The EDD E3 ubiquitin ligase ubiquitinates and up-regulates β-catenin

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

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INTRODUCTION

The Wnt signaling pathway regulates numerous processes during normal animal development and controls adult stem cell self-renewal (Reya and Clevers, 2005; Clevers, 2006). Hyperactivation of the canonical Wnt pathway is a hallmark of many human cancers, including the majority of sporadic and hereditary colorectal tumors (Polakis, 2000; Moon *et al.*, 2004). As β-catenin is a major key component of the canonical Wnt pathway, its protein levels and activity are tightly regulated (Bienz and Clevers, 2003; Clevers, 2006). In the absence of Wnt stimulation, cytoplasmic β-catenin is targeted to a multiprotein complex, termed the "β-catenin degradation complex," that includes axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK-3β), and casein kinase I (CK1). GSK-3β and CK1 phosphorylate β-catenin at several serine/threonine residues (Kimelman and Xu, 2006), and the phosphorylated protein is then recognized and ubiquitinated by E3 ligases. The best studied is $SCF^{\beta\text{-TrCP}}$, a multiprotein complex that marks β-catenin for proteasomal degradation (Aberle *et al.*, 1997) and is regulated by the canonical Wnt pathway (Yost *et al.*, 1996; Latres *et al.*, 1999). Wnt signaling is initiated by the binding of Wnt family members to a receptor complex consisting of the Frizzled transmembrane receptors, together with the coreceptors low-density lipoprotein receptor–related proteins 5/6 (LRP5/6). The receptor activation leads to disassembly of the β-catenin degradation complex and, as a consequence, unphosphorylated β-catenin is no longer degraded but accumulates and translocates into the nucleus. In the nucleus, β-catenin functions as a transcription cofactor for members of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) families to activate Wnt target genes. Aberrant stabilization of β-catenin triggers up-regulation of Wnt target genes such as *c-myc* and *cyclin D1* (Giles *et al.*, 2003; Clevers, 2006; Kikuchi *et al.*, 2006) and is believed to be the basis for tumorigenesis. In addition to the β-catenin degradation complex, several other mechanisms have been shown to repress the transcriptional activity of β-catenin (Liu *et al.*, 2001; Matsuzawa and Reed, 2001; Rosin-Arbesfeld *et al.*, 2003; Cong and Varmus, 2004; Bachar-Dahan *et al.*, 2006; Sierra *et al.*, 2006; Finkbeiner *et al.*, 2008);

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Abbreviations used: ABC, active β-catenin; AHR, aryl hydrocarbon receptor; APC, adenomatous polyposis coli; ARA3, aryl hydrocarbon receptor–associated 3; CK1, casein kinase I; CRC, colorectal cancer; DAPI, 4′,6-diamidino-2-phenylindole; EDD, E3 ubiquitin ligase identified by differential display; EGTA, ethylene glycol tetraacetic acid; GFP, green fluorescent protein; GSK-3β, glycogen synthase kinase 3β; GST, glutathione *S*-transferase; HA, hemagglutinin; IB, immunoblot; IF, immunofluorescence; IP, immunoprecipitation; LEF, lymphoid enhancer factor; LRP, lipoprotein receptor–related protein; PBS, phosphate-buffered saline; pEGFP, plasmid enhanced GFP; RIPA, radioimmunoprecipitation assay; RNAi, RNA interference; siRNA, small interfering RNA; TCF, T-cell factor; UBC, ubiquitin-conjugating enzyme.

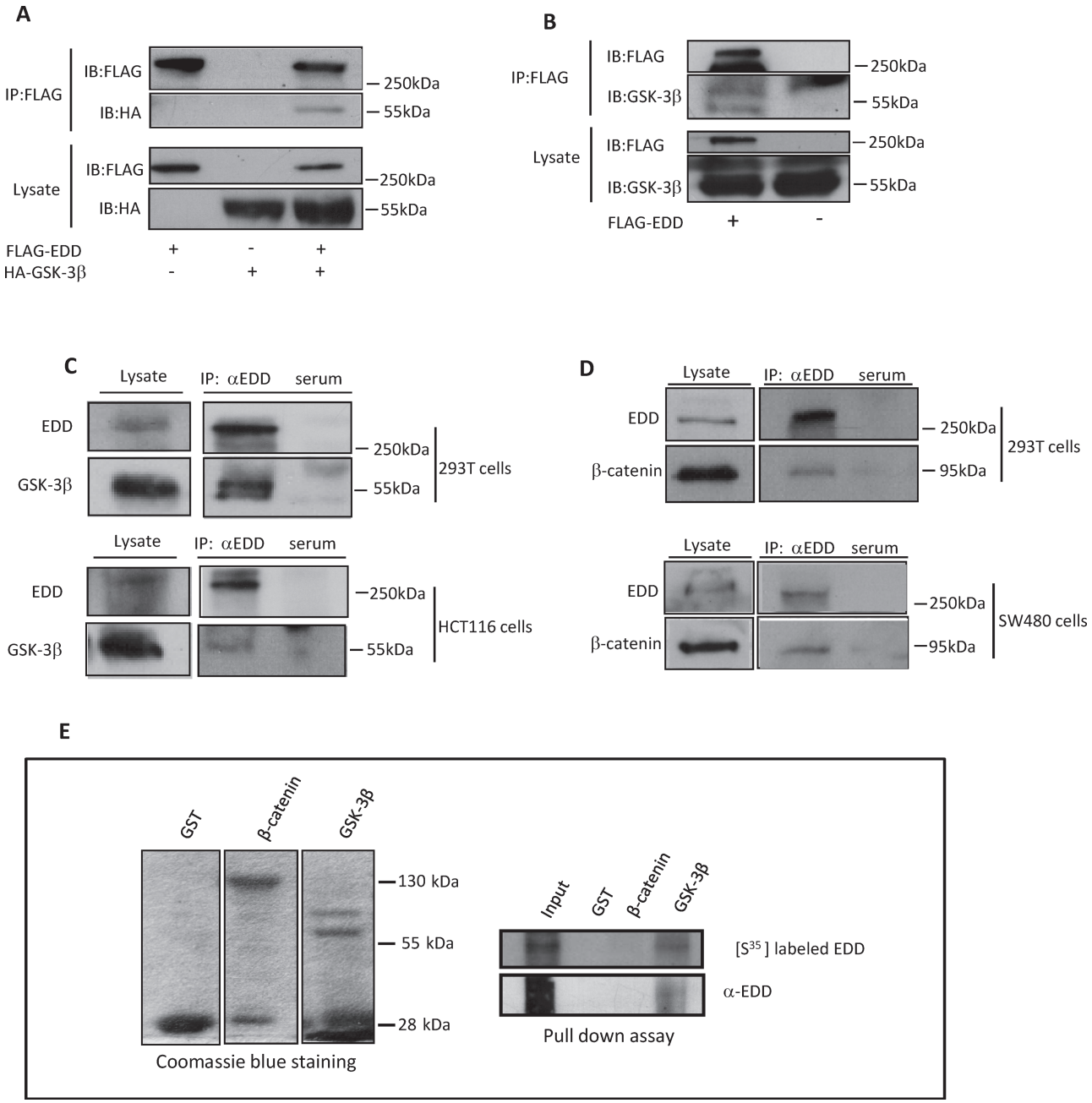


FIGURE 1: In vivo and in vitro interaction between EDD and GSK-3β or β-catenin. (A) HEK293T cells were transfected with FLAG-EDD and HA–GSK-3β and immunoprecipitated with anti-FLAG. Western blot was performed using anti-FLAG and anti-HA antibodies. (B) HEK293T cells were transfected with FLAG-EDD, and immunoprecipitation was performed as before. Western blot was performed using anti-FLAG and anti–GSK-3β antibodies. (C) Lysates from HEK293T (top) or HCT116 (bottom) cells were immunoprecipitated using a rabbit anti-EDD antibody or control unimmuned serum and subjected to Western blotting using anti-GSK-3β and goat anti-EDD antibodies. (D) Nuclear fraction of HEK293T (top) or total lysate from SW480 (bottom) cells were immunoprecipitated with a rabbit anti-EDD antibody or control unimmuned 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 *Escherichia coli* bacteria (left). FLAG-EDD protein translated in a rabbit reticulocyte system in the presence or absence of [S^{35}]methionine was pulled down using the recombinant proteins as indicated (right). The [S^{35}]methionine-labeled reaction was exposed to an x-ray film (top), and the nonradioactive reaction was immunoblotted using an anti-EDD antibody (bottom). IB, immunoblot; IP, immunoprecipitation.

however, the regulation of nuclear β-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 (Li and Ye, 2008). In addition, ubiquitination is beginning to emerge as an important controlling factor in transcriptional switches (Muratani and Tansey, 2003). In the Wnt signaling pathway, ubiquitination is known to mark β-catenin for degradation, thus reducing signaling levels. However, in this report we provide evidence indicating that the E3 ubiquitin ligase identified by differential display (EDD) serves as a positive regulator of β-catenin–mediated signaling. We show that EDD interacts with both GSK-3β and β-catenin, leading to β-catenin ubiquitination. Expression of EDD results in up-regulation of β-catenin protein levels and activity, suggesting a positive role for ubiquitination by EDD in the Wnt signaling pathway and cancer development.

RESULTS

EDD forms cellular complexes with GSK-3β and β-catenin

In our studies of the Wnt signaling pathway, we have tried to identify the potential functional partner(s) of GSK-3β, a core component of this oncogenic pathway. To do so, lysates from HEK293T cells transfected with FLAG–GSK-3β 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 coprecipitated with FLAG–GSK-3β 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-3β–binding proteins: ARA3, 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 assays in order to verify the cellular interaction between EDD and GSK-3β. HEK293T cells were cotransfected with constructs encoding FLAG-EDD in the presence or absence of hemagglutinin (HA)–tagged GSK-3β. Both HA–GSK-3β (Figure 1A) and endogenous GSK-3β (Figure 1B) coimmunoprecipitated with FLAG-EDD, confirming the interaction between the two proteins. Next, we examined whether endogenous EDD can form a complex with endogenous GSK-3β. Indeed, EDD–GSK-3β complex was observed in both HEK293T cells (top) and the colorectal cell line HCT116, where β-catenin is constitutively active (bottom). As β-catenin is a core component of the canonical Wnt pathway, we tested whether β-catenin can interact with EDD as well. EDD is known to be a nuclear protein (Henderson *et al.*, 2002); thus we used the nuclear fraction of HEK293T cells to try to detect such an interaction. Our results show that endogenous nuclear β-catenin immunoprecipitated with endogenous EDD (Figure 1D, top). A similar result was obtained when using the

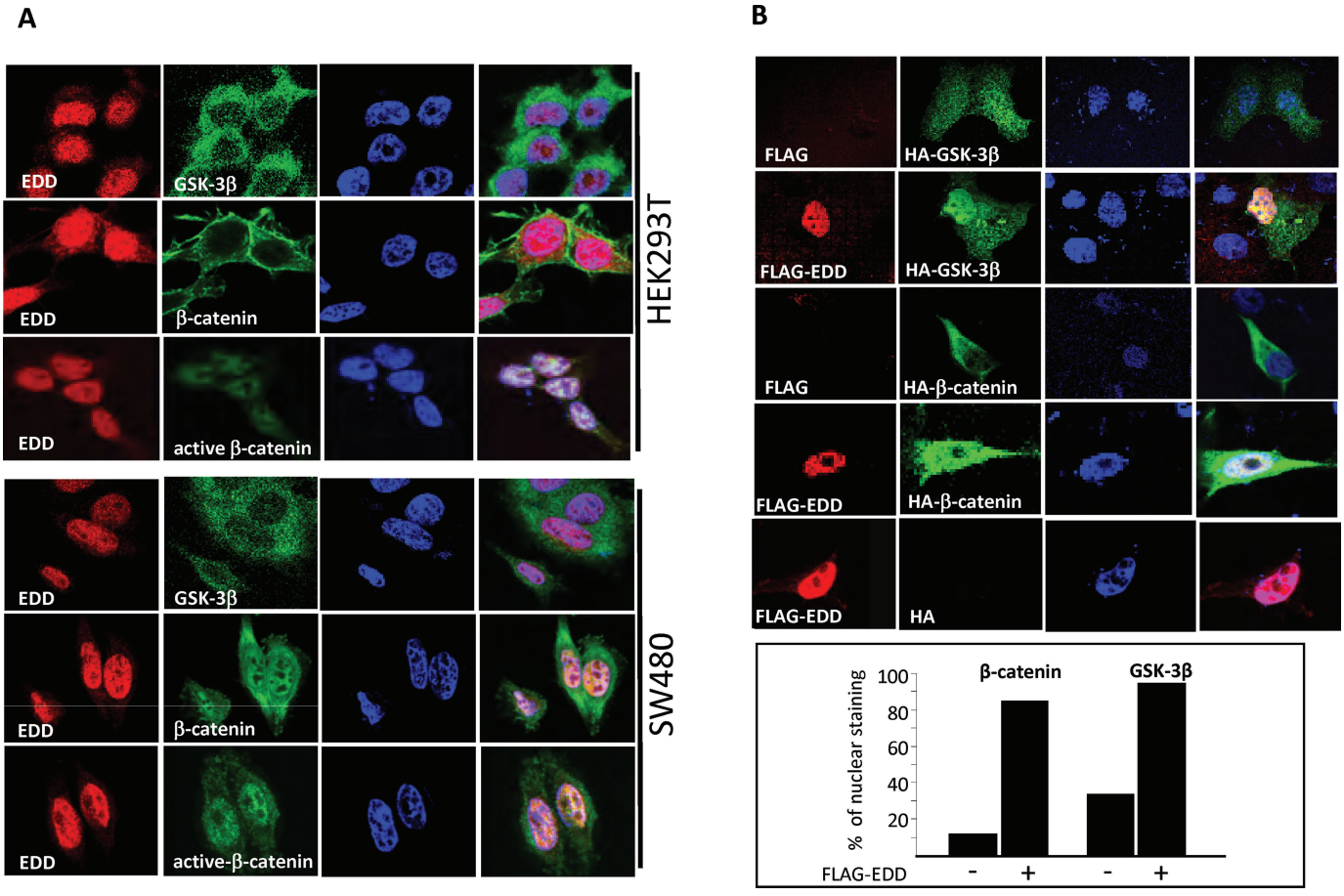
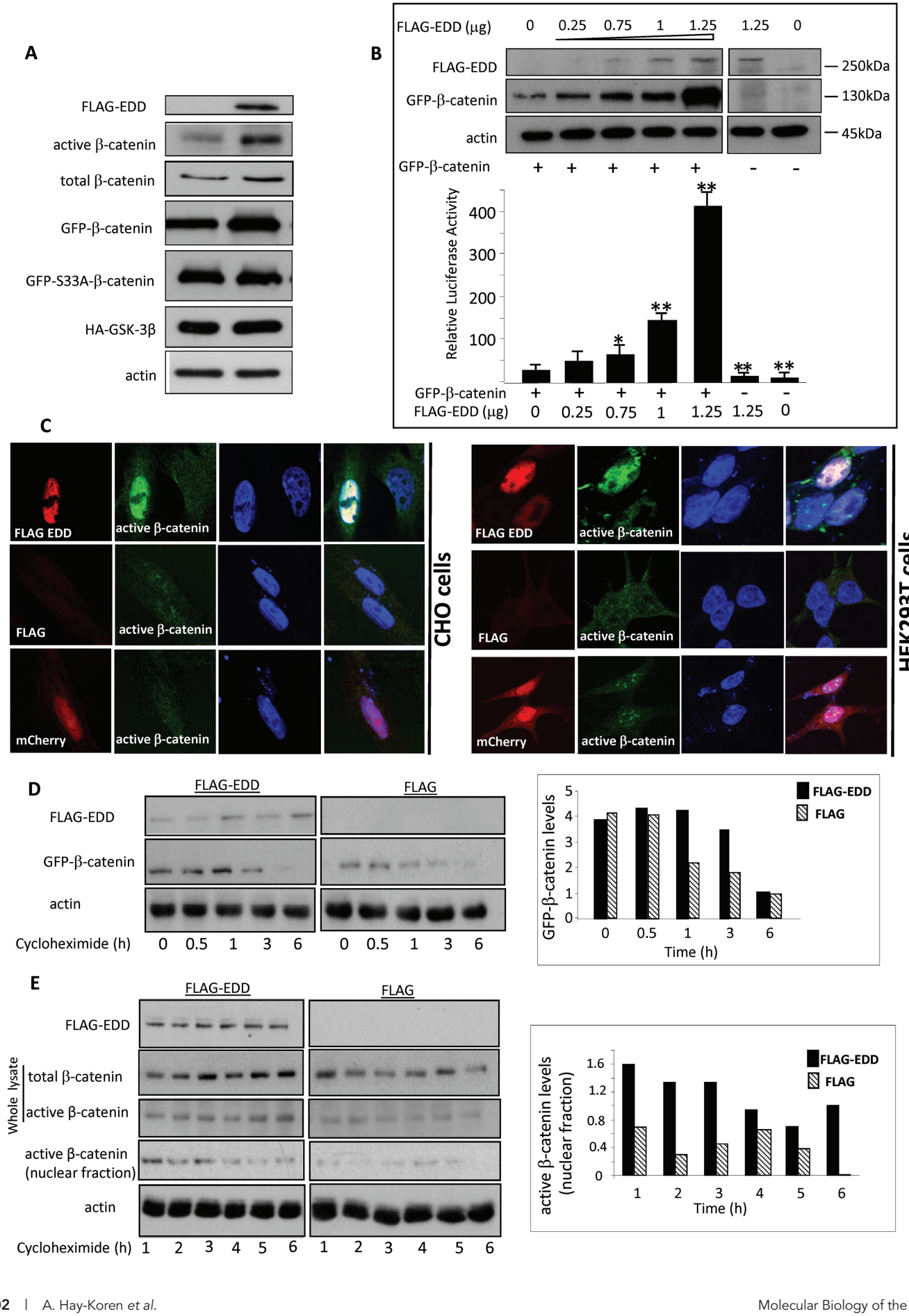


FIGURE 2. Nuclear accumulation of GSK-3β and β-catenin following EDD overexpression. (A) HEK293T (top) and SW480 (bottom) cells were stained using anti-EDD, anti–GSK-3β, anti–β-catenin, and anti–active β-catenin antibodies as indicated. DAPI was used to mark the nucleus. (B) HEK293T cells were cotransfected with HA–GSK-3β, HA–β-catenin, or an empty HA vector and either FLAG-EDD or an empty FLAG vector as indicated. At 48 h posttransfection, cells were fixed and reacted with anti-FLAG and anti-HA antibodies (top). DAPI was used to mark the nucleus. Transfected cells were counted, and percentage of β-catenin or GSK-3β nuclear localization was calculated (bottom).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active β-catenin levels (nuclear fraction)
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colorectal cell line SW480, which expresses high levels of nuclear β-catenin due to a truncated APC protein (Figure 1D, bottom). To examine whether the interaction between EDD and GSK-3β or β-catenin is direct, in vitro pull-down experiments were next performed. GSK-3β or β-catenin proteins were bacterially produced, purified, and tested for their ability to bind EDD translated in the TNT reticulocyte system. As shown in Figure 1E (right), GSK-3β bound EDD, suggesting that the interaction between EDD and GSK-3β is direct. β-Catenin, on the other hand, did not directly interact with EDD. Coomassie staining of bacterially produced proteins is shown in the left panel.

Overexpression of EDD enhances GSK-3β's and β-catenin's nuclear accumulation

Immunofluorescence was used to determine the subcellular localization of endogenous EDD, GSK-3β, and β-catenin in both HEK293T and SW480 cells (Figure 2A). In these experiments, we used anti-total β-catenin and anti-ABC antibodies. The anti-ABC specifically detects dephosphorylated nuclear β-catenin, which is transcriptionally active in mediating the Wnt pathway, as opposed to β-catenin, which functions in cell–cell adhesion (van Noort *et al.*, 2002). As seen in HEK293T cells (top), endogenous EDD shows nuclear staining and colocalizes with the active nuclear form of β-catenin. However, EDD does not colocalize with GSK-3β that is mostly cytoplasmic or with the membranal pool of β-catenin. Unlike HEK293T, SW480 cells express mainly nuclear β-catenin, which is transcriptionally active. In these cells (bottom), endogenous EDD colocalizes with the nuclear β-catenin. To test whether overexpression of EDD affects the subcellular localization of GSK-3β or β-catenin, immunofluorescence assay was performed in HEK293T cells transfected with FLAG-EDD and HA–GSK-3β or HA–β-catenin (Figure 2B, top). As can be seen, FLAG-EDD is nuclear, whereas HA–GSK-3β and HA–β-catenin are mostly cytoplasmic. However, when coexpressed with FLAG-EDD, the subcellular distribution of both HA–GSK-3β or HA–β-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-3β and β-catenin. To quantify the nuclear accumulation of GSK-3β or β-catenin induced by EDD overexpression, cotransfected cells were counted (n = 200) and the percentage of nuclear staining with or without FLAG-EDD was determined (Figure 2B, bottom). Expression of FLAG-EDD resulted in more than a threefold increase in the levels of nuclear GSK-3β or nuclear β-catenin. Taken together, these results indicate that EDD alters the subcellular distribution of both GSK-3β and β-catenin, leading to their nuclear accumulation.

EDD up-regulates the active form of β-catenin and enhances Wnt signaling

Next, the effect of EDD overexpression on β-catenin and GSK-3β levels was examined (Figure 3A). HEK293T cells were cotransfected with FLAG-EDD or the FLAG empty vector, along with HA–GSK-3β, green fluorescent protein (GFP)–β-catenin, or GFP–S33A-β-catenin, a constitutively active β-catenin mutant that is insensitive to GSK-3β–mediated phosphorylation and proteasomal degradation (Kolligs *et al.*, 1999). Overexpression of EDD led to a significant increase in the levels of the active β-catenin fraction as compared with the levels of the total β-catenin protein pool. Although GSK-3β levels were unchanged by EDD overexpression, GSK-3β phosphorylation of β-catenin seemed to be important for EDD's function, as the GFP–S33A-β-catenin protein was hardly affected by EDD (Figure 3A). As HEK293T cells exhibit low levels of Wnt signaling due to the activity of the β-catenin degradation complex, GFP–β-catenin was expressed in these cells to induce activation of signaling. The cells were cotransfected with GFP–β-catenin and with increasing amounts of FLAG-EDD. Results show that overexpression of EDD led to increased levels of β-catenin in a dose-dependent manner (Figure 3B, top). Accordingly, EDD overexpression resulted in enhanced β-catenin activity that was measured by the TCF/β-catenin–dependent transcription system (pTOPFLASH/pFOPFLASH) (Figure 3B, bottom). Next, immunofluorescence analysis was performed in CHO (Figure 3C, left) and HEK293T (Figure 3C, right) cells, both exhibiting low levels of β-catenin and Wnt signaling activity. The data presented support our Western blot observation as overexpression of EDD resulted in increased levels of the ABC protein.

To better understand the mechanism by which EDD affects β-catenin, we next examined β-catenin stability by following its degradation rate after cycloheximide addition (Figure 3, D and E). HEK293T cells were cotransfected with GFP–β-catenin and either FLAG-EDD or an empty FLAG vector (Figure 3D). At 24 h posttransfection, 80 μg/ml cycloheximide was added, and the levels of GFP–β-catenin were measured at the indicated times. Data showed that 1 h following cycloheximide addition, the levels of β-catenin were significantly decreased in the vector-transfected cells, while in the presence of EDD the decrease was observed only after 6 h. The levels of

FIGURE 3. Overexpression of EDD enhances β-catenin levels, activity, and stability. (A) HEK293T cells were cotransfected with FLAG-EDD or an empty FLAG vector and either GFP–β-catenin, GFP–S33A-β-catenin, or HA–GSK-3β. Forty-eight hours later the cells were harvested and subjected to Western blot analysis using anti-FLAG, anti-GFP, anti–β-catenin, anti–active β-catenin (anti-ABC), anti-HA, and anti-actin antibodies. (B) Luciferase assay (bottom) was performed in HEK293T cells transfected with GFP–β-catenin and increasing amounts of FLAG-EDD along with pTOPFLASH/pFOPFLASH reporters and β-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 significant 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 (top) using anti-FLAG, anti-GFP, and anti-actin antibodies. (C) CHO (left) and HEK293T (right) cells were transfected with FLAG-EDD, empty FLAG, or mCherry vectors. At 48 h posttransfection, cells were fixed and reacted with anti-FLAG and anti-ABC antibodies. DAPI was used to mark the nucleus. (D) HEK293T cell were cotransfected with GFP–β-catenin and either FLAG-EDD or an empty FLAG vector. Cycloheximide was added to the cells 24 h posttransfection. 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). Densitometric analysis of GFP–β-catenin was performed using TINA software (right). (E) Cycloheximide stability assay was repeated with endogenous β-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–β-catenin, anti-ABC, and anti-actin antibodies. Densitometric analysis of ABC in the nuclear fraction was performed using TINA software (right).

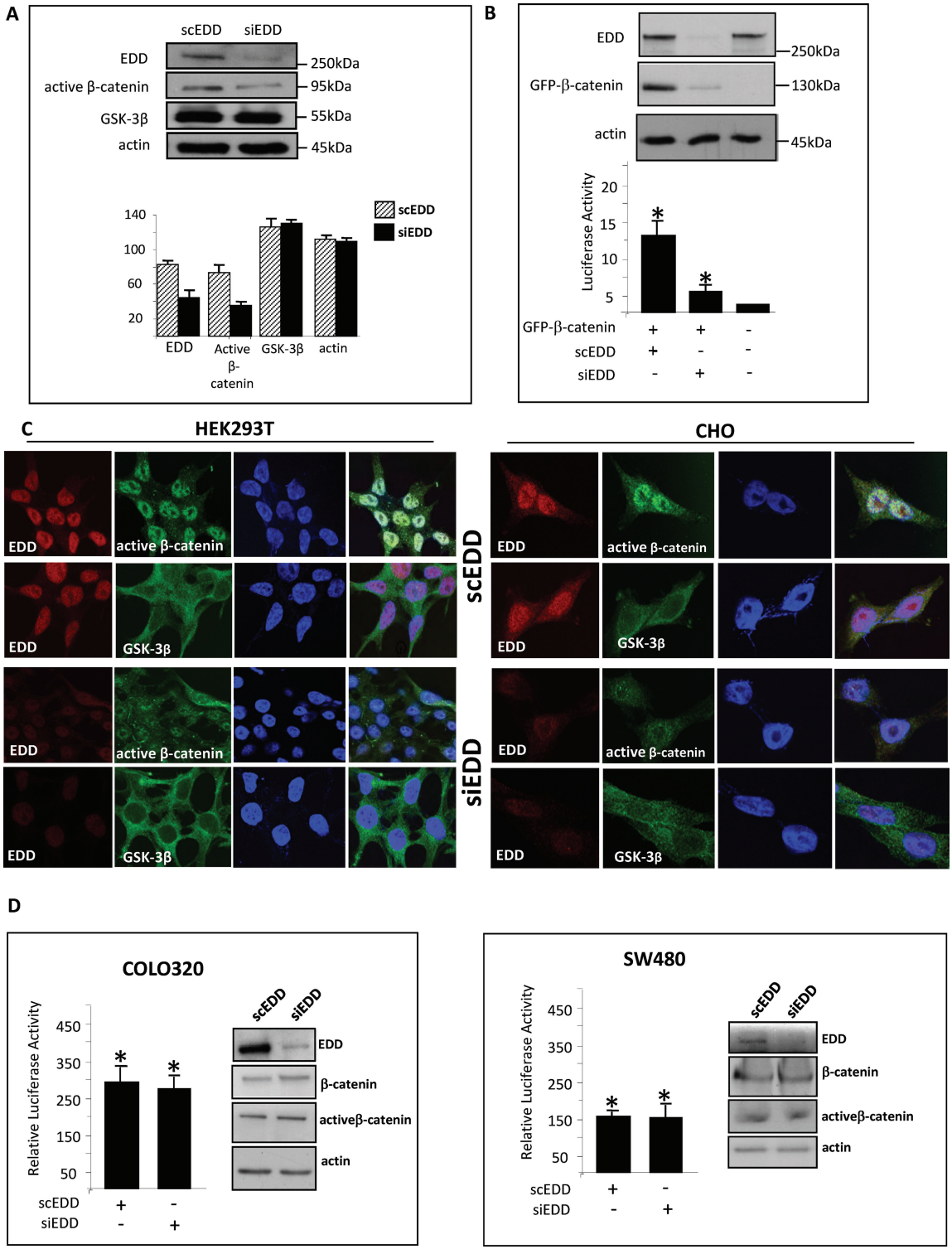


FIGURE 4. EDD depletion decreases β-catenin levels and activity in HEK293T cells. (A) HEK293T cells were subjected to EDD depletion using siRNA oligonucleotides for 72 h. Cells were harvested and Western blot analysis was performed using anti-EDD, anti–active β-catenin (anti-ABC), anti–GSK-3β, and anti-actin antibodies (top). Densitometric analysis of all proteins was performed using TINA software (bottom). (B) TOPFLASH luciferase assay (bottom) was performed in HEK293T cells depleted of EDD and transfected with GFP–β-catenin as indicated, along with pTOPFLASH reporter and β-gal plasmid. The lysates that were used in the TOPFLASH assay were subjected to Western blot analysis (top) using anti-EDD, anti-GFP, and anti-actin antibodies. (C) HEK293T (left) and CHO (right) cells were transfected with siEDD or scEDD oligonucleotides as indicated. At 72 h posttransfection, cells were fixed and reacted with anti-EDD and either anti-ABC or anti–GSK-3β antibodies. DAPI was used to mark the nucleus. (D) Activity of β-catenin was measured in COLO320 (left) and SW480 (right) cells transfected with scEDD or siEDD constructs, along with pTOPFLASH/pFOPFLASH reporters and pCMV-Renilla. Experiments were repeated four times in triplicates. Lysates were also subjected to Western blot analysis using anti-EDD, anti–β-catenin, anti-ABC, and anti-actin antibodies.

GFP–β-catenin were quantified using a computer-assisted densitometer (right panel). A similar assay was performed to measure the effect of EDD on endogenous β-catenin stability (Figure 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 β-catenin was tested by antibodies against both the total and ABC forms. Because there are high levels of membrane-bound β-catenin, which may have diluted the effect of EDD on cytosolic or nuclear β-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 β-catenin were measured using the anti-ABC antibody. The TINA software was used to quantify these results (right panel). Indeed, the degradation rate of endogenous β-catenin was reduced in the presence of EDD. Altogether, these results imply that EDD up-regulates β-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 did not decrease through the entire course of the experiment.

Silencing of EDD decreases β-catenin levels and activity in HEK293T cells

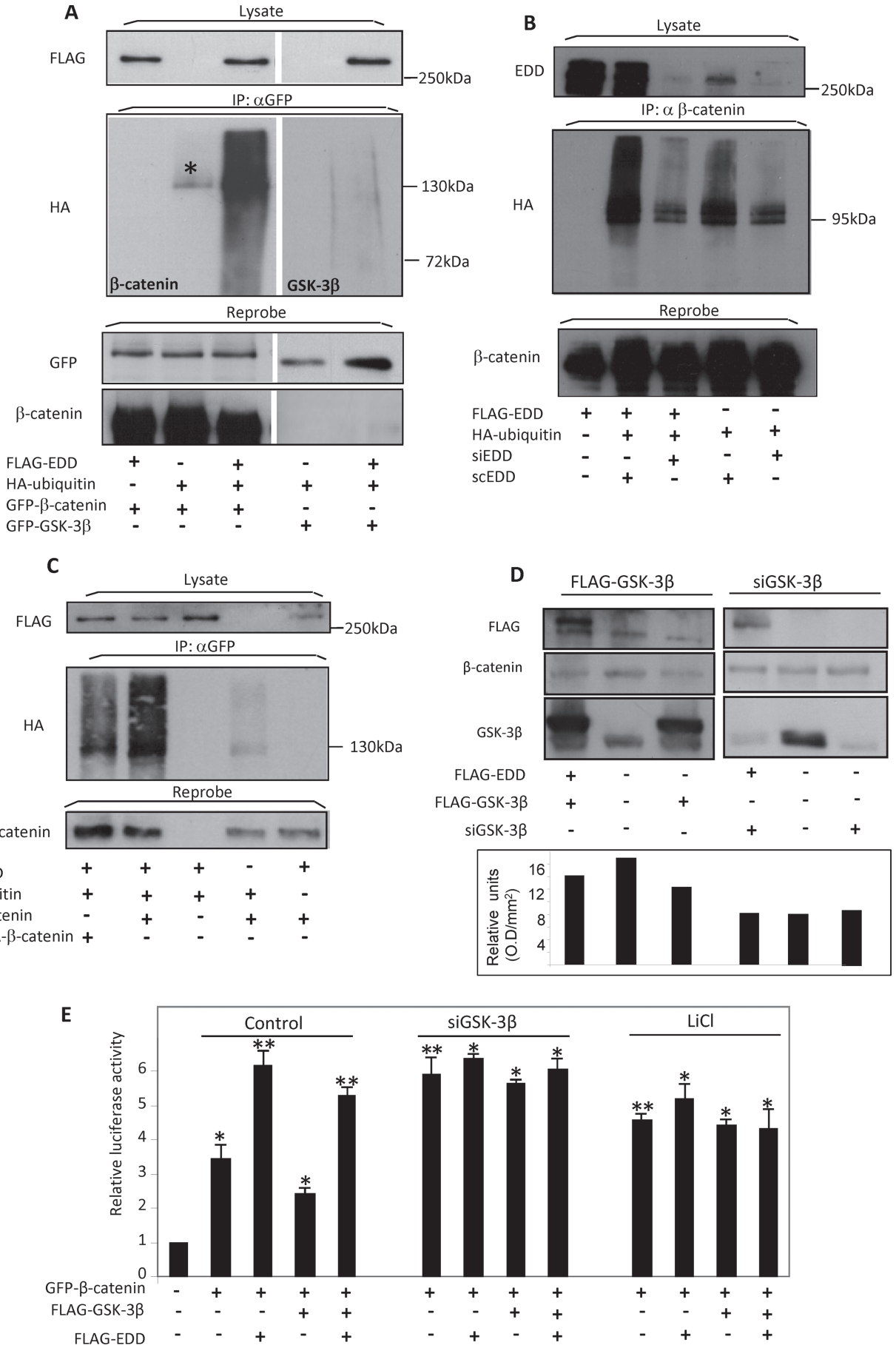
To further verify the specificity of EDD's effect on β-catenin–mediated signaling, RNA interference was used to inhibit EDD expression in HEK293T, CHO, COLO320, and SW480 cells. HEK293T cells were transfected with siRNA EDD oligonucleotides for 72 h, and the levels of endogenous EDD, ABC, and GSK-3β were measured (Figure 4A). EDD depletion led to decreased levels of ABC, whereas the levels of GSK-3β remained unchanged (top). The experiment was repeated three times, and protein levels were quantified by densitometric computer analysis (bottom). Next, we measured the effect of EDD depletion on the levels and activity of GFP–β-catenin (Figure 4B). Results show that silencing of endogenous EDD resulted in inhibited β-catenin/TCF–mediated transcription (bottom) and decreased levels of GFP–β-catenin protein, accordingly (top). The effect of EDD depletion was also tested using immunofluorescence assay (Figure 4C). HEK293T (left panel) and CHO (right panel) cells were transfected with siRNA oligonucleotides as indicated. Our results show that the nuclear accumulation of ABC was dramatically reduced when EDD was silenced in both cell types. The subcellular localization of GSK-3β remained unaffected. As mentioned above, in HEK293T cells the β-catenin degradation complex is intact. However, in COLO320 and SW480 cells, a mutation in the APC gene results in a truncated protein that prevents assembly of this degradation complex. Consequently, these cells express high levels of β-catenin protein and exhibit high levels of β-catenin–mediated transcription (Rowan *et al.*, 2000; Rosin-Arbesfeld *et al.*, 2003). EDD silencing in the CRC cell lines COLO320 and SW480 was performed by transfection with siRNA constructs for 48 h (Figure 4D). In both cell lines, depletion of EDD affected the levels of neither total or active β-catenin, nor their activity. These results imply that an intact β-catenin degradation complex is required for EDD's function.

EDD ubiquitinates β-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 (Honda *et al.*, 2002). Because the data presented so far indicated that EDD enhances β-catenin expression, we examined whether the effect on β-catenin levels is related to EDD's ubiquitination activity. To test whether EDD ubiquitinates β-catenin or GSK-3β, HEK293T cells were transfected with FLAG-EDD, HA-ubiquitin, and either GFP–β-catenin or GFP–GSK-3β (Figure 5A). The proteasomal inhibitor MG132 was added to the cells before 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, β-catenin, and ubiquitin were overexpressed simultaneously, suggesting that EDD polyubiquitinates β-catenin in vivo. GSK-3β, however, did not appear to be ubiquitinated by EDD. The HA blot was reprobed using anti-GFP or anti–β-catenin antibodies to confirm that the protein identified by the HA antibody is indeed β-catenin. As the experiment was performed in the presence of MG132, GFP–β-catenin ubiquitinated by the EDD-independent ubiquitin pathway is also observed (marked by an asterisk). To confirm the specificity of EDD's effect on β-catenin ubiquitination, overexpressed or endogenous EDD was silenced in HEK293T cells expressing HA-ubiquitin (Figure 5B). Silencing of either endogenous or overexpressed EDD resulted in reduced ubiquitination of endogenous β-catenin, indicating a specific ubiquitination of β-catenin by EDD. Because our results implied that GSK-3β is involved in EDD's effect on β-catenin, we compared the ability of EDD to ubiquitinate wild-type GFP–β-catenin and the constitutively active β-catenin (S33A) mutant. This mutated form of β-catenin, which is insensitive to GSK-3β–mediated phosphorylation, exhibited impaired ubiquitination relative to the wild-type form (Figure 5C). Similar results were obtained when the GFP–β-catenin protein was replaced by a Myc tag (unpublished data). We then examined the involvement of GSK-3β in EDD-mediated β-catenin up-regulation by transfecting HEK293T cells with FLAG-EDD and either overexpression or silencing of GSK-3β (Figure 5D). While, as expected, overexpression of GSK-3β resulted in decreased β-catenin protein levels (left panel), coexpression of both GSK-3β and EDD abolished GSK-3β's inhibitory effect on β-catenin. GSK-3β depletion, on the other hand, did not affect β-catenin levels (right panel), possibly due to the functional redundancy between GSK-3α and GSK-3β in Wnt/β-catenin signaling (Doble *et al.*, 2007). Importantly, in cells depleted of GSK-3β, EDD-induced up-regulation of β-catenin could not be detected, supporting our hypothesis that GSK-3β is required for this function. Next, we tested the effect of GSK-3β repression on EDD-induced β-catenin activity using the luciferase pTOPFLASH/pFOPFLASH assay (Figure 5E). Interestingly, although overexpression of GSK-3β inhibited the Wnt pathway as predicted, the addition of EDD impaired GSK-3β's ability to reduce β-catenin/TCF–mediated transcription (left section). Furthermore, the repression of GSK-3β by either siRNA (middle section) or LiCl (right section) led to enhanced Wnt signaling stimulation but compromised EDD's ability to activate β-catenin. Taken together, these results indicate that GSK-3β has a role in EDD-mediated activation of β-catenin and the Wnt signaling pathway activity.

β-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, polyubiquitination via lysine (K)48 is often coupled to degradation of proteins by the 26S proteasome system, whereas polyubiquitination via lysine (K)63 acts as a nonproteolytic signal in several intracellular pathways such as recruitment and activation of substrates (Pickart and Fushman, 2004). To determine the nature of EDD-mediated β-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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FLAG-GSK-3β - - - + + - - + + - - + +
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was carried out as described above. Our results indicate that EDD's ability to ubiquitinate β-catenin was impaired when the ubiquitin mutant K29R was used (Figure 6A). This result points to the involvement of a Lys29 polyubiquitin chain in EDD-mediated ubiquitination of β-catenin. Because different polyubiquitinations can lead to either protein degradation or other cellular processes, we performed the ubiquitination assay with (top) or without (bottom) 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 β-catenin for degradation. Next, we tested the effect of the different ubiquitin mutants on β-catenin levels (Figure 6B). HEK293T cells were cotransfected with FLAG-EDD, GFP–β-catenin, and HA-ubiquitin or the different mutants. The levels of GFP–β-catenin were determined using Western blot analysis (top) and quantified by a computer-assisted densitometer (bottom). The use of either K29R or K11R mutants led to decreased GFP–β-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 β-catenin's activity. The luciferase assay results show that GFP–β-catenin activity was highest when both ubiquitin and EDD were overexpressed. K29R and K11R mutants significantly impaired β-catenin–mediated transcription relative to the wild-type ubiquitin (Figure 6C). Overall, these results imply that EDD ubiquitinates β-catenin using Lys29 and/or Lys11 polyubiquitin chains and thus enhances its protein levels and activity.

DISCUSSION

The canonical Wnt/β-catenin signaling pathway regulates cell fate and proliferation in embryonic development and regeneration and tissue homeostasis in adults (Logan and Nusse, 2004; Reya and Clevers, 2005). Up-regulation of this pathway is linked to a number of different diseases, including cancer, Alzheimer's disease, and bone density syndromes (Moon *et al.*, 2004). Activation of β-catenin, a key factor of the canonical Wnt pathway, is a critical step in the pathogenesis of many human cancers. β-Catenin expression is known to be regulated primarily by proteasomal degradation, mediated by CK-1 and GSK-3β phosphorylation.

Using immunoprecipitation experiments, we identified three potential GSK-3β–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 β-catenin degradation complex together with GSK-3β, 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 (Dunham *et al.*, 2006). 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 β-catenin in mice intestinal tumors (Kawajiri *et al.*, 2009) and may also down-regulate the Wnt pathway during development (Mathew *et al.*, 2009).

The E3 ubiquitin ligase *EDD* is the mammalian orthologue of the *Drosophila melanogaster* hyperplastic discs gene (*hyd*) (Callaghan *et al.*, 1998); a loss-of-function mutation of *hyd* causes imaginal disc overgrowth in *Drosophila* (Mansfield *et al.*, 1994). Reduced expression of *EDD* has been observed in several human cancers (Mori *et al.*, 2002; Fuja *et al.*, 2004), indicating that *EDD* may function as a tumor suppressor gene. However, in breast and ovarian cancers, *EDD* is overexpressed (Clancy *et al.*, 2003; O'Brien *et al.*, 2008), 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-3β and forms a cellular complex with β-catenin. Moreover, EDD ubiquitinates β-catenin in a GSK-3β–dependent manner and up-regulates the expression levels and activity of β-catenin, possibly through increasing its stability. We demonstrate that endogenous EDD colocalizes with nuclear active β-catenin (ABC), and when overexpressed, EDD leads to increased nuclear accumulation of both β-catenin and GSK-3β. 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-3β, thus recruiting it into the nucleus. Nuclear phosphorylation by GSK-3β has been shown to regulate nuclear export, DNA binding, and transcriptional activity of different substrates (Beals *et al.*, 1997; Alt *et al.*, 2000; Ginger *et al.*, 2000; Grimes and Jope, 2001; Morisco *et al.*, 2001). By modulating GSK-3β's nuclear localization, EDD may affect GSK-3β–dependent phosphorylation of different substrates, thus influencing a wide range of cellular processes, including β-catenin–mediated transcription.

Our data indicate that EDD ubiquitinates β-catenin, leading to enhanced β-catenin protein levels and activity. The regulation of Wnt signaling by ubiquitination is generally related to β-catenin degradation. Nevertheless, a number of recent studies have shown that the ubiquitin system is involved in activation of Wnt target genes. For example, Trabid and Cezanne, both deubiquitinating (DUB) enzymes, antagonize the ubiquitination of APC, resulting in activation of β-catenin/TCF–mediated transcription (Tran *et al.*, 2008). In addition, Sierra *et al.* (2006) have demonstrated that in

FIGURE 5. EDD ubiquitinates β-catenin. (A) In vivo ubiquitination was performed by transfecting HEK293T cells with FLAG-EDD, HA-ubiquitin, GFP–β-catenin, or GFP–GSK-3β as indicated. Cells were harvested 48 h posttransfection following 5 h 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 reprobed with anti-GFP or anti–β-catenin antibodies. FLAG-EDD was detected in the lysates using an anti-FLAG antibody. Asterisk indicates the GFP–β-catenin protein that was ubiquitinated by the cellular ubiquitin pathway. (B) HEK293T cells were subjected to siRNA transfection as described before. The cells were transfected 24 h later 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–β-catenin antibody. The immunoprecipitation membrane was stripped and reprobed with a mouse anti–β-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–β-catenin, or GFP–S33A-β-catenin as indicated. In vivo ubiquitination was performed as in (A). The immunoprecipitation membrane was stripped and reprobed with an anti–β-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 subjected to either GSK-3β overexpression or depletion using siRNA oligonucleotides as described. Cells were then harvested and analyzed by Western blotting (top) using anti-FLAG, anti–β-catenin, and anti–GSK-3β antibodies and quantified using the TINA software (bottom). (E) β-Catenin/TCF–mediated transcription was measured as described above using the pTOPFLASH/pFOPFLASH assay. The cells were transfected with GFP–β-catenin, FLAG–GSK-3β, and FLAG-EDD as indicated. GSK-3β expression was inhibited by either siGSK-3β RNA oligonucleotides or LiCl treatment (30 mM for 24 h).

vitro transcriptional activation by recombinant β-catenin/LEF-1 complexes requires ubiquitin.

Our results indicate that the ubiquitin mutants K48R and K63R do not have much effect on EDD-mediated β-catenin ubiquitination or protein levels and activity. The ubiquitin mutants K29R and K11R, on the other hand, decreased EDD-mediated ubiquitination and its effects on β-catenin. Trabid and Cezanne were shown to cleave K29- and K11-linked ubiquitin chains, respectively (Bremm *et al.*, 2010; Virdee *et al.*, 2010). Although Trabid was first reported to activate the Wnt pathway by cleavage of the K63-linked chains (Tran *et al.*, 2008), it was recently demonstrated to be 40-fold more active toward the K29-linked chains (Virdee *et al.*, 2010). 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 β-catenin polyubiquitination and stabilization, thus activating the

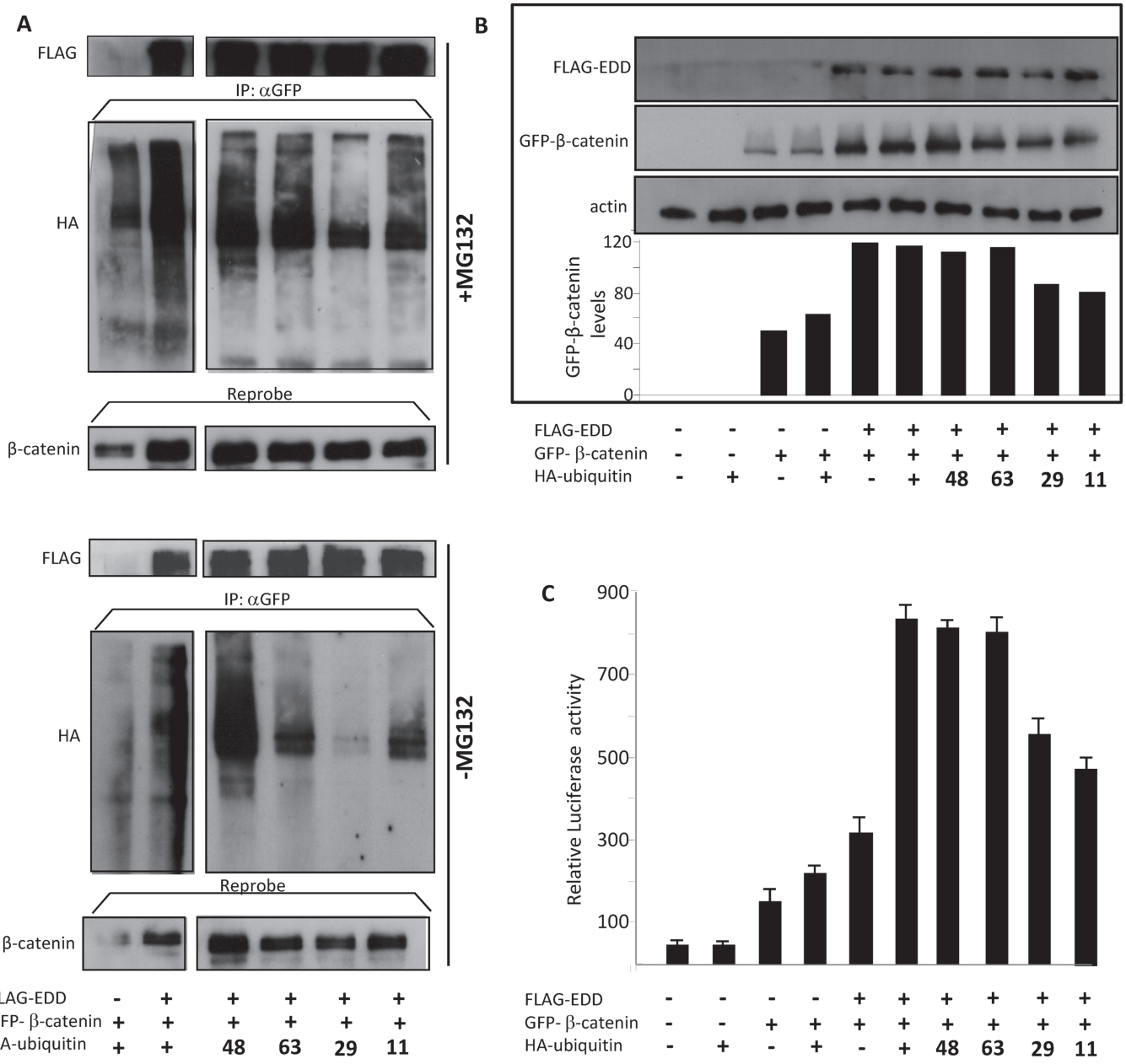

FIGURE 6. β-Catenin ubiquitination by EDD involves Lys29 and Lys11 ubiquitin chains. (A) In vivo ubiquitination was performed by transfecting HEK293T cells with FLAG-EDD, GFP–β-catenin, and HA-ubiquitin or the ubiquitin mutants as indicated. Cells were harvested 48 h posttransfection following 5 h of incubation with MG132 (top) or DMSO (bottom). 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 reprobed with an anti–β-catenin antibody. FLAG-EDD was detected in the lysates using an anti-FLAG antibody. (B) HEK293T cells were transfected with FLAG-EDD, GFP–β-catenin, and HA-ubiquitin as indicated. At 48 h posttransfection, cells were harvested and subjected to Western blot analysis using anti-FLAG, anti-GFP, and anti-actin antibodies. The experiment was repeated three times, and representative blots are presented (top). Densitometric analysis of GFP–β-catenin was performed using TINA software (bottom). (C) β-Catenin/TCF–mediated transcription was measured as described above using the pTOPFLASH/pFOPFLASH assay. HEK293T cells were transfected with FLAG-EDD, GFP–β-catenin, HA-ubiquitin, or the ubiquitin mutants as indicated.

Wnt signaling pathway (Shekhar *et al.*, 2008). As both EDD and Rad6B are involved in DNA damage response (Prakash *et al.*, 1993; Henderson *et al.*, 2002; Honda *et al.*, 2002; Ulrich, 2002), it is possible that Rad6B functions as an E2 ubiquitin–conjugating enzyme (UBC) in combination with EDD to ubiquitinate β-catenin. We are currently testing this hypothesis and examining whether EDD/Rad6B-mediated ubiquitination of β-catenin can result in cancerous phenotypes.

Our data demonstrate that the constitutively active form of β-catenin (the S33A mutant, which is GSK-3β insensitive) was only moderately ubiquitinated by EDD compared with the wild-type form. Furthermore, in COLO320 and SW480 cells, where GSK-3β cannot phosphorylate β-catenin, silencing of EDD had no effect on β-catenin/TCF–mediated transcription. Although GSK-3β is a negative regulator of the Wnt pathway (Kimelman and Xu, 2006), our results show that GSK-3β is involved in EDD-mediated activation of β-catenin and that phosphorylation of β-catenin by GSK-3β is required for its EDD-induced ubiquitination. Together these results imply that GSK-3β may have a dual role in regulation of the Wnt pathway: on one hand, as part of the destruction complex leading to degradation of β-catenin and, on the other hand, as a binding partner of the EDD protein that functions to stabilize β-catenin.

Overall, our results reveal a correlation between EDD and up-regulation of β-catenin both in the expression levels and transcriptional activity. In a recent study, EDD was shown to colocalize with APC in the cytoplasm (Ohshima *et al.*, 2007), which somewhat conflicts with our findings and other reports showing that EDD is a nuclear protein (Henderson *et al.*, 2002; O'Brien *et al.*, 2008). This article also demonstrated that EDD up-regulates APC and down-regulates β-catenin. Our data, however, clearly show that overexpression of EDD up-regulates β-catenin and, moreover, that EDD depletion results in decreased levels of β-catenin. The apparent discrepancy between the results of Ohshima *et al*. (2007) and our findings may result from the different cell lines and experimental conditions used in the EDD RNA interference (RNAi) experiments.

The data presented in this work suggest that the ubiquitin system, mediated through EDD E3 ligase, is involved in stabilization of β-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.

MATERIALS AND METHODS

Cell culture and transfection

HEK293T, CHO, and the human colorectal cancer cell lines COLO320, HCT116, and SW480 were maintained in DMEM supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. Cells were transfected either by $CaPO_4$ precipitation or using the DNA transfection reagent jetPEI (Polyplus Transfection, Illkirch, France) following the manufacturer's protocols.

Plasmids and reagents

The FLAG-EDD expression vector was a generous gift from Michael J. Weber (University of Virginia Health Sciences Center, Charlottesville). GFP–β-catenin was constructed by inserting the β-catenin cDNA into plasmid enhanced GFP (pEGFP)-C1 (Clontech, Palo Alto, CA) using *Bam*HI and *Xba*I restriction sites. This expression vector was used as a template for mutagenesis of β-catenin by the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to replace serine (Mathew *et al.*, 2009) to alanine to generate GFP–S33A-β-catenin.

The HA-GSK expression vector was kindly provided by T. C. Dale (Developmental Biology, Chester Beatty Laboratories, Institute of Cancer Research, London, UK). FLAG–GSK-3β was kindly provided by Hagit Eldar-Finkelman (Tel-Aviv University, Tel-Aviv, Israel), and HA–β-catenin was kindly provided by Arnona Gazit (Tel-Aviv University). GFP–GSK-3β was constructed by inserting the GSK-3β 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 H. Clevers (Center for Biomedical Genetics, Utrecht, Netherlands) and were described previously (Korinek *et al.*, 1997). pSV-β-Galactosidase Control Vector and pCMV-Renilla were purchased from Promega, Madison, WI. HA-ubiquitin was a generous gift from Dirk Bohmann (University of Rochester Medical Center, Rochester, NY). Ubiquitin mutants K48R, K63R, K29R, and K11R expression vectors were kindly provided by Yosef Yarden (Weizmann Institute of Science, Rehovot, Israel). Control vectors were pEGFP-C1, mCherry (Clontech), pCMV-FLAG (Sigma-Aldrich, Rehovot, Israel), and pCDNA3-HA (Invitrogen, Camarillo, CA). Cycloheximide and LiCl (Sigma-Aldrich) were used at concentrations as indicated.

Antibodies

The following antibodies were used: mouse anti–β-catenin (immunoblot [IB], 1:5000; immunofluorescence [IF], 1:300; BD Transduction Laboratories, Lexington, KY), rabbit anti–β-catenin (immunoprecipitation [IP], 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-ABC (IB, 1:1000; IF, 1:150; Millipore, Temecula, CA), mouse anti–GSK-3β (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, Cambridge, MA), rabbit anti-EDD (IF, 1:100; Bethyl Laboratories, Montgomery, TX), mouse anti-FLAG (1:5000; Sigma), and 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 ImmunoResearch, West Grove, PA. For IF, Alexa red and green (1:500; Molecular Probes, Camarillo, CA) were used.

Luciferase reporter assays

To assay TCF-mediated transcription cells, they were seeded at 1×10^5 cells per well in a 24-well plate 24 h before transfection. Cells were transfected with the indicated vectors, along with pTOPFLASH/pFOPFLASH and β-gal or pCMV-Renilla plasmids. At 48 h posttransfection, the cells were harvested and subjected to luciferase assay according to the 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 radioimmunoprecipitation assay (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 × *g* for 15 min at 4°C. Following 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 h

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 (described previously). Cell lysates were incubated with anti-FLAG M2-agarose affinity gel (Sigma), with rotation for 18 h at 4°C. Alternatively, cell lysates were incubated with the specific antibody for 1 h at 4°C before 18-h 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 glutathione *S*-transferase (GST), GST–β-catenin, or 6XHis GSK-3β (kindly provided by Hagit Eldar-Finkelman). The EDD protein was produced in a rabbit reticulocyte TNT system (Promega) using [S^{35}] methionine or in the presence of nonlabeled amino acids mixture. Glutathione (Sigma) or Ni-NTA (Qiagen) beads were used to precipitate the GST-fusion proteins or the 6XHis GSK-3β, respectively. For each experiment, 10 μg recombinant proteins was incubated with 25 μl reticulocyte lysates and the appropriate beads for 3 h 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, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid [EGTA]) containing protease inhibitor cocktail (Sigma). To extract cytosolic fraction, cells were centrifuged for 10 min at 7000 × *g*. Pellets were then resuspended in high-salt buffer (20 mM HEPES, pH 7.9, 0.42 mM NaCl, 1 mM EDTA, 1 mM EGTA). Nuclear fraction was collected following centrifugation of 5 min at 12,000 × *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 h, and blocked with bovine serum albumin for 1 h. 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 confocal microscopy.

In vivo ubiquitination assay

HEK293T cells were transfected with HA-ubiquitin constructs together with the indicated plasmids. Five hours before harvesting, 25 μM proteasomal inhibitor MG132 (CalBiochem, La Jolla, CA) was added. Forty-eight hours after transfection, cells were harvested and lysed in RIPA buffer. Cell lysates were immunoprecipitated with anti-GFP or anti–β-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–β-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 (O'Brien *et al.*, 2008) was cloned into the *Bgl*II–*Hin*dIII site of pSUPER. The EDD-RNAi was 5′-GCAGTGTTCCTGCCTTCTT-3′. The sequence of the control scrambled EDD-RNAi was 5′-GGTCCGTCTTCTCGTC-TAT-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 (Essex, UK) (siGENOME SMART pool M-007189-02-0005, Human UBR5) as well as transfection reagent Dharmafect-1. Transfection was performed according to the 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 10 nM siRNA oligonucleotides using INTERFERin (Polyplus Transfection) according to the manufacturer's order. Nontargeting RNA oligonucleotides (Thermo Scientific Dharmacon) were used for both scEDD and scGSK controls.

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