Putative tumor suppressor EDD interacts with and up-regulates APC

Ryuichi Ohshima[1], Tomohiko Ohta[2,*], Wenwen Wu[2], Ayaka Koike[2], Tsuguo Iwatani[2], Michelle Henderson[3], Colin K. W. Watts[3] and Takehito Otsubo[1]

[1]*Division of Gastroenterological Surgery, and*
[2]*Division of Breast and Endocrine Surgery, Department of Surgery, St. Marianna University School of Medicine, Kawasaki, 216-8511 Japan*
[3]*Cancer Research Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, New South Wales 2010, Australia*

Adenomatous polyposis coli (APC), whose mutation causes colorectal cancers, is a key player in the Wnt signaling pathway. While the role of APC in inhibition of β-catenin/LEF1-dependent activation of transformation-inducing genes has been intensively studied and well established, regulation of APC expression at the protein level is only partially understood. Here we report that APC is up-regulated by EDD, the mammalian orthologue of *Drosophila melanogaster* "hyperplastic discs" gene (*hyd*) that is considered to be a putative tumor suppressor. Screening of APC immuno-complexes by mass spectrometry identified EDD as a putative APC-interacting protein. Exogenously expressed and endogenous APC interacted with EDD *in vivo*. Indirect immunofluorescent analyses demonstrated that APC and EDD co-localized in the cytoplasm of the cell. Over-expression of EDD enhanced the protein expression level of APC and its binding partner Axin, resulting in inhibition of Wnt signaling downstream of β-catenin. Conversely, siRNA knock-down of EDD down-regulated APC at the protein level without altering its mRNA level, causing enhanced protein expression of β-catenin. Thus, through protein–protein interaction, EDD stabilizes APC and up-regulates APC's function to inhibit β-catenin, suggesting that EDD could act as a colorectal tumor suppressor.

Introduction

EDD is the mammalian orthologue of the *Drosophila melanogaster* "hyperplastic discs" gene (*hyd*) (Callaghan *et al*. 1998) that encodes a large 2799 amino acid protein with several putative functional domains including a UBR box and a homologous to E6-AP carboxyl terminus (HECT) domain, supporting its role as an E3 ubiquitin ligase (Callaghan *et al*. 1998; Henderson *et al*. 2002; Tasaki *et al*. 2005). Because loss-of-function mutations of *hyd* cause imaginal disc overgrowth in *Drosophila* (Mansfield *et al*. 1994) and because reduced expression of EDD was observed in several human cancers (Fuja *et al*. 2004), it has been proposed that *EDD* is a putative tumor suppressor gene. In addition, *EDD* is frequently mutated in microsatellite-unstable gastric and colorectal cancers (Mori *et al*. 2002).

Communicated by: Keiichi I. Nakayama
**Correspondence*: E-mail: to@marianna-u.ac.jp

Adenomatous polyposis coli (*APC*) was discovered as a gene responsible for familial adenomatous polyposis (FAP) that predisposes patients to multiple colorectal polyps and cancers with high penetrance rates (Nishisho *et al*. 1991). Inactivation of the gene encoding the APC protein is common to most sporadic colorectal cancers and is an early event in tumorigenesis (Fodde 2002). APC acts as a scaffold protein for GSK3β, Axin and β-catenin to induce the GSK3β-mediated, phosphorylation-dependent degradation of β-catenin, a key Wnt signaling effecter (Bienz & Clevers 2000; Bienz 2002). APC inactivation leads to nuclear β-catenin accumulation, resulting in the constitutive activation of Wnt target genes through the activation of TCF/LEF transcription factors. APC also participates in the regulation of cytoskeletal networks to regulate cell adhesion and motility through interaction with proteins including Asef, IQGAP, kinesin-2 and *Drosophila* discs large (DLG) (Akiyama & Kawasaki 2006). While the mechanisms regulated by APC have been intensively studied, little is known about the

DOI: 10.1111/j.1365-2443.2007.01138.x

mechanisms that regulate APC at its protein level, except for extracellular Wnt signals. In this report, we show that EDD interacts with and up-regulates APC at the protein level, suggesting a role for EDD as a colorectal tumor suppressor.

Results

To search for candidate functional partner(s) of APC, we screened APC immunocomplexes by mass spectrometry. HEK 293T cells were transfected with Myc-APC and FLAG-mouse Axin (mAxin), and APC complexes were immunoprecipitated with anti-Myc antibody followed by elution with a competing Myc peptide. The elution was digested with trypsin and subjected to LC/MS/MS analysis. With eight peptides displaying a probability-based Mowse score of 66 (Supplementary Table S1), one of the most interesting candidate proteins identified was EDD. Because *EDD* is a putative tumor suppressor gene frequently mutated in microsatellite-unstable gastric and colorectal cancers, we further investigated EDD and its relationship with APC.

An interaction between APC and EDD was first confirmed by transient transfection followed by immunoprecipitation–Western analysis (Fig. 1). We did not use colorectal cancer cell lines for any of our experiments because they potentially contain APC mutations or a dysfunctional Wnt pathway. Control plasmid or plasmid directed to express Myc-APC was transfected into HEK 293T cells, and anti-Myc immunocomplexes were analyzed for the presence of endogenous EDD. EDD was clearly detected with Myc-APC (Fig. 1A, lanes 3 and 4) when compared to control samples (lane 1). Isolating an excess amount of Myc-APC did not increase the amount of associated EDD protein (lane 4), indicating limiting amounts of available EDD protein. To isolate endogenous APC–EDD complexes under physiological conditions, untreated HEK 293T cell lysate was immunoprecipitated with antibodies to EDD, and precipitates were analyzed by immunoblotting with an anti-APC antibody Ab1 (Fig. 1B). APC was readily co-immunoprecipitated with EDD (lane 2), but not with control IgG (lane 1). Together, the results indicate that EDD physically interacts with the APC complex *in vivo*.

Although there are conflicting reports concerning the localization of endogenous APC, re-examination of various anti-APC antibodies has revealed that APC predominantly resides in the cytoplasm (Brocardo *et al.* 2005). In contrast, EDD predominantly localized to the nucleus especially following DNA damage or when exogenously over-expressed, where it can interact with the progesterone receptor and TopBP1 (Henderson *et al.*

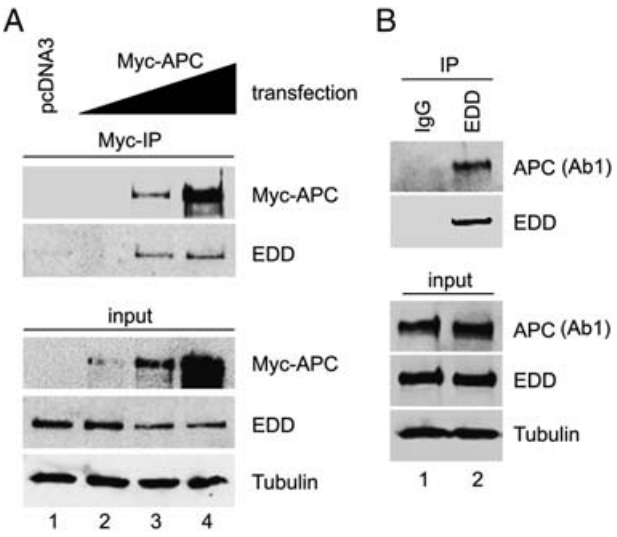

Figure 1 EDD interacts with APC. (A) Exogenously over-expressed APC interacts with EDD. HEK 293T cells were transfected with plasmid encoding Myc-APC (lane 2: 2 μg, lane 3: 4 μg, lane 4: 8 μg). Cells were harvested 36 h post-transfection, and cell extracts were subjected to immunoprecipitation with 3 μg of anti-Myc antibody followed by immunoblotting with anti-Myc or anti-EDD antibody. Total cell lysates (15 μL) were also analyzed (input, lower panels). Tubulin was immunoblotted as a loading control. IP: immunoprecipitate. (B) Endogenous APC interacts with EDD. Lysates prepared from HEK 293T cells were immunoprecipitated with 6 μg of anti-EDD antibody (lane 2) or control IgG (lane 1) as in (A). Total cell lysates (input, lower panels) or the immunoprecipitates (IP) (upper panels) were subjected to immunoblotting with the indicated antibodies.

2002; Honda *et al.* 2002). However, EDD was also reported to be reproducibly observed in the perinuclear cytoplasm of histology sections from cancer patients (Fuja *et al.* 2004). Therefore, we tested whether EDD and APC proteins co-localize in HEK 293T cells by co-staining formalin-fixed cells with anti-APC and anti-EDD antibodies. We first tested the same primary antibodies that were used for immunoprecipitation and immunoblotting. APC (red) and EDD (green) both localized to the cytoplasm, demonstrating enhanced perinuclear staining (Fig. 2A, upper panels). The yellow areas in the merged image demonstrate co-localization. The same results were observed with HeLa, MCF and T-47D cells (lower panels and data not shown). Because the anti-APC antibody Ab1 was previously shown to stain nuclear artifacts when used for immunofluorescent analyses (Brocardo *et al.* 2005), we further tested APC-EDD co-localization using the recommended anti-APC antibodies Ali12-28 and Ab7. APC–EDD co-localization

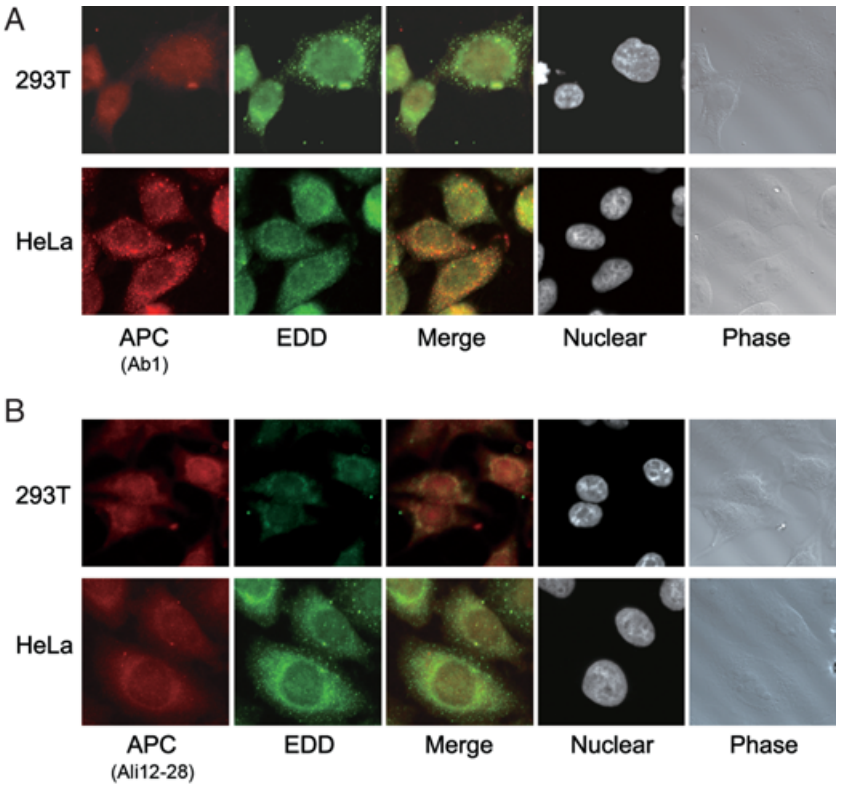

Figure 2 Co-localization of APC with EDD in the cytoplasm. Proliferating HEK 293T (upper panels) or HeLa (lower panels) cells were fixed with formalin and stained with anti-APC Ab1 (A) or Ali12-28 (B) together with anti-EDD followed by Rhodamine- or FITC-conjugated secondary antibodies. The nucleus was counterstained with TO-PRO-3. Merge indicates the overlayed images of the two proteins (APC and EDD). Phase indicates phase contrast microscopic observation.

was reproducibly observed in HEK-293T, HeLa, MCF-7 and T-47D cells (Fig. 2B, and data not shown). The results suggest that EDD predominantly localizes to the cytoplasm in at least some types of cancer cells, where it can potentially interact with APC.

Because EDD contains UBR and HECT domains and is thought to be an E3 ubiquitin ligase (Honda *et al.* 2002; Tasaki *et al.* 2005), we first speculated that EDD may ubiquitinate and destabilize APC. However, during the course of our experiments, we noticed that EDD over-expression somewhat increased the amounts of both APC and mAxin (data not shown). To test this observation, constant amounts of Myc-APC and FLAG-mAxin were co-expressed in HEK 293T cells with increasing amounts of FLAG-EDD. The steady state levels of both Myc-APC and FLAG-mAxin increased upon co-expression of FLAG-EDD in a dose-dependent manner (Fig. 3A). In contrast, increasing amounts of FLAG-mAxin did not increase, but rather decreased, Myc-APC expression and caused a slight increase in FLAG-EDD expression (Fig. 3B). This result is consistent with the recently reported, though unexpected, role for Axin in facilitating the ubiquitin-proteasome-dependent degradation of APC (Choi *et al.* 2004). In this case, the role of EDD could be to protect APC from Axin-

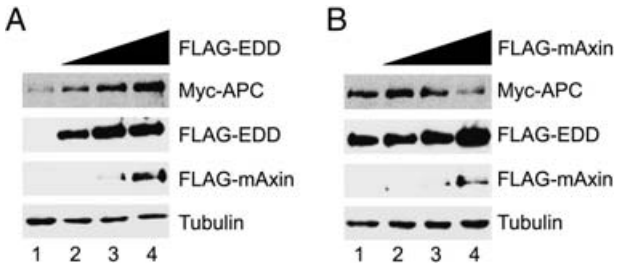

Figure 3 EDD up-regulates APC and Axin. (A) HEK 293T cells were transfected with plasmids encoding Myc-APC and FLAG-mAxin (lanes 1–4, 4 μg each) and increasing amounts of FLAG-EDD (lane 2: 2 μg, lane 3: 4 μg, lane 4: 8 μg). Total plasmid DNA was adjusted to 15 μg per plate by adding the parental pcDNA3 vector. The steady state level of each protein in the cell lysate was analyzed by immunoblot using anti-Myc, anti-FLAG or anti-tubulin antibody. (B) Steady state levels of the indicated proteins were analyzed as in (A), except that the amount of plasmid expressing FLAG-mAxin, instead of FLAG-EDD, was increased.

mediated down-regulation. However, this protection is not through the degradation of Axin by EDD E3 ligase activity because EDD also up-regulates mAxin expression (Fig. 3A). One interpretation is that the APC–Axin–EDD

complex is a stable form, whereas the APC–Axin complex is unstable and is a better substrate for its E3 ligase.

In order to eliminate the possibility that the observed effects on protein expression were artifacts due to protein over-expression, we next tested the effect of the EDD expression level on APC under physiological conditions using RNA interference of EDD. MCF-7, T-47D and HeLa cells were transfected with EDD-specific siRNA. Cells were successfully silenced for EDD expression (0.15-, 0.34- and 0.04-fold, respectively) compared with their controls (Fig. 4A, top panel). The expression of APC was dramatically reduced upon EDD knock-down in MCF-7 and HeLa cells (0.63- and 0.61-fold, respectively, including slower migrating proteins) and was slightly reduced in T-47D cells (0.80-fold). Interestingly, APC from EDD knock-down cells migrated slower on the gel (lanes 2 and 6) than APC from control cells (lanes 1 and 5). This may indicate that APC is covalently modified while being destabilized.

The observed elevation of APC levels by EDD (Fig. 3A) was obtained using exogenous APC and EDD proteins expressed from the CMV promoter. This suggested that the up-regulation occurred at the protein level, not at the mRNA level. Alternatively, the reduced expression of APC in EDD knock-down cells still could be due to down-regulation of APC at the mRNA level. Therefore, we tested if APC mRNA was affected by EDD knock-down. RT-PCR analysis of mRNA from cells treated with EDD siRNA demonstrated that APC mRNA was not down-regulated by EDD knock-down when compared to control samples (Fig. 4B). Together, the results indicate that EDD stabilizes APC at the protein level.

Stabilization or increased expression of a protein in cells does not always result in up-regulation of the protein's function. Therefore, we next needed to analyze the effect of EDD on APC function. The most well-studied function of APC is its role in β-catenin degradation in the Wnt signaling pathway. Hence, the effect of EDD on the protein expression level of β-catenin was analyzed. The steady state level of endogenous β-catenin was increased upon FLAG-EDD over-expression in HEK 293T cells as compared with cells transfected with parental pcDNA3 vector (Fig. 5A). In order to confirm this observation, we tested the effect of EDD siRNA knock-down on β-catenin expression. MCF-7 cells were transfected with EDD-specific or control siRNA, and β-catenin protein levels were examined by immunoblotting. As expected, β-catenin levels were enhanced by EDD knock-down (Fig. 5B). These results indicate that EDD-stabilized APC is functionally active and inhibits the Wnt signaling pathway.

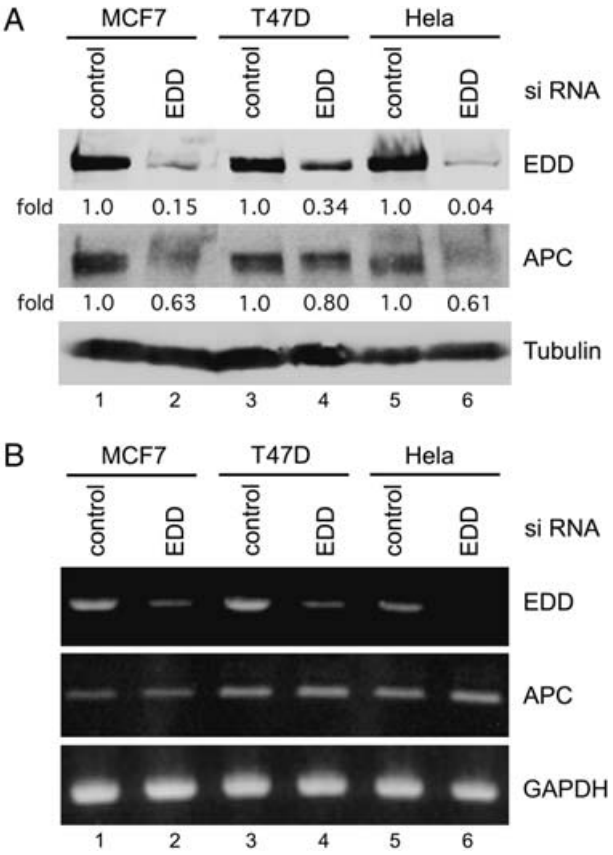

Figure 4 EDD knock-down destabilizes APC. (A) Knock-down of EDD with siRNA down-regulates the expression level of APC protein. EDD siRNA (lanes 2, 4 and 6) and control siRNA (lanes 1, 3 and 5) were transfected into either MCF-7, T-47D or HeLa cells. Cells were harvested 48 h post-transfection, and total cell lysates were subjected to immunoblotting with the indicated antibodies. Tubulin served as a loading control. The amounts of protein expression were quantified with the lumino-image analyzer LAS3000 (Fuji film, Tokyo, Japan) and are indicated as a ratio to the total amount of protein expressed in each control. (B) RT-PCR analysis demonstrated that APC mRNA expression was not affected by EDD knock-down. Total RNA was isolated from cells transfected with siRNA as in (A) and the cDNAs for APC, EDD and control GAPDH were amplified by RT-PCR. The PCR products for APC (393 bp), EDD (2679 bp) and GAPDH (445 bp) were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized by UV transillumination.

Discussion

The results presented here clearly demonstrate an interaction between the well-known colorectal tumor suppressor APC and the putative tumor suppressor EDD. Somatic frameshift mutations including biallelic alterations in a microsatellite sequence in the coding region of *EDD* occur in 27.8% and 23.3% of microsatellite-unstable

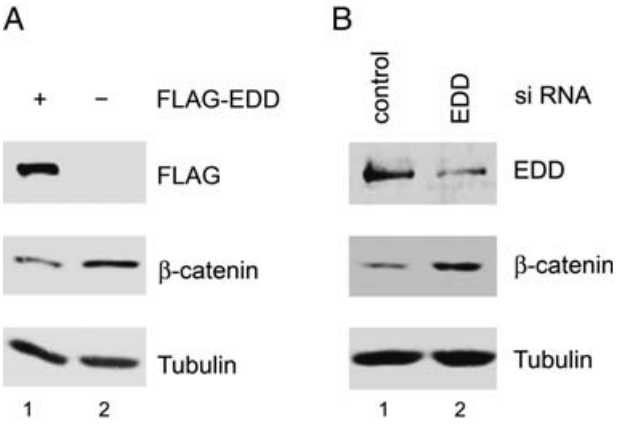

Figure 5 EDD down-regulates β-catenin. (A) Over-expression of EDD inhibits the expression level of β-catenin protein. HEK 293T cells were either transfected with plasmids encoding FLAG-EDD (lane 1) or the parental pcDNA3 vector (lane 2). The steady state level of each protein in the cell lysate was analyzed by immunoblot using anti-FLAG, anti-β-catenin or anti-tubulin antibody as indicated. (B) Knock-down of EDD with siRNA enhances the expression level of β-catenin protein. Control siRNA (lanes 1) and EDD siRNA (lanes 2) were transfected into MCF-7 cells. Cells were harvested 48 h post-transfection, and total cell lysates were subjected to immunoblotting with the indicated antibodies.

gastric and colorectal cancers, respectively (Mori *et al.* 2002). One simple and important question evoked from our results is whether the mutation of *EDD* could cause defective APC function in such alimentary tract cancers. In this regard, it could be interesting to test whether APC function is retained in *EDD*-mutated gastric or colorectal cancers. Another important aspect of EDD in relation to the Wnt signaling pathway is that hyd/EDD inhibits the expression of the *hedgehog* (*Hh*) gene, a morphogen that regulates epithelial/mesenchymal interactions during embryonic development (Lee *et al.* 2002). Indeed, EDD was found to be essential for embryonic development and vascularization in an *EDD* gene knockout mouse model (Saunders *et al.* 2004). Loss of hyd/EDD function leads to the ectopic expression of *Hh* and decapentaplegic (*Dpp*) genes by independent mechanisms, resulting in non-autonomous overgrowth of the eye disc and premature photoreceptor differentiation (Lee *et al.* 2002). It recently has been highlighted that the Hh pathway and the Wnt pathway antagonize each other during intestinal cell proliferation (van den Brink & Hardwick 2006). While Wnt signals maintain intestinal precursor cell proliferation at the base of the crypt in the colon, Indian hedgehog (Ihh) inhibits Wnt signaling and induces differentiation at the top of the crypt. Therefore, EDD may integrate the two pathways in the maintenance of intestinal cell growth and differentiation. Although how EDD stabilizes APC remains to be determined, the up-regulation of APC by EDD could be an important mechanism underlying the prevention of gastric and colorectal cancer development.

Experimental procedures

Mass spectrometric analysis

Proteins interacting with Myc-APC and FLAG-mAxin complexes were immunoprecipitated as described in Fig. 1A. The complexes were eluted off the beads in 30 μL of 25 mM ammonium bicarbonate containing 10 μg/mL of Myc peptide and digested with 7.4 μg/mL of trypsin for 20 h at 30 °C. The peptide fragments were subjected to nanoscale capillary liquid chromatography-tandem mass spectrometric (LC/MS/MS) analysis as described (Nishikawa *et al.* 2004). The acquired collision-induced dissociation spectra were analyzed by MASCOT software.

Plasmids

FLAG-tagged mammalian expression plasmid for EDD, pcDNA3-FLAG-EDD, was previously described (Henderson *et al.* 2002; Honda *et al.* 2002). Myc-APC and FLAG-mAxin were generous gifts from Dr Tetsu Akiyama at University of Tokyo.

Cell culture and transfections

MCF-7, T-47D breast carcinoma cells, HeLa cervical carcinoma cells and HEK 293T transformed human kidney cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and 1% antibiotic-antimycotic agent (Life Technologies, Inc, Grand Island, NY) in 5% CO_2 at 37 °C. HEK 293T cells were transfected using the standard calcium phosphate precipitation method. Total plasmid DNA was adjusted to 15 μg per 100-mm dish by adding the parental pcDNA3 vector.

Antibodies

Mouse monoclonal antibodies to APC (Ab1 and Ab7, Calbiochem, Darmstadt, Germany; Ali12-28, Santa Cruz Biotechnology, Santa Cruz, CA), Myc (9E10, BabCo, Richmond, CA), FLAG (M2; Sigma, St. Louis, MO), β-catenin (8E7, Upstate, Temucula, CA) and α- and β-tubulin (DMIA + BMIB; Neomarkers, Fremont, CA) as well as goat polyclonal antibodies to EDD (N-19, Santa Cruz Biotechnology) and control IgG (Sigma, St. Louis, MO) were purchased commercially.

Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were lysed in 1 mL of buffer A [50 mM Tris–HCl (pH 7.5), 0.5% NP-40, 150 mM NaCl, 50 mM

NaF, 1 mM dithiothreitol (DTT), 1 mM $NaVO_3$, 1 mM PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 10 μg/mL trypsin inhibitor and 150 μg/mL benzamidine] at 4 °C for 30 min. Extracts were then clarified by centrifugation at 23 000 ***g*** at 4 °C for 10 min. The supernatants (0.5 mL) were mixed with appropriate antibody, and the antibody-bound proteins were precipitated with protein A-agarose beads (7.5 μL). The proteins bound to the beads were washed 3 times with buffer A and boiled in Laemmli SDS-loading buffer with 0.1 M DTT. Half of the sample was resolved by 7.5% SDS-PAGE, followed by immunoblotting with anti-Myc or anti-EDD goat polyclonal antibody. For straight immunoblotting, cells were lysed and clarified as described above, and 15 μL of each sample was resolved by SDS-PAGE, followed by immunoblotting.

Indirect immunocytochemistry

Proliferating cells were fixed with 3% formalin for 15 min and permeabilized with 0.2% Triton X-100 for 5 min. Cells were washed with phosphate-buffered saline (PBS), blocked with 0.5% BSA in PBS, and stained with the indicated antibodies. Primary antibodies were diluted in the blocking buffer at the following concentrations: anti-APC Ab1 (4 μg/mL), Ab7 (5 μg/mL), Ali12-28 (4 μg/mL) and anti-EDD (4 μg/mL). For secondary antibodies, Rhodamine-conjugated anti-mouse IgG or FITC-conjugated anti-goat IgG (Jackson Immuno Research, West Grove, PA) were used at a 1 : 50 dilution. The nucleus was counterstained with 0.5 mM TO-PRO-3 (Molecular probe, Carlsbad, CA), cells were then mounted with fluorescent mounting medium (BioLad, Hercules, CA) and they were examined with a confocal laser scan microscope (LSM 510, Carl Zeiss, Jena, Germany).

RNAi

SMART pool® EDD siRNA mix and control siRNA mix were purchased from Dharmacon Research, Inc (Lafayette, CO), and RNA duplexes (final concentration 50 nM) were transfected into cells with Oligofectamine® (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were harvested 48 h post-transfection and subjected to immunoblotting or RT-PCR.

RT-PCR

Total RNA was isolated from cells using the guanidine isothiocyanate-based TRIzol solution (Life Technologies, Gaithersburg, MD). It was then converted to single strand cDNA using the PrimeScript™-RTase (Takara, Shiga, Japan) according to the manufacturer's instructions. The cDNAs for APC, EDD and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified by PCR using PrimeSTAR®Max DNA polymerase (Takara) with the following primers.

APC: 5′-GAGACAGAATGGAGGTGCTGC-3′ and 5′-CAGGACTGCACTCTCCAGAACG-3′, EDD: 5′-GCAC-CATGACGTCCATCCATTTCGTG-3′ and 5′-GATATT-GTCGACAGGCCTCATAGTCACAGCG-3′, GAPDH: 5′-GACAACTTTGGTATCGTGGA-3′ and 5′-TACCAG-GAAATGAGCTTGAC-3′.

Acknowledgements

We would like to thank Dr Tetsu Akiyama at University of Tokyo for plasmids expressing APC and mAxin. This study was supported by grants from Japan Society for the Promotion of Science and Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Akiyama, T. & Kawasaki, Y. (2006) Wnt signalling and the actin cytoskeleton. *Oncogene* **25**, 7538–7544.

Bienz, M. (2002) The subcellular destinations of APC proteins. *Nat. Rev. Mol. Cell Biol.* **3**, 328–338.

Bienz, M. & Clevers, H. (2000) Linking colorectal cancer to Wnt signaling. *Cell* **103**, 311–320.

Brocardo, M., Näthke, I.S. & Henderson, B.R. (2005) Redefining the subcellular location and transport of APC: new insights using a panel of antibodies. *EMBO Rep.* **6**, 184–190.

Callaghan, M.J., Russell, A.J., Woollatt, E., Sutherland, G.R., Sutherland, R.L. & Watts, C.K. (1998) *Oncogene* **17**, 3479–3491.

Choi, J., Park, S.Y., Costantini, F., Jho, E.H. & Joo, C.K. (2004) Adenomatous polyposis coli is down-regulated by the ubiquitin-proteasome pathway in a process facilitated by Axin. *J. Biol. Chem.* **279**, 49188–49198.

Fodde, R. (2002) The APC gene in colorectal cancer. *Eur. J. Cancer* **38**, 867–871.

Fuja, T.J., Lin, F., Osann, K.E. & Bryant, P.J. (2004) Somatic mutations and altered expression of the candidate tumor suppressors CSNK1 epsilon, DLG1, and EDD/hHYD in mammary ductal carcinoma. *Cancer Res.* **64**, 942–951.

Henderson, M.J., Russell, A.J., Hird, S., Muñoz, M., Clancy, J.L., Lehrbach, G.M., Calanni, S.T., Jans, D.A., Sutherland, R.L. & Watts, C.K. (2002) EDD, the human hyperplastic discs protein, has a role in progesterone receptor coactivation and potential involvement in DNA damage response. *J. Biol. Chem.* **277**, 26468–26478.

Honda, Y., Tojo, M., Matsuzaki, K., Anan, T., Matsumoto, M., Ando, M., Saya, H. & Nakao, M. (2002) Cooperation of HECT-domain ubiquitin ligase hHYD and DNA topoisomerase II-binding protein for DNA damage response. *J. Biol. Chem.* **277**, 3599–3605.

Lee, J.D., Amanai, K., Shearn, A. & Treisman, J.E., (2002) The ubiquitin ligase hyperplastic discs negatively regulates hedgehog and decapentaplegic expression by independent mechanisms. *Development* **129**, 5697–5706.

Mansfield, E., Hersperger, E., Biggs, J. & Shearn, A. (1994) Genetic and molecular analysis of hyperplastic discs, a gene whose product is required for regulation of cell proliferation in *Drosophila melanogaster* imaginal discs and germ cells. *Dev. Biol.* **165**, 507–526.

Mori, Y., Sato, F., Selaru, F.M. *et al.* (2002) Instabilotyping reveals unique mutational spectra in microsatellite-unstable gastric cancers. *Cancer Res.* **62**, 3641–3645.

Nishikawa, H., Ooka, S., Sato, K., Arima, K., Okamoto, J., Klevit, R.E., Fukuda, M. & Ohta, T. (2004) Mass spectrometric

and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase. *J. Biol. Chem.* **279**, 3916–3924.

Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Hori, A., Koyama, K., Utsunomiya, J., Baba, S. & Hedge, P. (1991) Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. *Science* **253**, 655–659.

Saunders, D.N., Hird, S.L., Withington, S.L., Dunwoodie, S.L., Henderson, M.J., Biben, C., Sutherland, R.L., Ormandy, C.J. & Watts, C.K. (2004) Edd, the murine hyperplastic disc gene, is essential for yolk sac vascularization and chorioallantoic fusion. *Mol. Cell. Biol.* **24**, 7225–7234.

Tasaki, T., Mulder, L.C., Iwamatsu, A., Lee, M.J., Davydov, I.V., Varshavsky, A., Muesing, M. & Kwon, Y.T. (2005) A family of mammalian E3 ubiquitin ligases that contain the UBR box motif and recognize N-degrons. *Mol. Cell. Biol.* **25**, 7120–7136.

van den Brink, G.R. & Hardwick, J.C. (2006) Hedgehog Wnteraction in colorectal cancer. *Gut* **55**, 912–914.

Received: 5 July 2007
Accepted: 23 August 2007

Supplementary material

The following supplementary material is available for this article online:

Table S1 EDD peptides identified by LC/MS/MS analyses