -catenin stability by following its degradation rate after cycloheximide addition (Fig. 3D, E). HEK293T cells were co-transfected with GFP- $\beta$ -catenin and either FLAG-EDD or an empty FLAG vector (Fig. 3D). Twenty-four hours post transfection, 80  $\mu$ g/ml cycloheximide was added, and the levels of GFP- $\beta$ -catenin were measured at the indicated times. Data showed that one hour following cycloheximide addition, the levels of  $\beta$ -catenin were significantly decreased in the vector transfected cells, while in the presence of EDD the decrease was observed only after six hours. The levels of GFP- $\beta$ -catenin were quantified using a computer-assisted densitometer (right panel). A similar assay was performed to measure the effect of EDD on endogenous  $\beta$ -catenin stability (Fig. 3E). Cells were transfected with FLAG-EDD or an empty FLAG vector and harvested at the indicated times following cycloheximide addition. The degradation rate of endogenous  $\beta$ -catenin was tested by antibodies against both the total and active  $\beta$ -catenin forms. Since there are high levels of membrane bound  $\beta$ -catenin which may have diluted the effect of EDD on cytosolic or nuclear  $\beta$ -catenin, EDD's effect could not be detected. To overcome this problem, a portion of the same lysates was subjected to nuclear fractionation and the levels of endogenous  $\beta$ -catenin were measured using the anti-active  $\beta$ -catenin antibody. The TINA software was used to quantify these results (right panel). Indeed, the degradation rate of endogenous  $\beta$ -catenin was reduced in the presence of EDD. Altogether, these results imply that EDD up-regulates  $\beta$ -catenin activity and levels, most likely by increasing protein stability via posttranslational modifications. It is interesting to note that the FLAG-EDD protein seems to be a fairly stable protein as its expression levels do not decrease through the entire course of the experiment.

#### Silencing of EDD decreases $\beta$ -catenin levels and activity in HEK293T cells

To further verify the specificity of EDD's effect on  $\beta$ -catenin-mediated signaling, RNA interference was used to inhibit EDD expression in HEK293T, CHO, COLO320 and SW480 cells. HEK293T cells were transfected with siRNA EDD oligonucleotides for 72 hours and the levels of endogenous EDD, active  $\beta$ -catenin and GSK-3 $\beta$  were measured (Fig. 4A). EDD depletion led to decreased levels of active  $\beta$ -catenin, whereas the levels of GSK-3 $\beta$  remained unchanged (upper panel). The experiment was repeated three times and protein levels were quantified by densitometric computer analysis (lower panel). Next, we measured the effect of EDD depletion on the levels and activity of GFP- $\beta$ -catenin (Fig. 4B). Results show that silencing of endogenous EDD resulted in inhibited  $\beta$ -catenin/TCF-mediated transcription (lower panel) and decreased levels of GFP- $\beta$ -catenin protein, accordingly (upper panel). The effect of EDD depletion was also tested using immunofluorescence assay (Fig. 4C). HEK293T (left panel) and CHO (right panel) cells were transfected with siRNA oligonucleotides as indicated. Our results show that the nuclear accumulation of active  $\beta$ -catenin was dramatically reduced when EDD was silenced in both cell types. The subcellular localization of GSK-3 $\beta$  remained unaffected. As mentioned above, in HEK293T cells the  $\beta$ -catenin degradation complex is intact. However, in COLO320 and SW480 cells a mutation in the APC gene results in a truncated protein which prevents assembly of this degradation complex. Consequently, these cells express

high levels of  $\beta$ -catenin protein and exhibit high levels of  $\beta$ -catenin-mediated transcription<sup>26 14</sup>. EDD silencing in the CRC cell lines COLO320 and SW480 was performed by transfection with siRNA constructs for 48h. In both cell lines, depletion of EDD affected neither the levels of total or active  $\beta$ -catenin, nor their activity. These results imply that an intact  $\beta$ -catenin degradation complex is required for EDD's function.

#### EDD ubiquitinates $\beta$ -catenin

EDD is an E3 ubiquitin ligase containing UBA (Ubiquitin Associated), UBR (Ubiquitin-Protein Ligase E3 Component N-Recognin) and HECT (Homologous to the E6-AP Carboxyl Terminus) domains, all known to be involved in ubiquitination or ubiquitin binding<sup>27</sup>. Since the data presented so far indicated that EDD enhances  $\beta$ -catenin expression, we examined whether the effect on  $\beta$ -catenin levels is related to EDD's ubiquitination activity. To test whether EDD ubiquitinates  $\beta$ -catenin or GSK-3 $\beta$ , HEK293T cells were transfected with FLAG-EDD, HA-ubiquitin and either GFP- $\beta$ -catenin or GFP-GSK-3 $\beta$  (Fig. 5A). The proteasomal inhibitor MG132 was added to the cells prior to harvesting in order to visualize the ubiquitinated proteins. Cell lysates were immunoprecipitated with an anti-GFP antibody and proteins were detected using an anti-HA antibody. A smear of protein bands was observed only when EDD,  $\beta$ -catenin and ubiquitin were over-expressed simultaneously, suggesting that EDD poly-ubiquitinates  $\beta$ -catenin *in-vivo*. GSK-3 $\beta$ , however, did not appear to be ubiquitinated by EDD. The HA blot was reprobbed using an anti-GFP or anti- $\beta$ -catenin antibodies to confirm that the protein identified by the HA antibody is indeed  $\beta$ -catenin. As the experiment was performed in the presence of MG132, GFP- $\beta$ -catenin ubiquitinated by the EDD-independent ubiquitin pathway is also observed (marked by an asterisk). To confirm the specificity of EDD's effect on  $\beta$ -catenin ubiquitination, over-expressed or endogenous EDD was silenced in HEK293T cells expressing HA-ubiquitin (Fig. 5B). Silencing of either endogenous or over-expressed EDD resulted in reduced ubiquitination of endogenous  $\beta$ -catenin, indicating a specific ubiquitination of  $\beta$ -catenin by EDD. Since our results implied that GSK-3 $\beta$  is involved in EDD's effect on  $\beta$ -catenin, we compared the ability of EDD to ubiquitinate wild type GFP- $\beta$ -catenin and the constitutively active  $\beta$ -catenin (S33A) mutant. This mutated form of  $\beta$ -catenin, which is insensitive to GSK-3 $\beta$ -mediated phosphorylation, exhibited impaired ubiquitination relative to the wild type form (Fig. 5C). Similar results were obtained when the GFP- $\beta$ -catenin protein was replaced by a Myc tag (not shown). We then examined the involvement of GSK-3 $\beta$  in EDD-mediated  $\beta$ -catenin up-regulation by transfecting HEK293T cells with FLAG-EDD and either over-expression or silencing of GSK-3 $\beta$  (Fig. 5D). While, as expected, over-expression of GSK-3 $\beta$  resulted in decreased  $\beta$ -catenin protein levels (left panel), co-expression of both GSK-3 $\beta$  and EDD abolished GSK-3 $\beta$ 's inhibitory effect on  $\beta$ -catenin. GSK-3 $\beta$  depletion, on the other hand, did not affect  $\beta$ -catenin levels (right panel), possibly due to the functional redundancy between GSK-3 $\alpha$  and GSK-3 $\beta$  in Wnt/ $\beta$ -catenin signaling<sup>28</sup>. Importantly, in cells depleted of GSK-3 $\beta$ , EDD-induced up-regulation of  $\beta$ -catenin could not be detected, supporting our hypothesis that GSK-3 $\beta$  is required for this function. Next, we tested the effect of GSK-3 $\beta$  repression on EDD-induced  $\beta$ -catenin activity using the luciferase pTOPFLASH/pFOPFLASH assay (Fig. 5E). Interestingly, although over-expression of GSK-3 $\beta$  inhibited the Wnt pathway as predicted, the addition of EDD impaired GSK-3 $\beta$ 's ability to reduce  $\beta$ -catenin/TCF mediated transcription (left section). Furthermore, the repression of GSK-3 $\beta$  by either siRNA (middle section) or LiCl (right section), led to enhanced Wnt signaling stimulation, but compromised EDD's ability to activate  $\beta$ -catenin.

Taken together, these results indicate that GSK-3 $\beta$  has a role in EDD-mediated activation of  $\beta$ -catenin and the Wnt signaling pathway activity.

#### $\beta$ -catenin ubiquitination by EDD involves Lys29 and Lys11-ubiquitin chains

The utilization of different ubiquitin chains is a key element in determining the ubiquitinated protein fate. For example, poly-ubiquitination via lysine (K)48 is often coupled to degradation of proteins by the 26S proteasome system, whereas poly-ubiquitination via lysine (K)63 acts as a non-proteolytic signal in several intracellular pathways such as recruitment and activation of substrates<sup>29</sup>. In order to determine the nature of EDD-mediated  $\beta$ -catenin ubiquitination, we used a number of ubiquitin mutants (HA-Ubiquitin K48R, K63R, K29R and K11R). An *in-vivo* ubiquitination assay in which the HA-ubiquitin had been replaced by the different ubiquitin mutants was carried out as described above. Our results indicate that EDD's ability to ubiquitinate  $\beta$ -catenin was impaired when the ubiquitin mutant K29R was used (Fig. 6A). This result points to the involvement of Lys29-poly-ubiquitin chain in EDD-mediated ubiquitination of  $\beta$ -catenin. Since different poly-ubiquitination can lead to either protein degradation or other cellular processes, we performed the ubiquitination assay with (upper panel) or without (lower panel) MG132. The ubiquitination pattern remained similar in the presence and absence of the proteasomal inhibitor; supporting our findings that EDD-mediated ubiquitination does not target  $\beta$ -catenin for degradation. Next, we tested the effect of the different ubiquitin mutants on  $\beta$ -catenin levels (Fig. 6B). HEK293T cells were co-transfected with FLAG-EDD, GFP- $\beta$ -catenin and HA-ubiquitin or the different mutants. The levels of GFP- $\beta$ -catenin were determined using western blot analysis (upper panel) and quantified by a computer-assisted densitometer (lower panel). The use of either K29R or K11R mutants led to decreased GFP- $\beta$ -catenin levels compared to wild type ubiquitin and to the other mutants. HEK293T cells were then transfected with the same constructs along with the pTOPFLASH/pFOPFLASH reporters to examine the mutants' effect on  $\beta$ -catenin's activity. The luciferase assay results show that GFP- $\beta$ -catenin activity was highest when both ubiquitin and EDD were over-expressed. K29R and K11R mutants significantly impaired  $\beta$ -catenin-mediated transcription relative to the wild type ubiquitin (Fig. 6C). Overall, these results imply that EDD ubiquitinates  $\beta$ -catenin using Lys29 and/or Lys11 poly-ubiquitin chains, thus enhances its protein levels and activity.

#### **Acknowledgments**

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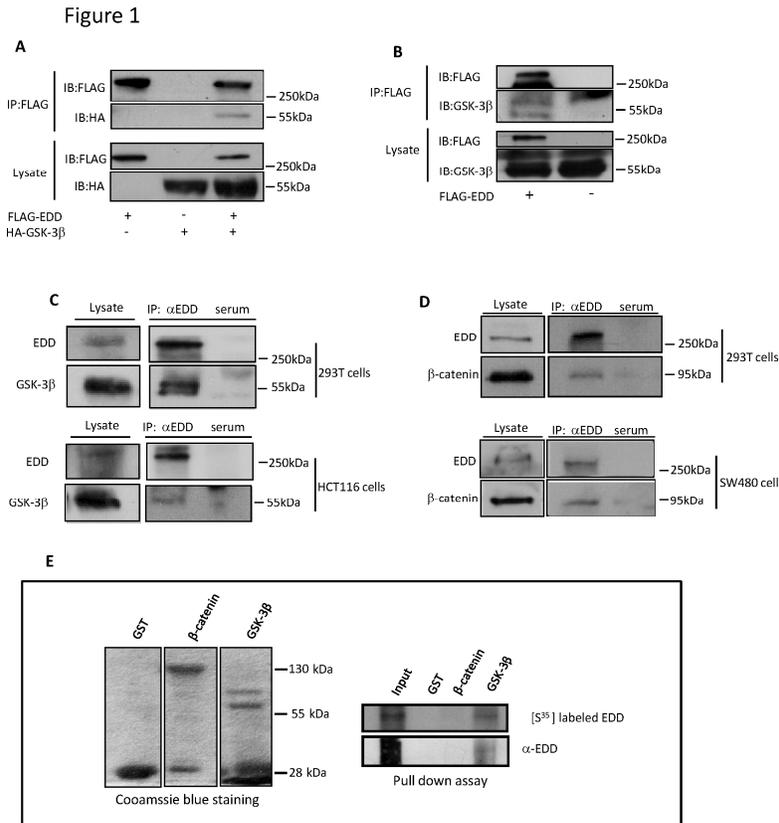
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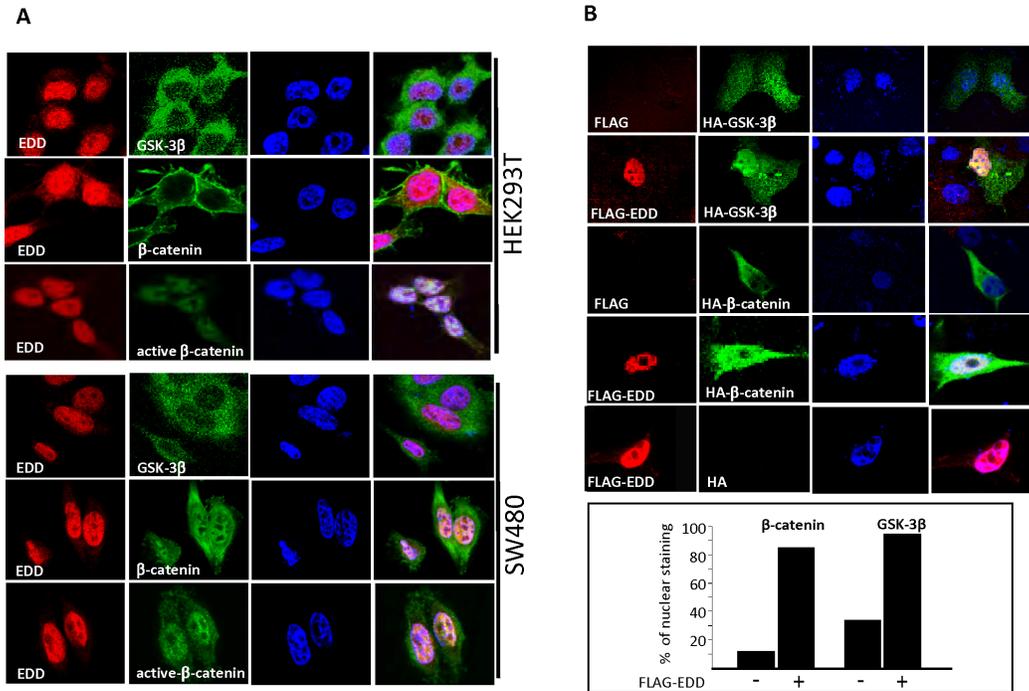
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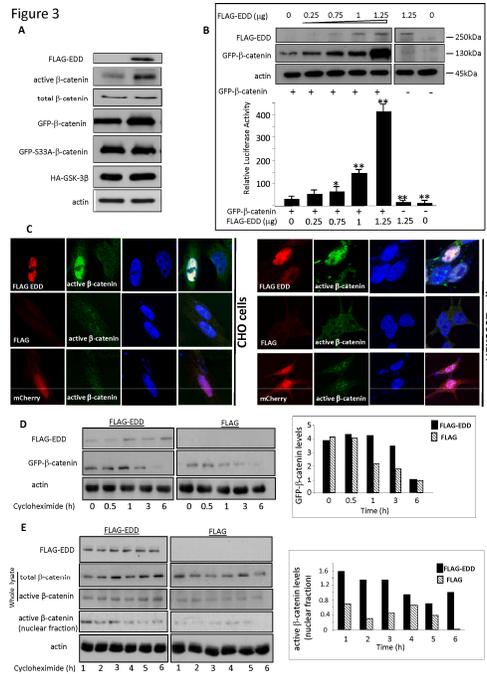
### Figure 1. *In vivo* and *in vitro* interaction between EDD and GSK-3β or β-catenin.

(A) HEK293T cells were transfected with FLAG-EDD and HA-GSK-3β and immunoprecipitated with anti-FLAG. Western blot was performed using anti-FLAG and anti-HA antibodies. (B) HEK293T cells were transfected with FLAG-EDD and immunoprecipitation was performed as before. Western blot was performed using anti-FLAG and anti-GSK-3β antibodies. (C) Lysates from HEK293T (upper panel) or HCT116 (lower panel) cells were immunoprecipitated using a rabbit anti-EDD antibody or control unimmunized serum and subjected to western blotting using anti-GSK-3β and goat anti-EDD antibodies. (D) Nuclear fraction of HEK293T (upper panel) or total lysate from SW480 (lower panel) cells were immunoprecipitated with a rabbit anti-EDD antibody or control unimmunized serum and subjected to western blotting using anti-β-catenin and goat anti-EDD antibodies. (E) Coomassie brilliant blue staining of GST, 6XHis-GSK-3β and GST-β-catenin recombinant proteins that were produced and purified from *E. coli* bacteria (left panel). FLAG-EDD protein translated in a rabbit reticulocyte system in the presence or absence of [<sup>35</sup>S]methionine was pulled down using the recombinant proteins as indicated (right panel). The [<sup>35</sup>S]methionine labeled reaction was exposed to an x-ray film (top) and the non-radioactive reaction was immunoblotted using an anti-EDD antibody (bottom). IB: immunoblot; IP: immunoprecipitation.

Figure 2

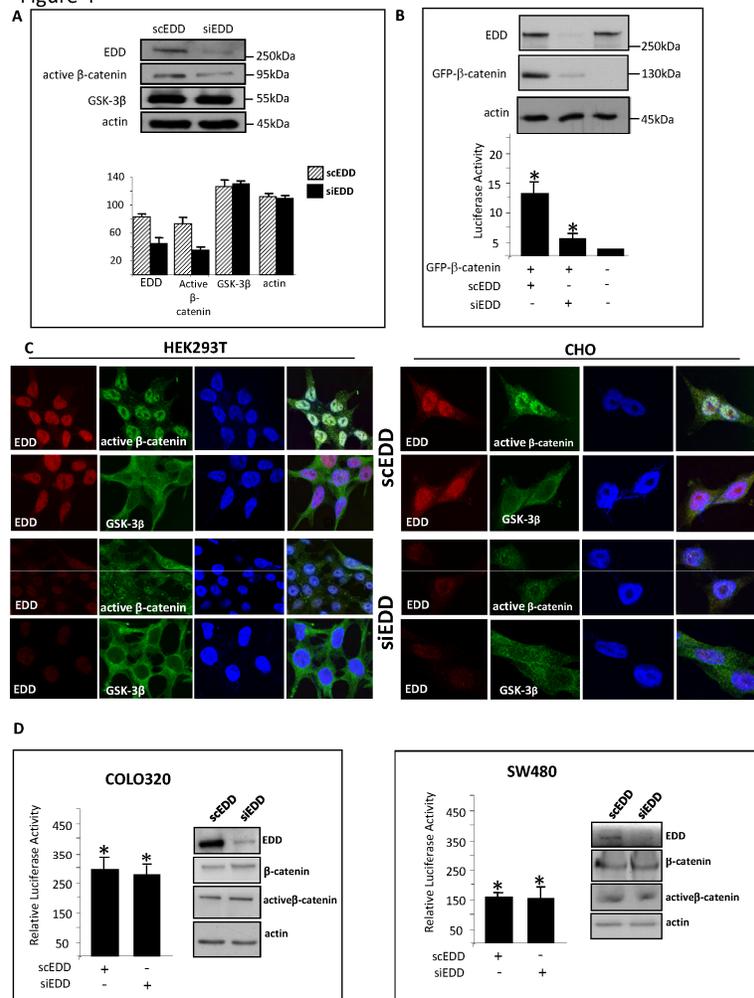


**Figure 2. Nuclear accumulation of GSK-3β and β-catenin following EDD over-expression.** (A) HEK293T (upper panel) and SW480 (lower panel) cells were stained using anti-EDD, anti-GSK-3β, anti-β-catenin and anti-active β-catenin antibodies as indicated. DAPI was used to mark the nucleus. (B) HEK293T cells were co-transfected with HA-GSK-3β, HA-β-catenin or an empty HA vector and either FLAG-EDD or an empty FLAG vector as indicated. Forty-eight hours post transfection, cells were fixed and reacted with anti-FLAG and anti-HA antibodies (upper panel). DAPI was used to mark the nucleus. Transfected cells were counted and percentage of β-catenin or GSK-3β nuclear localization was calculated (lower panel).



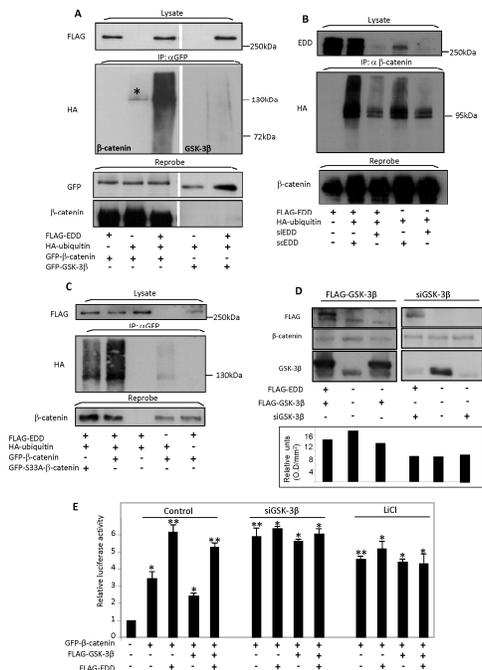
**Figure 3. Over-expression of EDD enhances  $\beta$ -catenin levels, activity and stability.** (A) HEK293T cells were co-transfected with FLAG-EDD or an empty FLAG vector and either GFP- $\beta$ -catenin, GFP-S33A- $\beta$ -catenin or HA-GSK-3 $\beta$ . Forty-eight hours later the cells were harvested and subjected to western blot analysis using anti-FLAG, anti-GFP, anti- $\beta$ -catenin, anti-active  $\beta$ -catenin, anti-HA and anti-actin antibodies. (B) Luciferase assay (lower panel) was performed in HEK293T cells transfected with GFP- $\beta$ -catenin and increasing amounts of FLAG-EDD along with pTOPFLASH/pFOPFLASH reporters and  $\beta$ -gal plasmid. Relative luciferase values are shown. Error bars in this and subsequent figures indicate standard deviations from the mean of two or three independent experiments (performed in duplicates). Asterisks indicate significance difference from control empty vector in Wnt signaling levels (\* $p < 0.05$ , \*\* $p < 0.01$ , Student's  $t$  test). The lysates that were used for the luciferase assay were subjected to western blot analysis (upper panel) using anti-FLAG, anti-GFP and anti-actin antibodies. (C) CHO (left panel) and HEK293T (right panel) cells were transfected with FLAG-EDD, empty FLAG or mCherry vectors. Forty-eight hours post transfection, cells were fixed and reacted with anti-FLAG and anti-active  $\beta$ -catenin antibodies. DAPI was used to mark the nucleus. (D) HEK293T cells were co-transfected with GFP- $\beta$ -catenin and either FLAG-EDD or an empty FLAG vector. Cycloheximide was added to the cells 24 hours post transfection. Cells were harvested at the indicated time points following cycloheximide addition and lysates were subjected to western blot analysis using anti-FLAG, anti-GFP and anti-actin antibodies (left panel). Densitometric analysis of GFP- $\beta$ -catenin was performed using TINA software (right panel). (E) Cycloheximide stability assay was repeated with endogenous  $\beta$ -catenin in a similar way. The lysates were either used directly for western blot analysis or subjected to nuclear fractionation. Total lysates or nuclear fractions were reacted with anti-FLAG, anti- $\beta$ -catenin, anti-active  $\beta$ -catenin and anti-actin antibodies. Densitometric analysis of active  $\beta$ -catenin in the nuclear fraction was performed using TINA software (right panel).

Figure 4



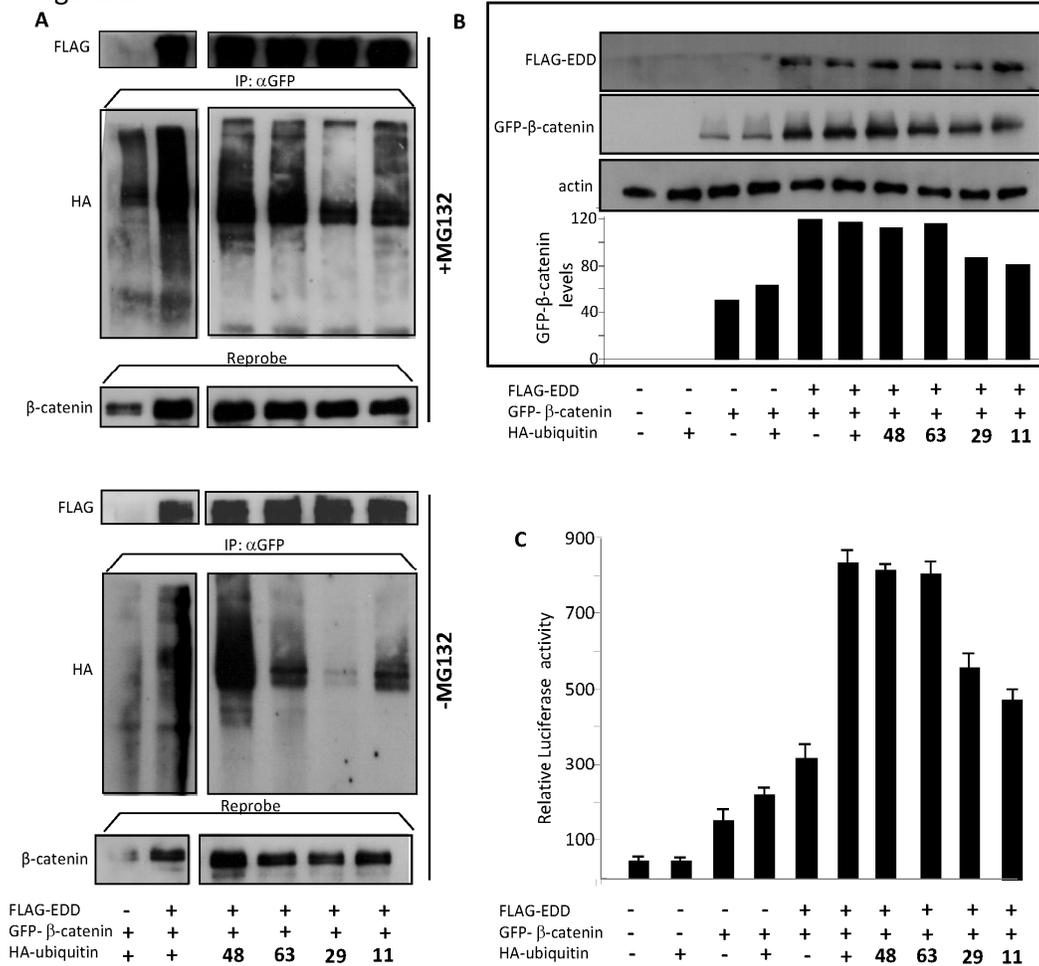
**Figure 4. EDD depletion decreases  $\beta$ -catenin levels and activity in HEK293T cells.** (A) HEK293T cells were subjected to EDD depletion using siRNA oligonucleotides for 72 hours. Cells were harvested and western blot analysis was performed using anti-EDD, anti-active  $\beta$ -catenin, anti-GSK-3 $\beta$  and anti-actin antibodies (upper panel). Densitometric analysis of all proteins was performed using TINA software (lower panel). (B) TOPFLASH luciferase assay (lower panel) was performed in HEK293T cells depleted of EDD and transfected with GFP- $\beta$ -catenin as indicated, along with pTOPFLASH reporter and  $\beta$ -gal plasmid. The lysates that were used in the TOPFLASH assay were subjected to western blot analysis (upper panel) using anti-EDD, anti-GFP and anti-actin antibodies. (C) HEK293T (left panel) and CHO (right panel) cells were transfected with siEDD or scEDD oligonucleotides as indicated. Seventy two hours post transfection, cells were fixed and reacted with anti-EDD and either anti-active  $\beta$ -catenin or anti-GSK-3 $\beta$  antibodies. DAPI was used to mark the nucleus. (D) Activity of  $\beta$ -catenin was measured in COLO320 (left panel) and SW480 (right panel) cells transfected with scEDD or siEDD constructs, along with pTOPFLASH/pFOPFLASH reporters and pCMV-Renilla. Experiments were repeated 4 times in triplicates. Lysates were also subjected to western blot analysis using anti-EDD, anti- $\beta$ -catenin, anti-active  $\beta$ -catenin and anti-actin antibodies.

Figure 5



**Figure 5. EDD ubiquitinates  $\beta$ -catenin.** (A) *In vivo* ubiquitination was performed by transfecting HEK293T cells with FLAG-EDD, HA-ubiquitin, GFP- $\beta$ -catenin or GFP-GSK-3 $\beta$  as indicated. Cells were harvested 48 hours post transfection following five hours of incubation with MG132. Lysates were then immunoprecipitated with an anti-GFP antibody and subjected to western blotting using an anti-HA antibody. The immunoprecipitation membrane was stripped and reprobbed with anti-GFP or anti- $\beta$ -catenin antibodies. FLAG-EDD was detected in the lysates using an anti-FLAG antibody. Asterisk indicates the GFP- $\beta$ -catenin protein that was ubiquitinated by the cellular ubiquitin pathway. (B) HEK293T cells were subjected to siRNA transfection as described before. Twenty four hours later the cells were transfected with FLAG-EDD and HA-ubiquitin as indicated. Five hours before harvesting, cells were incubated with MG132. *In vivo* ubiquitination was performed using a rabbit anti- $\beta$ -catenin antibody. The immunoprecipitation membrane was stripped and reprobbed with a mouse anti- $\beta$ -catenin antibody. FLAG-EDD and endogenous EDD were detected in the lysates using an anti-EDD antibody. (C) HEK293T cells were transfected with FLAG-EDD, HA-ubiquitin, GFP- $\beta$ -catenin or GFP-S33A- $\beta$ -catenin as indicated. *In vivo* ubiquitination was performed as in A. The immunoprecipitation membrane was stripped and reprobbed with an anti- $\beta$ -catenin antibody. FLAG-EDD was detected in the lysates using an anti-FLAG antibody. (D) HEK293T cells were transfected with FLAG-EDD or an empty FLAG vector and either subjected to GSK-3 $\beta$  overexpression or depletion using siRNA oligonucleotides as described. Cells were then harvested and analyzed by western blotting (upper panel) using anti-FLAG, anti- $\beta$ -catenin and anti-GSK-3 $\beta$  antibodies and quantified using the TINA software (lower panel). (E)  $\beta$ -catenin/TCF mediated transcription was measured as described above using the pTOPFLASH/pFOPFLASH assay. The cells were transfected with GFP- $\beta$ -catenin, FLAG-GSK-3 $\beta$  and FLAG-EDD as indicated. GSK-3 $\beta$  expression was inhibited by either siGSK-3 $\beta$  RNA oligonucleotides or LiCl treatment (30 mM for 24 h).

Figure 6



**Figure 6.  $\beta$ -catenin ubiquitination by EDD involves Lys29 and Lys11-ubiquitin chains.** (A) *In vivo* ubiquitination was performed by transfecting HEK293T cells with FLAG-EDD, GFP- $\beta$ -catenin and HA-ubiquitin or the ubiquitin mutants as indicated. Cells were harvested 48 hours post transfection following five hours of incubation with MG132 (upper panel) or DMSO (lower panel). Lysates were then immunoprecipitated with an anti-GFP antibody and subjected to western blotting using an anti-HA antibody. The immunoprecipitation membrane was stripped and re-probed with an anti- $\beta$ -catenin antibody. FLAG-EDD was detected in the lysates using an anti-FLAG antibody. (B) HEK293T cells were transfected with FLAG-EDD, GFP- $\beta$ -catenin and HA-ubiquitin as indicated. Forty-eight hours post transfection, cells were harvested and subjected to western blot analysis using anti-FLAG, anti-GFP and anti-actin antibodies. Experiment was repeated three times, and representative blots are presented (upper panel). Densitometric analysis of GFP- $\beta$ -catenin was performed using TINA software (lower panel). (C)  $\beta$ -catenin/TCF mediated transcription was measured as described above using the pTOPFLASH/pFOPFLASH assay. HEK293T cells were transfected with FLAG-EDD, GFP- $\beta$ -catenin, HA-ubiquitin, or the ubiquitin mutants as indicated.