The deubiquitinating enzyme Fam interacts with and stabilizes β-catenin

（脱ユビキチン化酵素Famによるβ-cateninの分解制御機構）

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Abstract

In the ubiquitin-proteasome pathway, the ubiquitinated substrates undergo either degradation by the proteasome or stabilization through the action of deubiquitinating enzyme. I have previously found that the deubiquitinating enzyme Fam is colocalized with AF-6, one of the effectors of the Ras small GTPase, at the cell-cell contact sites in epithelial cells and interacts with AF-6 *in vivo* and *in vitro*. Fam has deubiquitinating activity *in vitro* and prevents the ubiquitination of AF-6 in intact cells. The degradation of β-catenin, which accumulates at the cell-cell contact sites as a cadherin/catenin complex, is thought to be regulated by the ubiquitin-proteasome pathway. These observations prompted me to examine whether Fam regulates the stabilization of β-catenin. I here found that Fam interacted with β-catenin both *in vivo* and *in vitro*. The Fam-binding site of β-catenin mapped to the region close to the APC or Axin-binding site of β-catenin. APC and Axin directly bind to β-catenin and promote the degradation of β-catenin. This raises the possibility that Fam inhibits the binding of APC and/or Axin to β-catenin and represses the function of APC and/or Axin. Overexpression of Fam in mouse L cells resulted in elevation of β-catenin levels and in elongation of the half-life of β-catenin. In these L cells, Fam was colocalized with β-catenin at the dot-like structures in the cytoplasm. These results indicate that Fam interacts with and stabilizes β-catenin *in vivo* presumably through the deubiquitination of β-catenin.

Introduction

The ubiquitin-proteasome pathway plays an important role in the complete degradation of abnormal and short-lived regulatory proteins (reviewed by Hochstrasser 1995; Hershko and Ciechanover 1998). The ubiquitin-mediated degradation of certain regulatory proteins plays critical roles in various functions including cell-cycle, signal transduction, transcriptional regulation, tumor suppression, and endocytosis. In this pathway, substrates are ubiquitinated by the sequential action of three enzymes, which are a single ubiquitin (Ub) activating enzyme (E1), many species of Ub carrier proteins (E2s) and multiple families of Ub-protein ligases (E3s) or E3 multiprotein complexes. Multi-ubiquitinated substrates are then recognized by the proteasome and rapidly degraded into short peptides. The ubiquitination of substrates is a reversible reaction. The ubiquitinated substrates are deubiquitinated by so called deubiquitinating enzymes. A large superfamily of genes encoding the deubiquitinating enzymes, ubiquitin-specific proteases (Ubps), was identified (reviewed by Hochstrasser 1995; Wilkinson 1997; Hershko and Ciechanover 1998; D'Andrea and Pellman 1998). The deubiquitinating enzymes are of two main types. The first type is thought to remove Ub from Ub-conjugated degradation products produced by the proteasomes and the second one is thought to remove Ub from multi-ubiquitinated substrates. Recent genetic analyses indicate that the product of the *fat facets* gene (Faf) is an enzyme of the second type and that the deubiquitinating activity of Faf is essential for the regulation of the cell communication pathway necessary for normal eye development and

embryogenesis in *Drosophila* (Huang *et al.* 1995; Huang and Fischer-Vize 1996). Fam (fat facets in mouse) was identified as a murine ortholog of Faf. *In situ* hybridization analyses revealed that murine Fam transcripts exist in the rapidly expanding cell population of gastrulating and neurulating embryos and in post-mitotic cells of the central nervous system as well as in the apoptotic regions between digits (Wood *et al.* 1997).

I have recently identified Fam as an ALL-1 fusion partner from chromosome 6 (AF-6)-interacting protein (Taya *et al.* 1998). AF-6 was originally described as the fusion partner of the acute lymphoblastic leukemia-1 (ALL-1) protein (Prasad *et al.* 1993). My laboratory previously identified AF-6 as a Ras effector (Kuriyama *et al.* 1996). AF-6 has been shown to serve as one of the peripheral components of cell-cell adhesions, and thought to participate in the regulation of cell-cell adhesions downstream of Ras (Yamamoto *et al.* 1997). I have found that Fam is partially colocalized with AF-6 at the cell-cell contact sites of Madine-Darby canine kidney cell strain II (MDCKII) cells. AF-6 is ubiquitinated in intact cells, and Fam prevents the ubiquitination of AF-6 (Taya *et al.* 1998). AF-6 forms a complex with Fam and serves as one of the substrates for Fam. Thus, the degradation of peripheral components of cell-cell adhesions such as AF-6 appears to be regulated by Fam.

β-catenin has been shown to have two distinct roles in cell-cell adhesions and in nuclear signal transduction in embryos. β-catenin forms complexes with cadherin and α-catenin to establish a link with the actin cytoskeleton in cell-cell adhesions (reviewed by Tsukita *et al.* 1992; Takeichi

1995; Gumbiner 1996; Adams and Nelson 1998). In the cytoplasm, the stabilization of β-catenin is regulated by the Wnt signaling pathway. The activation of the Wnt signal results in the inactivation of glycogen synthase kinase-3β (GSK-3β), and leads to the accumulation of β-catenin in the cytoplasm and its translocation into the nucleus (reviewed by Miller and Moon 1996; Nusse 1997; Polakis 1997). In the absence of Wnt signal, GSK-3β is thought to phosphorylate β-catenin and subsequently to induce β-catenin degradation (Peifer *et al.* 1994; Papkoff *et al.* 1996; Yost *et al.* 1996; Rubinfeld *et al.* 1997). The degradation of β-catenin is regulated by the ubiquitin-proteasome pathway (Aberle *et al.* 1997; Orford *et al.* 1997). However, little is known about the regulatory mechanism underlying the stabilization of β-catenin in the ubiquitin-proteasome pathway.

In the light of these observations, I here examined the relationship between Fam and β-catenin. I found that Fam interacted with β-catenin both *in vivo* and *in vitro*, and that Fam could regulate the degradation of β-catenin.

Materials and Methods

Materials and chemicals

The Fam cDNA clone was kindly provided by Drs. Masami Kanai and S.A. Wood (University of Queensland, Australia). Madine-Darby canine kidney cell strain II (MDCKII) cells, EL cells which are L cells stably expressing E-cadherin, and various constructs of mouse E-cadherin, α-catenin and β-catenin were kindly

provided by Drs. A. Nagafuchi and S. Tsukita (Kyoto University, Kyoto, Japan) (Nagafuchi *et al.* 1994). The AF-6 cDNA was kindly provided by E. Canaani (Weizmann Institute of Science, Rehovot, Israel). The anti-GSK-3β and Axin antibodies were kindly provided by Drs. S. Kishida and A. Kikuchi (Hiroshima University, Hiroshima, Japan). The anti-Fam (1476-1918 aa) antibody was generated as described previously (Harlow *et al.* 1988). FITC-conjugated anti-rabbit IgG antibody, Texas red-conjugated anti-mouse IgG antibody, and Pro-mix L-[^{35}S] *in vitro* cell labelling mix were purchased from Amersham Corp. (Buckinghamshire, UK). The anti-α-catenin and β-catenin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). The anti-HA (hemagglutinin) monoclonal antibody (12CA5) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). The anti-maltose binding protein (MBP) polyclonal antibody was purchased from New England Biolabs Inc. (Beverly, MA). All materials used in the nucleic acid study were purchased from Takara Shuzo Corp. (Kyoto). Other materials and chemicals were obtained from commercial sources.

Plasmid constructions

The *Escherichia coli* expression plasmids pGEX-3X-AF-6 (1130-1612 aa), pMal-c2-Fam (1476-1918 aa), and the mammalian expression plasmid pEF-BOS-HA-Fam-CAT (1210-2410 aa) were produced as described (Taya *et al.* 1998). Glutathione-*S*-transferase (GST)-mouse E-cadherin involving the cytoplasmic domain, α-catenin (1-906 aa), and β-catenin (1-781 aa) were produced as described previously (Kuroda *et al.* 1998). To obtain maltose-binding protein (MBP)-mouse β-catenin (1-781

aa), (1-183 aa), (1-535 aa), (184-535 aa), or (536-781 aa), cDNA fragments of β-catenin were subcloned into pMal-c2 (New England Biolabs Inc.). The *Escherichia coli* expression plasmid pGEX-4T-1-Fam (1476-1918 aa) was constructed as follows. The fragment (1476-1918 aa) was cloned into the *Eco*RI and *Sal*I sites of pGEX-4T-1 (Pharmacia Biotech, Tokyo, Japan).

Cell culture

MDCKII cells were grown in Dulbecco's modified Eagles medium (DMEM) containing 10% calf serum, penicillin, and streptomycin in an air-5% CO_2 atmosphere at constant humidity. EL cells were grown in DMEM containing 10% fetal bovine serum (FBS) and 100 μg/ml of G418 in an air-5% CO_2 atmosphere at constant humidity. To obtain L cells stably expressing HA-Fam-CAT, L cells were transfected with pEF-BOS-HA-Fam-CAT along with pSVIISRα vector containing the neomycin resistance gene using Lipofectamine (GIBCO BRL, Rockville, MD) as described (Kuroda *et al.* 1998), and neomycin-resistant clones were selected. L cells expressing HA-Fam-CAT were grown in DMEM containing 10% FBS and 250 μg/ml of G418 in an air-5% CO_2 atmosphere at constant humidity.

Coimmunoprecipitation assay

MDCKII and EL cells were grown in 100 mm tissue culture dishes at a cell density of 1×10^6 cells/dish and cultured for 48 h. After being washed with phosphate-buffered saline (PBS), the cells were lysed with 1 ml of buffer A (50 mM Tris/HCl at pH 7.5, 1 mM EGTA, 50 mM NaCl, 0.5% (W/V) Triton X-100, 10 μM [*p*-amidino-phenyl] methanesulfonyl fluoride (*p*-APMSF), 10

μg/ml leupeptin). The lysate was removed from the dishes with a rubber policeman. The lysate was sonicated, incubated in a 1.5 ml tube for 15 min on ice and then clarified by centrifugation at 12,000 *g* for 30 min at 4°C. The soluble supernatant was incubated with 10 μl (total protein: 600 μg) of the anti-Fam antiserum, anti-β-catenin antiserum, or preimmuneserum. The immunocomplexes were then precipitated with protein A-Sepharose 4B (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The immunocomplexes were washed six times with buffer A, eluted by boiling in sample buffer for SDS-PAGE and subjected to immunoblot analysis with the anti-β-catenin antibody.

In vitro binding assay

GST or MBP fusion proteins were expressed in *E. coli* BL21(DE3) and purified according to the manufacturer's instructions. MBP-Fam (1476-1918 aa) (0.8 nmol) was mixed with glutathione-Sepharose 4B beads (30 μl) coated with 0.5 nmol of either GST, GST-AF-6 (1130-1612 aa), GST-E-cadherin, GST-α-catenin, or GST-β-catenin in 400 μl of buffer B (50 mM Tris/HCl at pH 8.0, 1 mM EDTA, 0.1% Triton X-100, 0.1% 3-[(3-Cholamidopropyl) dimethyl-ammonio]propanesulfonic acid (CHAPS)). The beads were washed six times with 100 μl of buffer B, and then washed three times with 100 μl of buffer B containing 50 mM NaCl. The bound MBP-Fam (1476-1918 aa) was co-eluted with GST fusion proteins by the addition of 100 μl of buffer B containing 10 mM glutathione three times. Portions (30 μl each) of the three fractions of the glutathione-eluates were subjected to SDS-PAGE, followed by immunoblot analysis with the anti-MBP antibody. To test the specificity of the binding, I

carried out a kinetic study of the binding of Fam to β-catenin. The indicated amount of MBP-Fam (1476-1918 aa) was mixed with glutathione-Sepharose 4B beads (30 μl) coated with 0.5 nmol of either GST, GST-AF-6 (1130-1612 aa), or GST-β-catenin in 400 μl of buffer B. *In vitro* binding assay and immunoblot analysis with the anti-MBP antibody were carried out as described above. The eluted MBP-Fam (1476-1918 aa) was visualized and estimated with a densitograph (ATTO, Tokyo). To determine which domain of β-catenin interacts with Fam, I prepared various deletion mutants of MBP-β-catenin such as β-catenin (1-781 aa), (1-183 aa), (1-535 aa), (184-535 aa), or (536-781 aa). MBP-β-catenin mutants (0.4 nmol) were mixed with glutathione-Sepharose 4B beads (30 μl) coated with 0.5 nmol of either GST or GST-Fam (1476-1918 aa) in 550 μl of buffer B. *In vitro* binding assay and immunoblot analysis with the anti-MBP antibody were carried out as described above.

Immunofluorescence and laser scanning confocal microscopy

L cells stably expressing HA-Fam-CAT and HA alone were plated on 13-mm-round glass coverslips in 35 mm tissue culture dishes at a cell density of 5×10^4 cells/dish and cultured for 48 h. The cells were fixed with 4% paraformaldehyde in PBS for 5 min. The fixed cells were incubated for 12 h at 4°C with rabbit polyclonal antibody against β-catenin and mouse monoclonal antibody against HA, and washed three times for 10 min with PBS. The cells were incubated for 1 h with FITC-conjugated anti-mouse IgG antibody and Texas red-conjugated anti-rabbit IgG antibody, and washed three times for 10 min with PBS. The accumulation of β-catenin and the expression of HA-Fam-CAT were

examined using a laser scanning confocal microscope (LSM510; Carl Zeiss, Inc., Thornwood, NY) equipped with an argon laser and a helium-neon laser for double fluorescence at 488 and 543 nm (emission filter; BP510-525 and LP590).

Pulse-chase analysis

L cells stably expressing HA-Fam-CAT or HA alone were seeded in 35 mm tissue culture dishes at a cell density of 1 x 10^5 cells/dish and cultured for 24 h. The cells were incubated for 1 h in methionine and cysteine-free medium, and were then incubated for 1 h in the presence of 50 μCi/ml of [^{35}S]methionine and [^{35}S]cysteine. The cells were washed with medium containing excess non-radioactive methionine and cysteine and were then incubated for various times. After the cells had been washed with ice-cold PBS, they were harvested in 200 μl of buffer C (10 mM Tris/HCl at pH 7.5, 1 mM EGTA, 150 mM NaCl, 1% (W/V) Nonidet P-40, 0.1% (W/V) deoxycholate, 0.1% (W/V) sodium dodecyl sulfate, 10 μM *p*-APMSF, 10 μg/ml leupeptin). An immunoprecipitation analysis with the anti-β-catenin antibody was performed as described above. The samples were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The [^{35}S]-labeled bands corresponding to labeled β-catenin were visualized with an image analyzer (BAS-2000; Fuji, Tokyo, Japan).

Effect of proteasome inhibitor

L cells stably expressing HA-Fam-CAT or HA alone were seeded in 100 mm tissue culture dishes at the cell density of 1 x 10^6 cells/dish and cultured for 24 h. The cells were then treated

with 25 μM ALLN (N-acetyl-Leu-Leu-norleucinal) or ALLM (N-acetyl-Leu-Leu-methional) for 4 h. The cells were washed twice with PBS and lysed in 200 μl of buffer D (50 mM Tris/HCl at pH 7.5, 100 mM KCl, 4 mM EGTA, 1 mM NaF, 1 mM sodium vanadate, 0.25% Triton X-100, 10 μM p-APMSF, 1 μg/ml leupeptin, 25 μM ALLN and 300 mM sucrose). The cell lysates were subjected to SDS-PAGE and immunoblotted with the anti-β-catenin antibody.

Other procedures

SDS-PAGE was performed as described (Laemmli 1970). Protein concentrations were determined with BSA as the reference protein as described (Bradford 1976). Immunoblot analyses were carried out as described (Harlow *et al.* 1988).

Results

Interaction of Fam and β-catenin

I have previously shown that Fam is partly colocalized with β-catenin at the cell-cell contact sites of confluent Madine-Darby canine kidney cell strain II (MDCKII) cells (**Fig. 1**) (Taya *et al.* 1998). This observation prompted me to examine whether Fam interacts with β-catenin *in vivo*. When Fam was immunoprecipitated with the anti-Fam antibody from MDCKII cells, β-catenin was coimmunoprecipitated with Fam (**Fig. 2A**). β-catenin was slightly coimmunoprecipitated with control preimmuneserum. Similar results were obtained when EL cells, which are mouse L fibroblasts stably expressing E-cadherin, were used instead of MDCKII cells (**Fig. 2B**). About 10 % of the

total Fam was immunoprecipitated and about 1.0 or 0.8% of the total β-catenin from MDCKII or EL cells was recovered with the immunoprecipitated Fam under the conditions, respectively. These results suggest that Fam directly or indirectly interacts with β-catenin *in vivo*.

To examine whether GST-β-catenin directly binds to MBP-Fam (1476-1918 aa) at the deubiquitinating catalytic domain, I carried out an *in vitro* binding assay. I have previously shown that MBP-Fam (1476-1918 aa) binds to GST-AF-6 (1130-1612 aa) (Taya *et al.* 1998). Affinity beads coated with GST, GST-AF-6 (1130-1612 aa), GST-E-cadherin, GST-α-catenin, or GST-β-catenin were mixed with MBP-Fam (1476-1918 aa). The bound MBP-Fam (1476-1918 aa) was then co-eluted with the GST fusion proteins by the addition of glutathione, and the eluted MBP-Fam (1476-1918 aa) was detected by immunoblot analysis with the anti-MBP antibody. MBP-Fam (1476-1918 aa) was detected in the eluates from the GST-AF-6 (1130-1612 aa) and GST-β-catenin affinity beads, but not in those from the GST, GST-E-cadherin, and GST-α-catenin affinity beads (Fig. 2A). To determine the specificity of the binding, a kinetic study of the binding of Fam to β-catenin was carried out. Affinity beads coated with GST, GST-AF-6 (1130-1612 aa), or GST-β-catenin were mixed with the indicated amounts of MBP-Fam (1476-1918 aa) (**Fig. 3**). The bound MBP-Fam (1476-1918 aa) was then co-eluted with the GST fusion proteins by the addition of glutathione, and the eluted MBP-Fam (1476-1918 aa) was detected by immunoblot analysis with the anti-MBP antibody. The apparent *Kd* values for the binding of MBP-Fam (1476-1918 aa) to GST-AF-6 (1130-1612 aa) or GST-β-catenin were estimated to be about 880 nM or 1180 nM under

the conditions, respectively (**Fig. 4**).

Next, I examined which domain of β-catenin interacts with Fam using various deletion mutants of MBP-β-catenin (**Fig. 5**). Affinity beads coated with GST or GST-Fam (1476-1918 aa) were mixed with deletion mutants of MBP-β-catenin, and the interacting proteins were then co-eluted with GST fusion proteins by the addition of glutathione. MBP-β-catenin (1-781 aa), (1-535 aa), and (184-535 aa) bound to GST-Fam (1476-1918 aa), but MBP-β-catenin (1-183 aa) and (536-781 aa) did not. MBP-β-catenin (1-781 aa), (1-535 aa), and (184-535 aa) did not bind to control GST (**Fig. 6**). The Fam-binding region (184-535 aa) of β-catenin contained the armadillo repeats 3-10. This region contains the binding sites for the adenomatous polyposis coli (APC) and Axin on β-catenin (Rubinfeld *et al.* 1995; Ikeda *et al.* 1998) (**Fig. 7**). Taken together, these results indicate that Fam (1476-1918 aa) directly binds to the region containing residues 184-535 of β-catenin *in vitro*.

Stabilization of β-catenin in L cells by Fam

I have previously shown that Fam has deubiquitinating activity *in vitro* and prevents the ubiquitination of AF-6 in intact cells (Taya *et al.* 1998) (**Fig. 8**). The degradation of β-catenin is regulated by the ubiquitin-proteasome pathway (Aberle *et al.* 1997; Orford *et al.* 1997). To examine the effect of Fam on the regulation of β-catenin stabilization, I employed mouse L cells, which do not express endogenous cadherin but express a low level of β-catenin. L cells were transfected with HA-Fam-CAT including the deubiquitinating catalytic domain of Fam (1210-2410 aa) along with pSVIISRα vector containing the

neomycin resistance gene and then G418-resistant colonies were isolated. I used Fam-CAT, because I could not detect the expression of full-length Fam, probably due to its low expression level. The expression of HA-Fam-CAT was detected by immunoblot analysis with the anti-HA antibody (**Fig. 9A**). To examine whether the stabilization of β-catenin is regulated by Fam, equal amounts of total cell protein from the clones stably expressing HA-Fam-CAT or HA alone were analyzed by immunoblot analysis with the anti-β-catenin antibody (**Fig. 9B**). In the clones expressing HA-Fam-CAT, β-catenin was more strongly detected than in the clones expressing HA alone. Whereas, the level of α-catenin, GSK-3β, or Axin was not changed in the clones expressing HA-Fam-CAT (**Fig. 9B**). These results suggest that Fam functions to stabilize β-catenin in L cells.

I determined the intracellular distribution of β-catenin in L cells stably expressing HA-Fam-CAT. L cells expressing HA-Fam-CAT (clone NO. 2) and HA alone (clone NO. 1) were mixed and cultured to normalize the conditions for the immunofluorescence study. The cells were fixed and were doubly stained with mouse monoclonal antibody against HA and rabbit polyclonal antibody against β-catenin. The expression of HA-Fam-CAT was predominantly detected at dot-like structures in the cytoplasm (**Fig. 10A**). β-catenin also accumulated in the cytoplasm but not in the nucleus or at the cell-cell contact sites in L cells expressing HA-Fam-CAT (**Fig. 10B**). In the clone expressing HA-Fam-CAT, the intensity of the β-catenin staining was more than that in the clone expressing HA alone. β-catenin was partly colocalized with HA-Fam-CAT at dot-like structures in the cytoplasm. These dot-like structures may be the

endoplasmic reticulum and/or Golgi complex. Similar results were obtained in several clones stably expressing HA-Fam-CAT (data not shown). These results indicate that Fam functions to stabilize β-catenin in the cytoplasm of L cells.

To further examine whether Fam regulates the turnover of β-catenin, I performed pulse-chase analysis of β-catenin in L cells stably expressing HA-Fam-CAT. The cells expressing HA-Fam-CAT (clone NO. 1) or HA alone (clone NO. 1) were labeled for 1 h in the presence of [^{35}S]methionine and [^{35}S]cysteine and were then chased with medium containing excess non-radioactive methionine and cysteine for various periods. After the cells had been harvested, immunoprecipitation analyses with the anti-β-catenin antibody were performed. The [^{35}S]-labeled bands corresponding to labeled β-catenin were visualized with an image analyzer (**Fig. 11**). Equivalent amounts of β-catenin were immunoprecipitated with the anti-β-catenin antibody from whole cell lysates and detected by immunoblot analysis with the anti-β-catenin antibody (data not shown). The [^{35}S]-labeled β-catenin decreased with a half-life of about 5 min in the control cells (**Fig. 12**). In the clone expressing HA-Fam-CAT, β-catenin exhibited a longer half-life of about 10 min. Similar results were obtained in several clones stably expressing HA-Fam-CAT (data not shown). Taken together, these results suggest that Fam elongates the half-life of β-catenin.

I examined the effects of ALLN, proteasome inhibitor, on the cells expressing HA-Fam-CAT (clone NO. 1) or HA alone (clone NO. 1). It is well known that the peptide aldehyde ALLN (*N*-acetyl-Leu-Leu-norleucinal) inhibits the proteasome-

dependent proteolysis pathway, and this leads to an accumulation of proteins that are usually metabolized by the proteasome pathway (Coux *et al.* 1996). It has been shown that the treatment of certain cell lines with ALLN resulted in the accumulation of the higher molecular weight β-catenin (Aberle *et al.* 1997; Orford *et al.* 1997; Taya *et al.* 1998). In the absence of Fam-CAT, the ubiquitinated β-catenin was detected as a band above β-catenin by immunoblot analysis (**Fig. 13**). In the L cells expressing HA-Fam-CAT, the amount of ubiquitinated β-catenin was reduced. These results strongly suggest that Fam deubiquitinates the ubiquitinated β-catenin or inhibits the ubiquitination of β-catenin.

Discussion

Interaction of Fam and β-catenin in vivo

β-catenin accumulates at the cell-cell contact sites as a cadherin/catenin complex in the epithelial cells. I have previously shown that Fam is localized at the cell-cell contact sites of MDCKII epithelial cells (Taya *et al.* 1998). I here found that Fam interacted with β-catenin *in vitro*. I also found that β-catenin was coimmunoprecipitated with Fam from MDCKII and EL cells, whereas E-cadherin or α-catenin was not coimmunoprecipitated with a Fam/β-catenin complex (data not shown). These observations suggest that Fam forms a complex with β-catenin. The reason why E-cadherin or α-catenin was not coimmunoprecipitated is not yet known. Fam may preferentially interact with β-catenin free from a cadherin/catenin complex

or a cadherin/catenin/Fam complex may be unable to be immunoprecipitated under my conditions. I here showed that the Fam-binding site mapped to the region close to the E-cadherin-binding site of β-catenin. This may explain why E-cadherin is not coimmunoprecipitated with the Fam/ β-catenin complex. It remains to be clarified whether Fam predominantly interacts with β-catenin in cell-cell adhesions or in the cytoplasm. When β-catenin was immunoprecipitated with the anti-β-catenin antibody from MDCKII or EL cells, Fam could not be coimmunoprecipitated with β-catenin (data not shown). E-cadherin and α-catenin were coimmunoprecipitated with β-catenin under the same conditions. The reason for this result is not known at present, but the anti-β-catenin antibody may immunoprecipitate β-catenin complexed with cadherin as well as free β-catenin, but not a Fam/β-catenin complex.

Stabilization of β-catenin by Fam

The turnover of β-catenin is regulated by the ubiquitin-proteasome pathway (Aberle *et al.* 1997; Orford *et al.* 1997). In the absence of Wnt signaling, the phosphorylation of β-catenin is promoted by various molecules such as GSK-3β, APC, or Axin. It is thought that the phosphorylated β-catenin is ubiquitinated by the sequential action of three enzymes (E1/E2/E3). Recently, it has been reported that F-box/WD40-repeat proteins regulate the ubiquitination of β-catenin as E3 complex (Jiang and Struhl 1998; Marikawa and Elinson 1998; Latres *et al.* 1999; Hart *et al.* 1999). The ubiquitinated β-catenin is recognized by the proteasome and rapidly degraded. However, little is known about the

regulatory mechanism underlying the deubiquitination of β-catenin in this pathway. I here showed that the deubiquitinating enzyme Fam interacted with β-catenin both *in vivo* and *in vitro*. I also found that the β-catenin level was elevated and β-catenin exhibited a longer half-life in L cells expressing Fam. My results suggest that Fam inhibits the degradation of β-catenin. Fam may directly release ubiquitin from the ubiquitinated β-catenin (**Fig. 14B**). Further studies are necessary to address this issue and to detect efficiently the ubiquitinated β-catenin *in vitro* or *in vivo*.

As described above, I showed that Fam (1476-1918 aa) directly bound to the residues 184-535 containing the armadillo repeats 3-10 of β-catenin *in vitro*. It has been reported that APC and Axin interact with the armadillo repeats 2-10 and 2-7 of β-catenin, respectively (Rubinfeld *et al.* 1995; Ikeda *et al.* 1998). APC and Axin directly bind to β-catenin and promote the degradation of β-catenin (Kishida *et al.* 1998; Hart *et al.* 1998). This raises the possibility that Fam inhibits the binding of APC and/or Axin to β-catenin and represses the function of APC and/or Axin, because the Fam-binding site of β-catenin maps to the region close to the APC or Axin-binding site of β-catenin (**Fig. 14A**).

Effect of Fam on the Wnt/β-catenin signaling pathway

I here generated L cells stably expressing HA-Fam-CAT and showed that overexpression of Fam-CAT resulted in an elevation of β-catenin levels and elongation of the half-life of β-catenin. I had expected β-catenin to accumulate in the nucleus, however it accumulated in the cytoplasm in these cells. It is thought

that β-catenin forms a complex with the transcriptional enhancer binding factor/T cell factor (LEF/TCF) family in the cytoplasm and then the complex is translocated into the nucleus (Behrens *et al.* 1996; Molenaar *et al.* 1996). LEF/TCF proteins interact with β-catenin containing all 13 armadillo repeats (Behrens *et al.* 1996; Huber *et al.* 1996; Molenaar *et al.* 1996). As described above, this region contains the binding sites for the Fam on β-catenin. I also found that β-catenin was partly colocalized with HA-Fam-CAT at the dot-like structures, which may be the endoplasmic reticulum and/or Golgi complex, in the cytoplasm. Thus, it is possible that Fam competes with LEF/TCF for the interaction with β-catenin. Therefore, β-catenin may not bind to LEF/TCF proteins or thereby be translocated into the nucleus. It has recently been reported that presenilin-1, identified as the product of causal gene for familial Alzheimer's disease, interacts with β-catenin and is colocalized with β-catenin at the Golgi complex (Murayama *et al.* 1998; Zhang *et al.* 1998). Presenilin-1 forms a complex with β-catenin, resulting in increased β-catenin stability (Zhang *et al.* 1998). The mechanism underlying the stabilization of β-catenin by Fam in the cytoplasm may be similar to that by presenilin-1.

Acknowledgments

I am grateful to Dr. Kozo Kaibuchi for his guidance and helpful discussion. He instruct me not only science but also society. I am very happy to study under him. I am grateful

to Drs. Naoyuki Inagaki, Masato Nakafuku, Shinya Kuroda, Hiroshi Qadota, and Mutsuki Amano for helpful discussion and their teaching. I am grateful to Dr. Takaharu Yamamoto for great help and kind advice.

I thank Drs. A. Nagafuchi and S. Tsukita (Kyoto University, Japan) for kindly providing MDCKII cells, EL cells, and various constructs of E-cadherin, α-catenin and β-catenin, Dr. E. Canaani (Weizmann Institute of Science, Rehovot, Israel) for kindly providing human AF-6 cDNA, and Drs. S. Kishida and A. Kikuchi (Hiroshima University, Japan) for kindly providing the anti-GSK-3β and Axin antibodies, Drs. Masami Kanai and Stepfen A. Wood (University of Queensland, Australia) for kindly providing Fam cDNA, Dr. Keiji Tanaka (Metropolitan Institute of Medical Science, Japan) for technical instructions of hydrolysis analysis, and Dr. Akihiro Iwamatsu (Kirin Brewery Co. Ltd., Japan) for peptide sequencing.

I thank member of Kaibuchi's laboratory for collaboration and helpful discussion. I thank Momoko Maeda and Akemi Takemura for secretarial assistance.

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Figure legends

Figure 1. Confocal microscope images of confluent MDCKII cells showing the distributions of Fam, ZO-1 and β-catenin. Confluent MDCKII cells doubly stained with a rabbit polyclonal antibody against Fam (**A** and **D**) and mouse monoclonal antibodies against ZO-1 (**B**) or β-catenin (**E**). Fam immunoreactivity is shown in green, and ZO-1 and β-catenin immunoreactivities are shown in red. ZO-1 was concentrated at the apical sections (**B**), whereas β-catenin was found at more basal sections (**E**). The yellow area indicates the colocalization of Fam and ZO-1 at the apical sites (**C**), or Fam and β-catenin at the basal sites (**F**). The results are representative of three independent experiments. Bar, 10 μm.

Figure 2. Interaction of Fam with β-catenin *in vivo*. MDCKII (**A**) or EL (**B**) cells were lysed and the lysate was incubated with the anti-Fam antiserum, anti-β-catenin antiserum, or preimmuneserum. The immunocomplexes were then precipitated with protein A-Sepharose 4B, and subjected to SDS-PAGE, followed by immunoblot analysis with the anti-β-catenin antibody. The arrowheads denote the positions of β-catenin. The results are representative of three independent experiments.

Figure 3. Interaction of MBP-Fam (1476-1918 aa) with GST-β-catenin *in vitro*. Affinity beads coated with GST, GST-AF-6 (1130-1612 aa), GST-E-cadherin, GST-α-catenin, or GST-β-catenin were mixed with MBP-Fam (1476-1918 aa). The interacting proteins were eluted with GST fusion proteins by

the addition of glutathione. The eluates were subjected to SDS-PAGE, followed by immunoblot analysis with the anti-MBP-antibody. The arrowheads denote the position of MBP-Fam (1476-1918 aa). The results are representative of three independent experiments.

Figure 4. The result of the kinetic study of the binding of MBP-Fam (1476-1918 aa) to GST-AF-6 (1130-1612 aa) or GST-β-catenin. Affinity beads coated with GST-AF-6 (1130-1612 aa) (**A**), or GST-β-catenin (**B**) were mixed with various amounts of MBP-Fam (1476-1918 aa). In vitro binding assay and immunoblot analysis with the anti-MBP antibody were carried out as described above. The bound MBP-Fam (1476-1918 aa) was visualized and estimated with a densitograph. The values are means ± SEM of triplicate experiments.

Figure 5. Domain diagram and various deletion mutants of MBP-β-catenin used for the *in vitro* binding assay. To determine which domain of β-catenin interacts with Fam, I prepared various deletion mutants of MBP-β-catenin such as β-catenin (1-781 aa), (1-183 aa), (1-535 aa), (184-535 aa), or (536-781 aa).

Figure 6. Interaction of GST-Fam (1476-1918 aa) with MBP-β-catenin (184-535 aa) *in vitro*. Affinity beads coated with GST or GST-Fam (1476-1918 aa) were mixed with indicated mutants of MBP-β-catenin, and the interacting proteins were then co-eluted with GST fusion proteins by the addition of glutathione. The eluates were subjected to SDS-PAGE, followed by immunoblot

analysis with the anti-MBP-antibody. The arrowheads denote the position of MBP-β-catenin (1-781 aa), (1-535 aa), and (184-535 aa). The results are representative of three independent experiments.

Figure 7. The APC, Axin and Fam-binding site of β-catenin. Fam directly bound to the armadillo repeats 3-10 of β-catenin *in vitro*. APC and Axin interact with the armadillo repeats 2-10 and 2-7 of β-catenin. APC and Axin directly bind to β-catenin and promote the degradation of β-catenin. This raises the possibility that Fam inhibits the binding of APC and/or Axin to β-catenin and represses the function of APC and/or Axin.

Figure 8. Hydrolysis of ^{125}I-labeled Ub-PEST by Fam. The ^{125}I-labeled Ub-PEST was incubated alone or with 1.6 μg of Fam purified by GST-AF-6 (1130-1612 aa) affinity column in the absence or presence of ubiquitin-aldehyde (Ub-CHO) for 1, 3, or 6 h at 37°C. After the incubation, the samples were subjected to SDS-PAGE, followed by Coomassie Brilliant Blue staining (**A**) and by autoradiography (**B**). The release of ubiquitin from Ub-PEST (**A**) or the production of the hydrolysis of ^{125}I-labeled PEST peptides from Ub-PEST (**B**). The results shown are representative of three independent experiments.

Figure 9. Stabilization of β-catenin in L cells by Fam. L cells were transfected with HA-Fam-CAT including the deubiquitinating catalytic domain of Fam (1210-2410 aa) along with pSVIISRα vector containing the neomycin resistance gene and then G418-resistant colonies were isolated. Equal amounts

of total protein (25 μg) from each clone expressing HA alone (2 clones) or HA-Fam-CAT (3 clones) were analyzed by immunoblot analysis with indicated antibodies. (**A**) The expression of HA-Fam-CAT was detected using the anti-HA antibody. (**B**) The amount of β-catenin, α-catenin, GSK-3β, or Axin was detected using these antibodies. The results are representative of three independent experiments.

Figure 10. Distribution of β-catenin in L cells stably expressing HA-Fam-CAT. L cells expressing HA-Fam-CAT and HA alone were mixed and cultured for 48 h. The cells were fixed with 4% paraformaldehyde in PBS for 5 min. The fixed cells were doubly stained with mouse monoclonal antibody against HA (**A**) and rabbit polyclonal antibody against β-catenin (**B**). The expression of HA-Fam-CAT and the accumulation of β-catenin were examined using a laser scanning confocal microscope. The arrowheads denote the expression cells of HA-Fam-CAT and the arrows denote those of HA alone. The results are representative of three independent experiments. Bar, 20 μm.

Figure 11. Elongation of the β-catenin half-life in L cells stably expressing HA-Fam-CAT. L cells expressing HA-Fam-CAT or HA alone were labeled for 1 h in the presence of [^{35}S]methionine and [^{35}S]cysteine and were then chased with medium containing excess non-radioactive methionine and cysteine for the indicated times. After the cells had been lysed, immunoprecipitation analyses with the anti-β-catenin antibody were performed. The [^{35}S]-labeled bands corresponding to labeled β-catenin were visualized with an

image analyzer.

Figure 12. Quantitative analysis of pulse-chase kinetics of β-catenin. The values are the percentage of the amount of β-catenin at 0 min (100%) and are means ± SEM of four experiments. ○, L cells expressing HA-Fam-CAT; ●, HA alone

Figure 13. Effect of proteasome inhibitor on the ubiquitinated β-catenin in L cells stably expressing HA-Fam-CAT. L cells stably expressing HA-Fam-CAT or HA alone were treated with the 25 μM ALLM or ALLN for 4 h. Equal amounts of total protein (25 μg) from the cells expressing HA alone or HA-Fam-CAT were analyzed by immunoblot analysis with the anti-β-catenin antibody. The arrowheads denote the position of endogenous β-catenin and the ubiquitinated β-catenin, respectively. The results are representative of three independent experiments.

Figure 14 Model for the stabilization of β-catenin by Fam. In the absence of the Wnt signaling pathway, the phosphorylation of β-catenin is promoted by various molecules such as GSK-3β, APC, or Axin. It has been thought that the phosphorylated β-catenin is ubiquitinated by the sequential action of three enzymes (E1/E2/E3). The ubiquitinated β-catenin is recognized by the proteasome and rapidly degraded. In this study, I suggest that Fam stabilizes β-catenin. Here I show two alternative hypotheses. (**A**) Fam may inhibit the binding of APC and/or Axin to β-catenin and repress the function of APC or Axin. (**B**) Fam may directly release ubiquitin from the ubiquitinated β-catenin. Ub; ubiquitin, E1; Ub activating enzyme, E2; Ub

carrier protein, E3; Ub-protein ligases.

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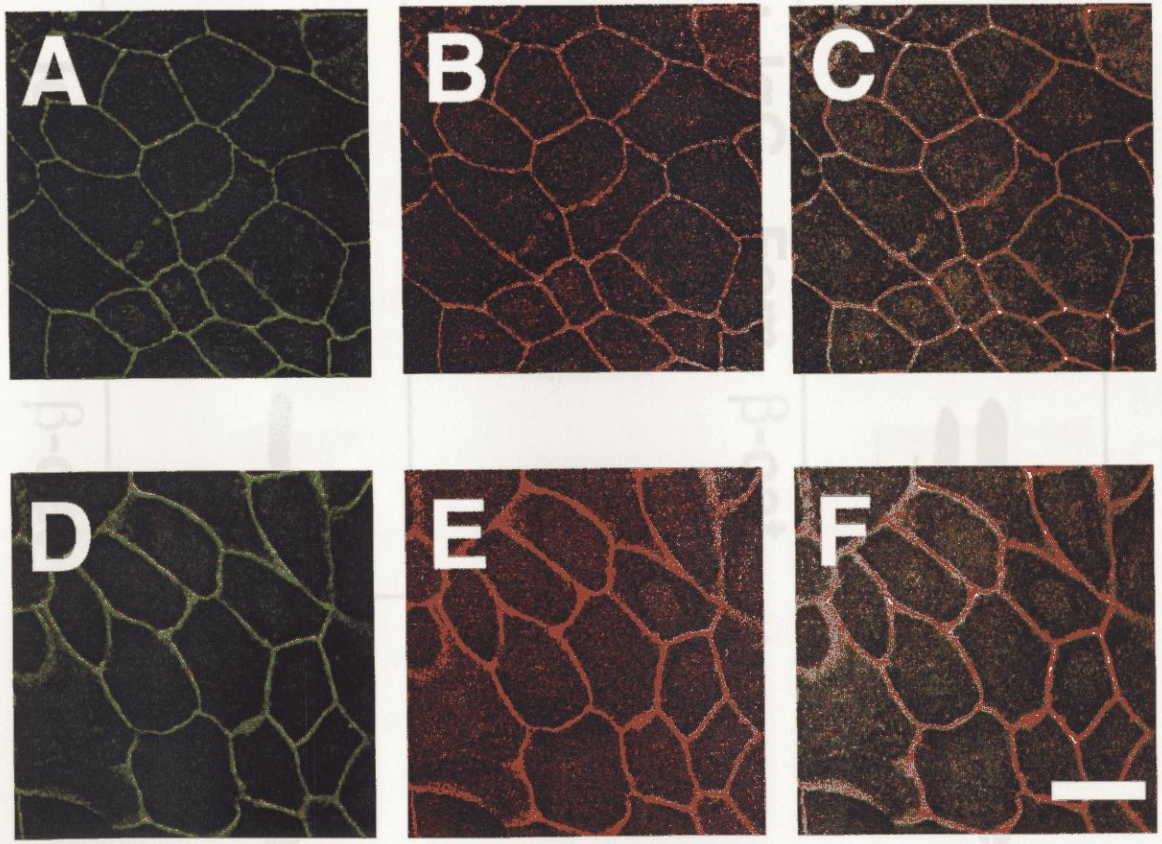
Figure 1



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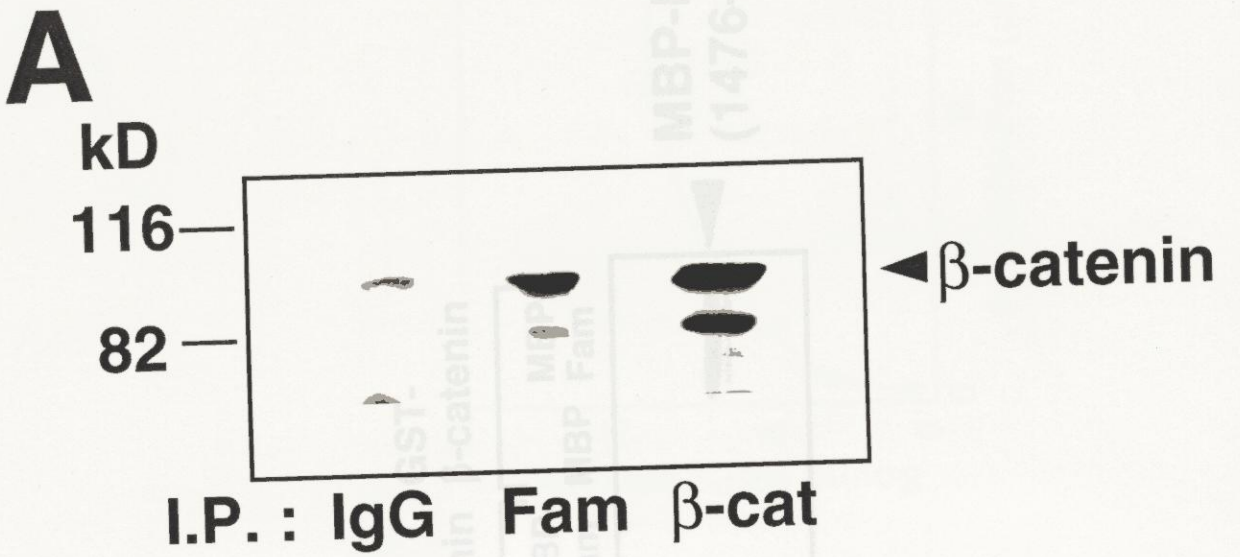
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kD
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82
β-catenin
I.P. : IgG Fam β-cat

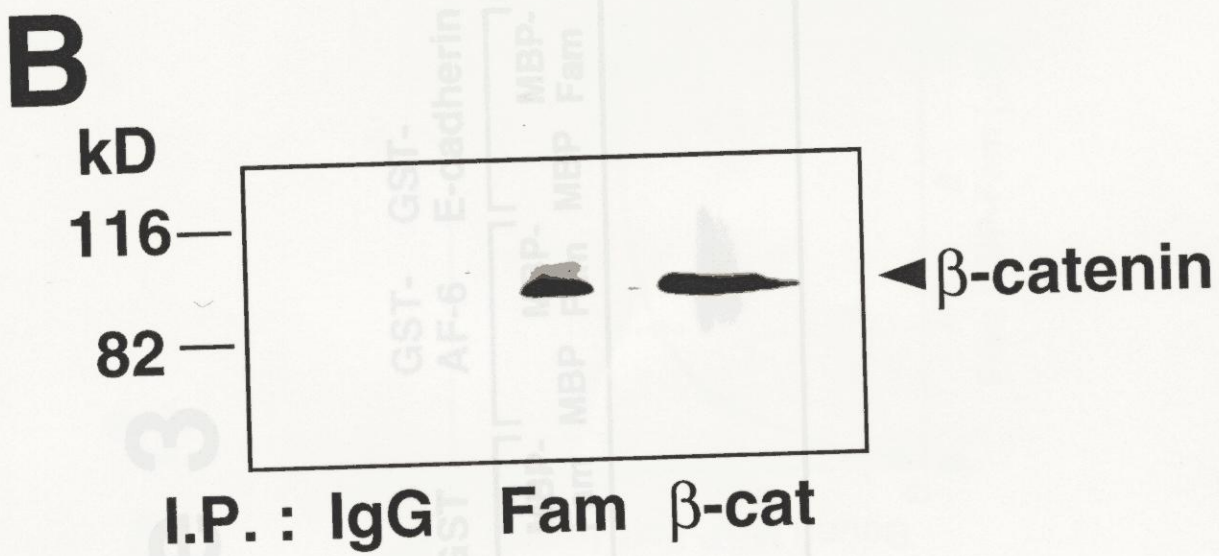
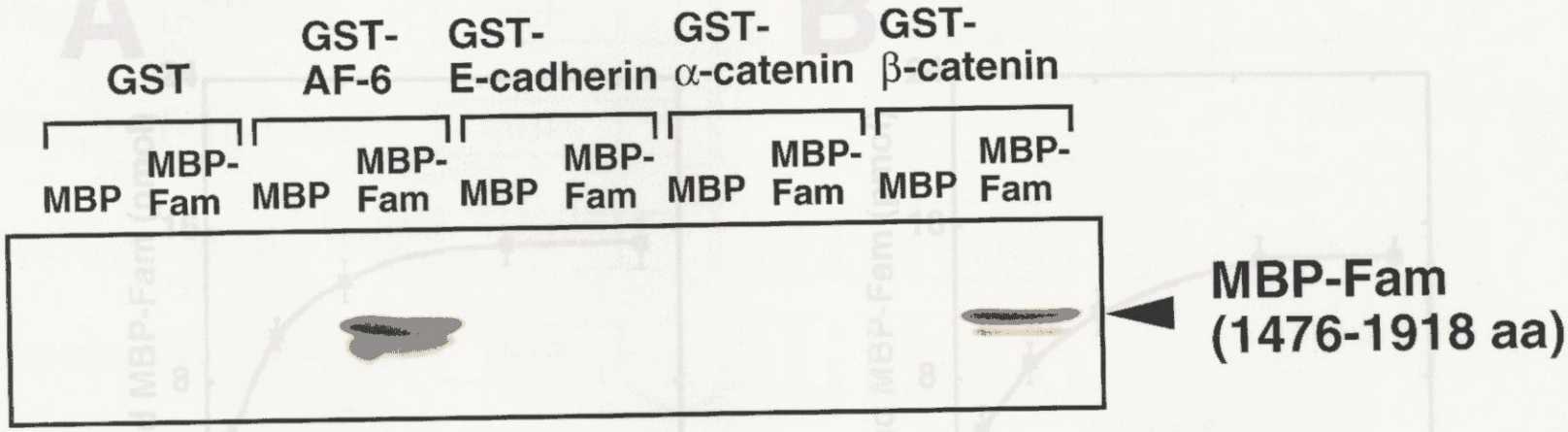

B
kD
116
82
β-catenin
I.P. : IgG Fam β-cat

Figure 3


GST
GST-
AF-6
GST-
E-cadherin
GST-
α-catenin
GST-
β-catenin
MBP
MBP-
Fam
MBP
MBP-
Fam
MBP
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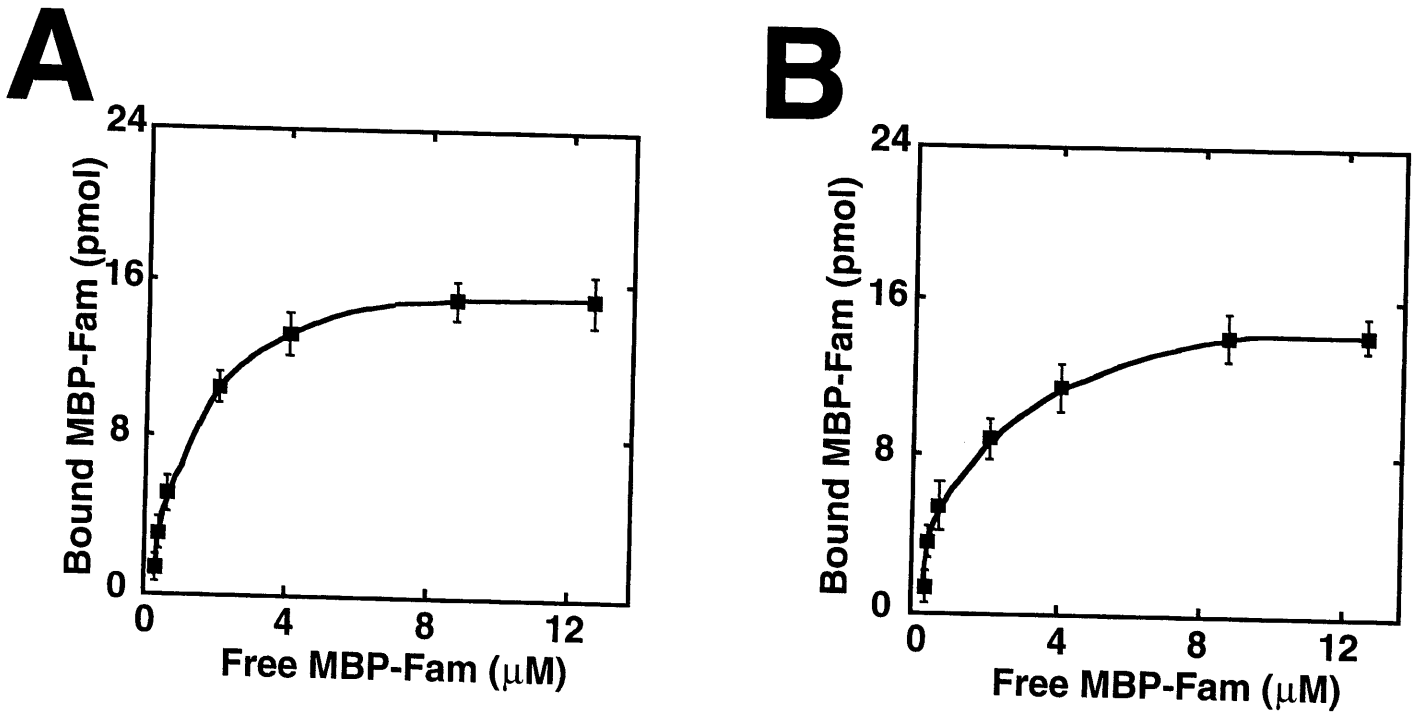
Fam
MBP
MBP-
Fam
MBP
MBP-
Fam
MBP-Fam
(1476-1918 aa)

Figure 4



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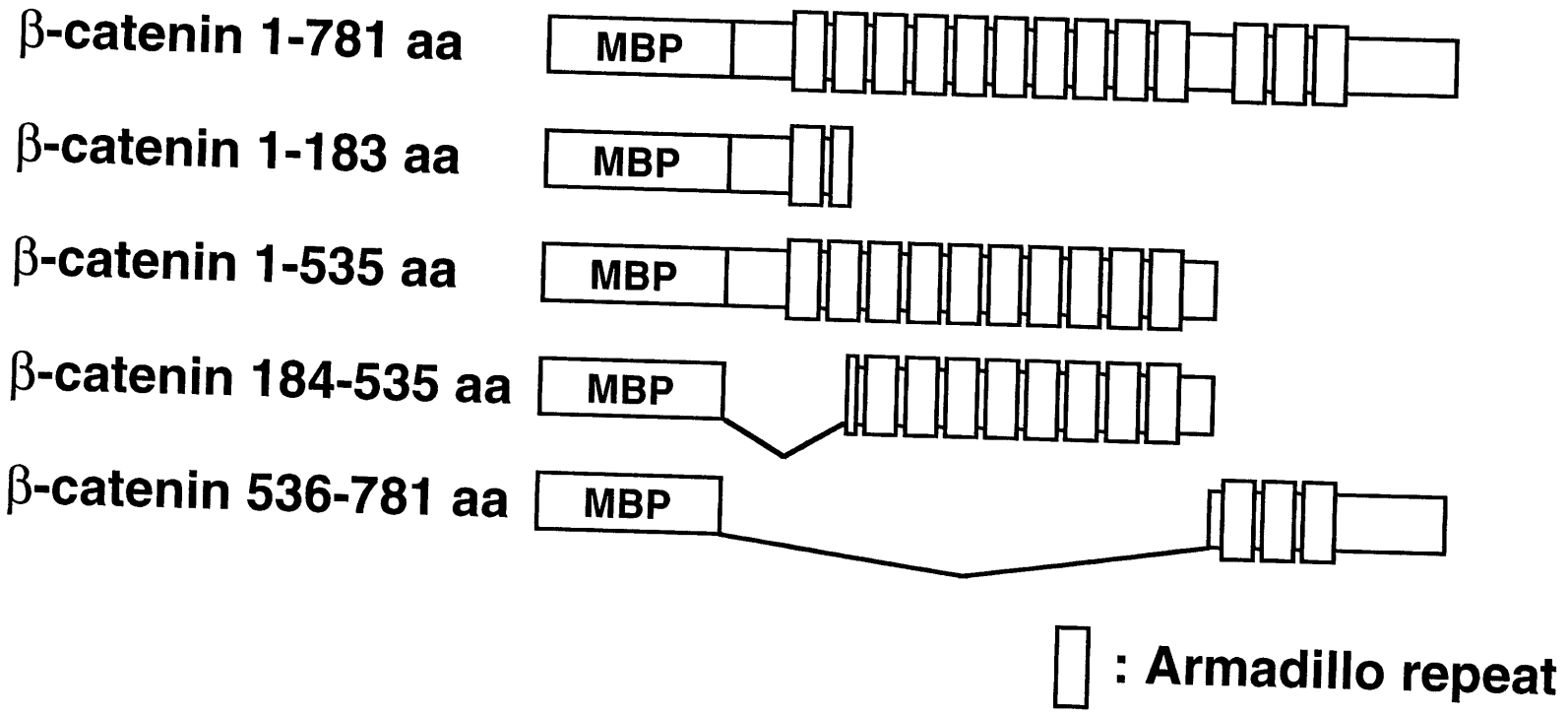

β-catenin 1-781 aa
MBP
β-catenin 1-183 aa
MBP
β-catenin 1-535 aa
MBP
β-catenin 184-535 aa
MBP
β-catenin 536-781 aa
MBP
: Armadillo repeat

Figure 6

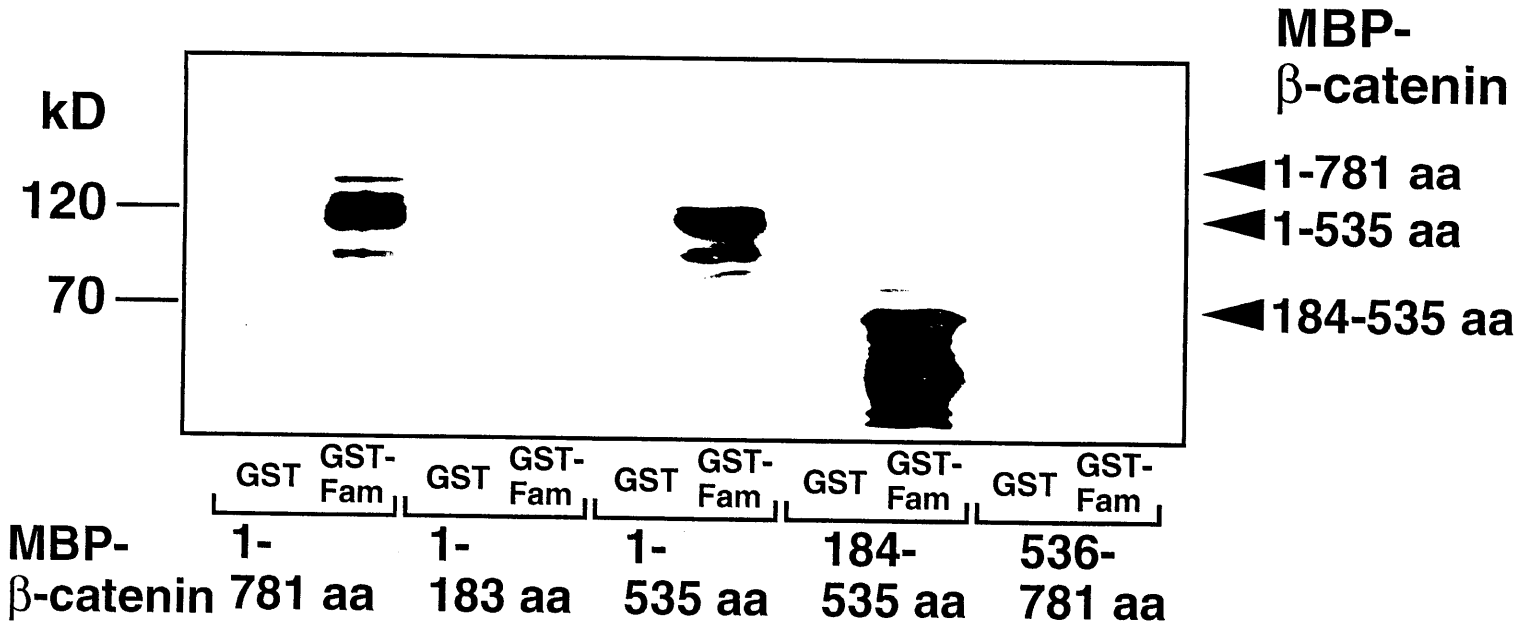

kD
120
70
MBP-
β-catenin
1-781 aa
1-535 aa
184-535 aa
GST
GST-Fam
GST
GST-Fam
GST
GST-Fam
GST
GST-Fam
GST
GST-Fam
MBP-
β-catenin
1-
781 aa
1-
183 aa
1-
535 aa
184-
535 aa
536-
781 aa

Figure 7

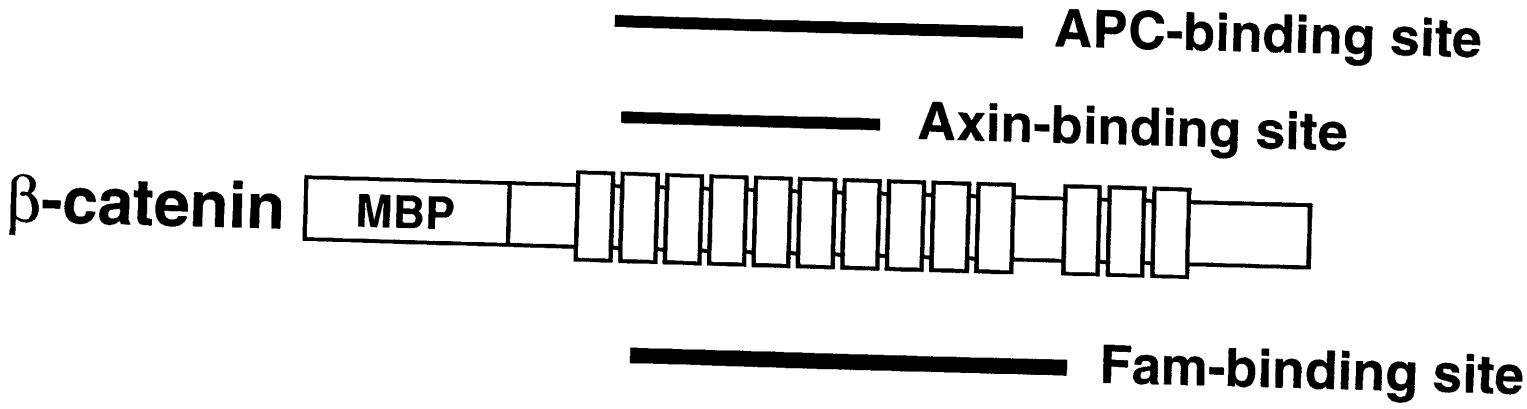
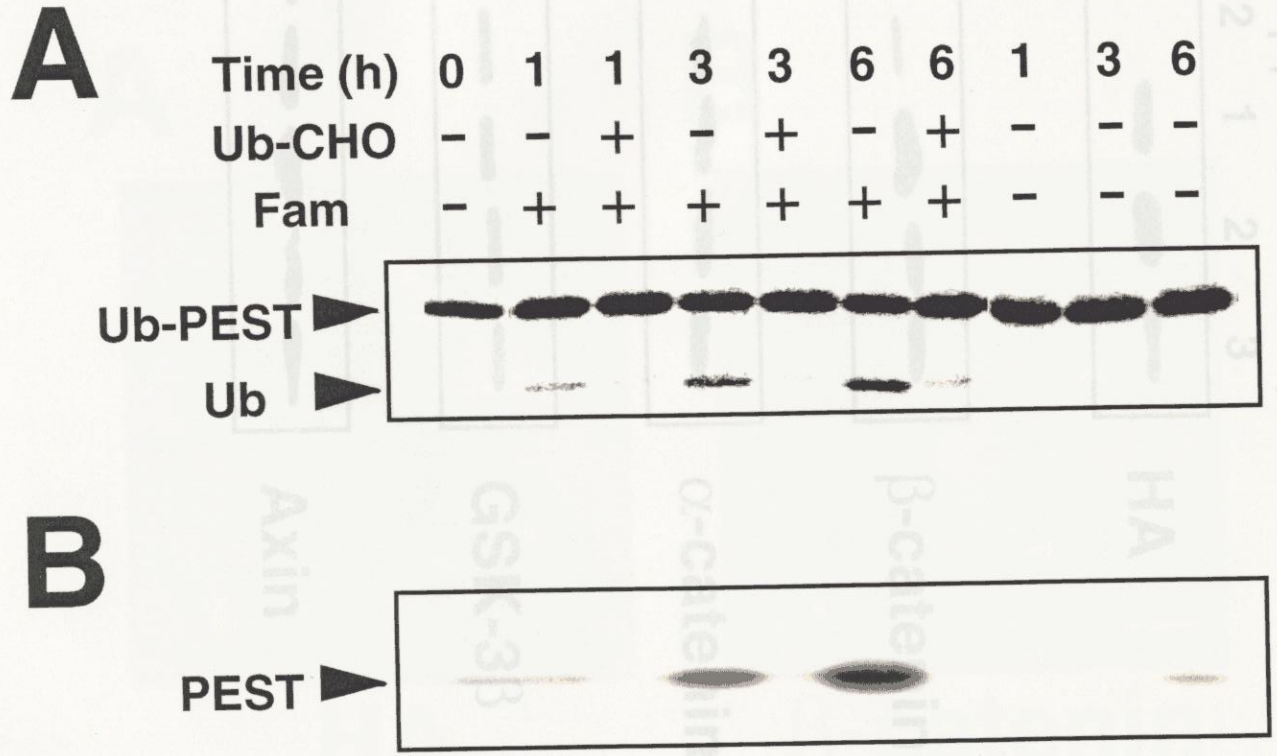

APC-binding site
Axin-binding site
β-catenin
MBP
Fam-binding site

Figure 8



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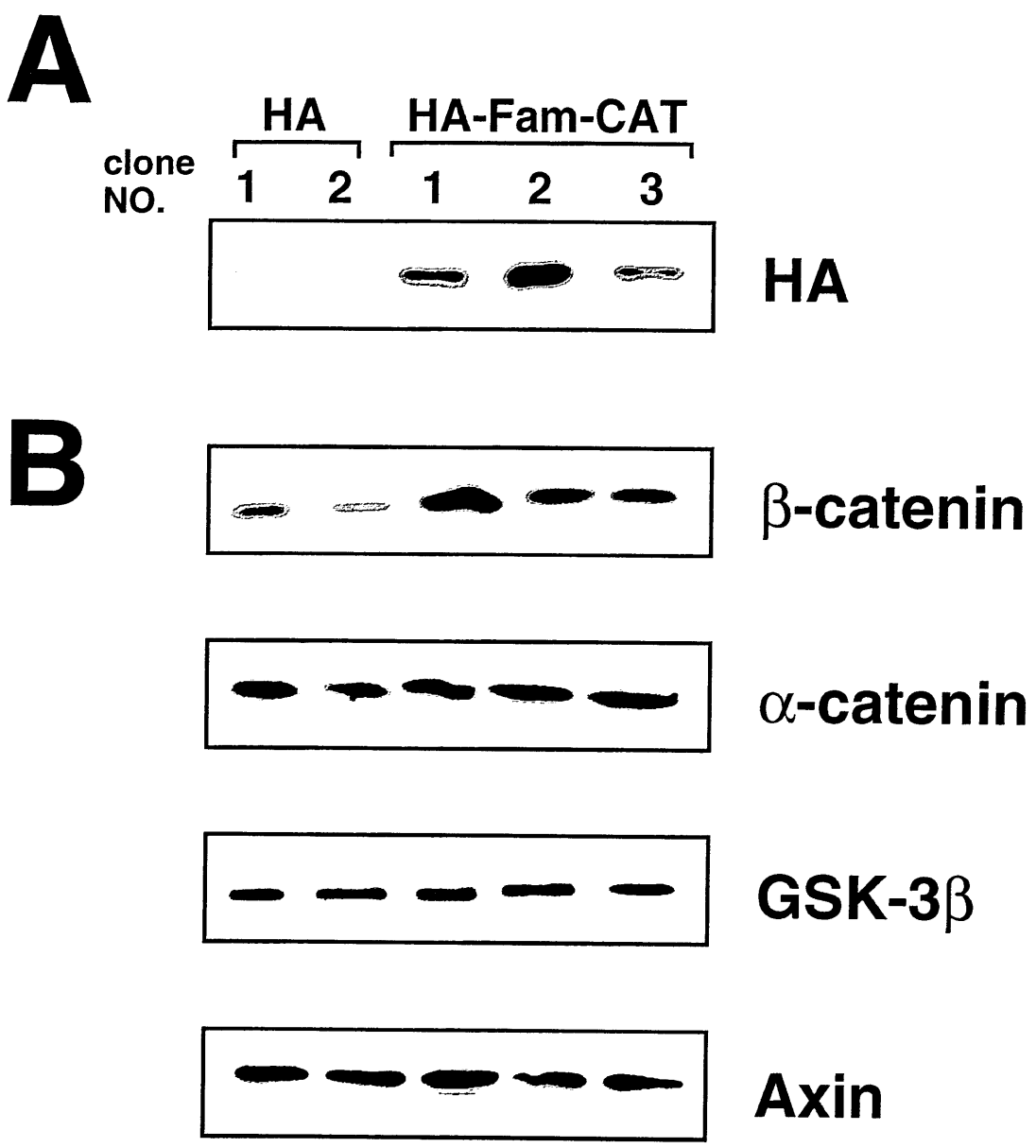
A
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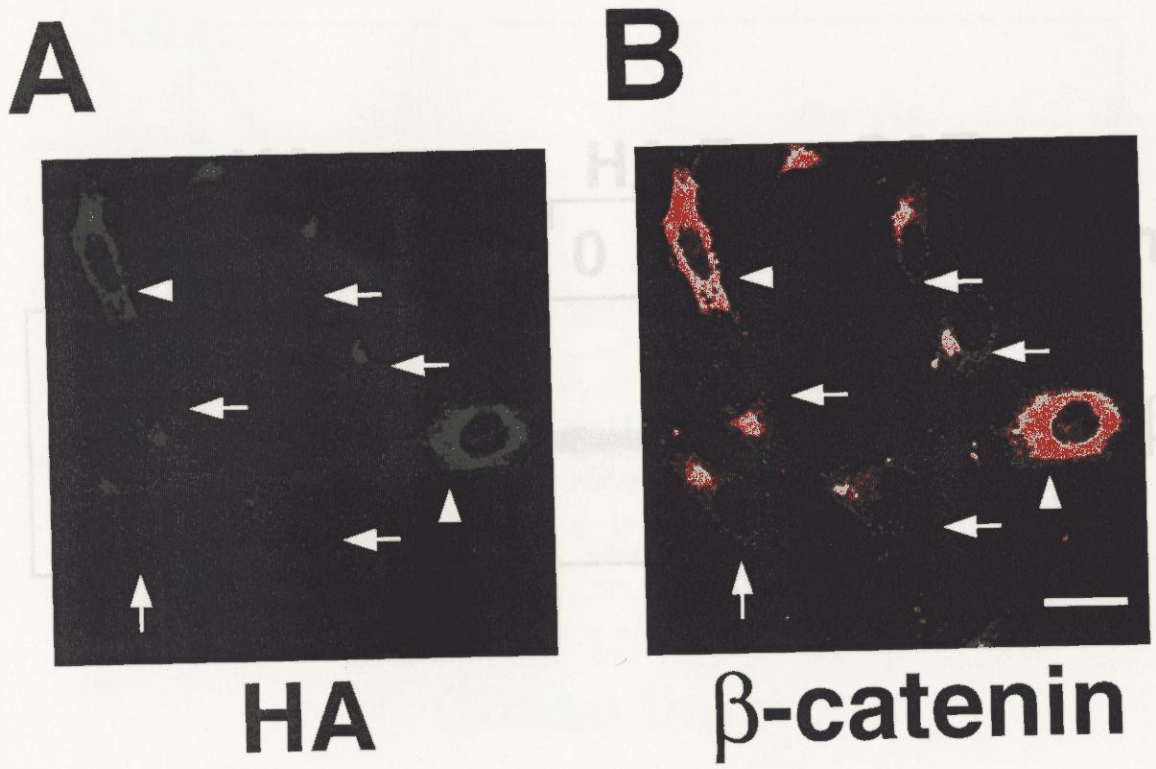
clone NO.
1
2
1
2
3
HA
B
β-catenin
α-catenin
GSK-3β
Axin

Figure 10



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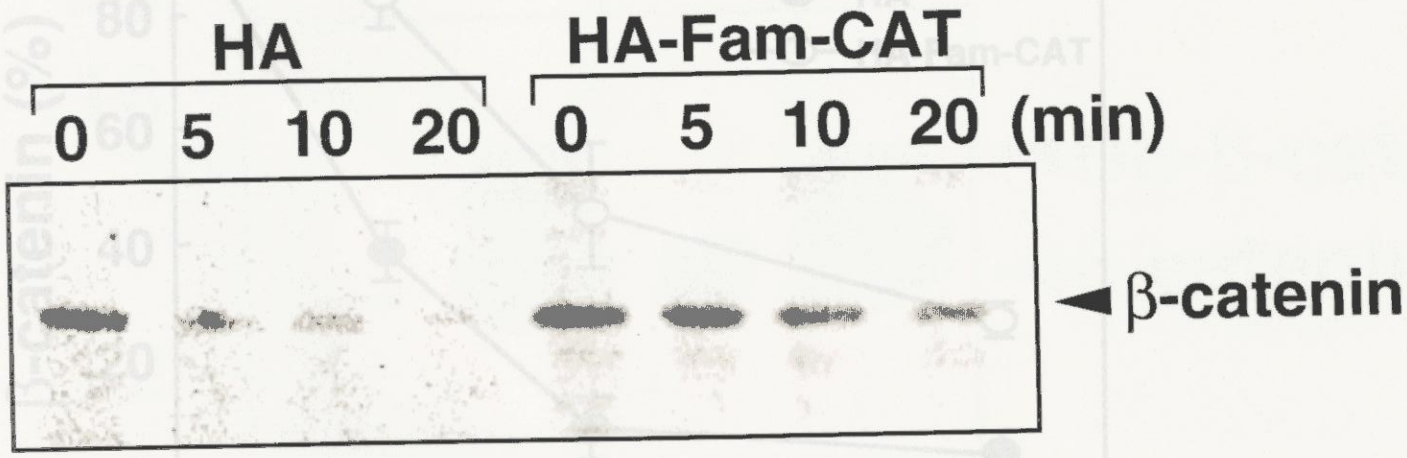


Figure 12

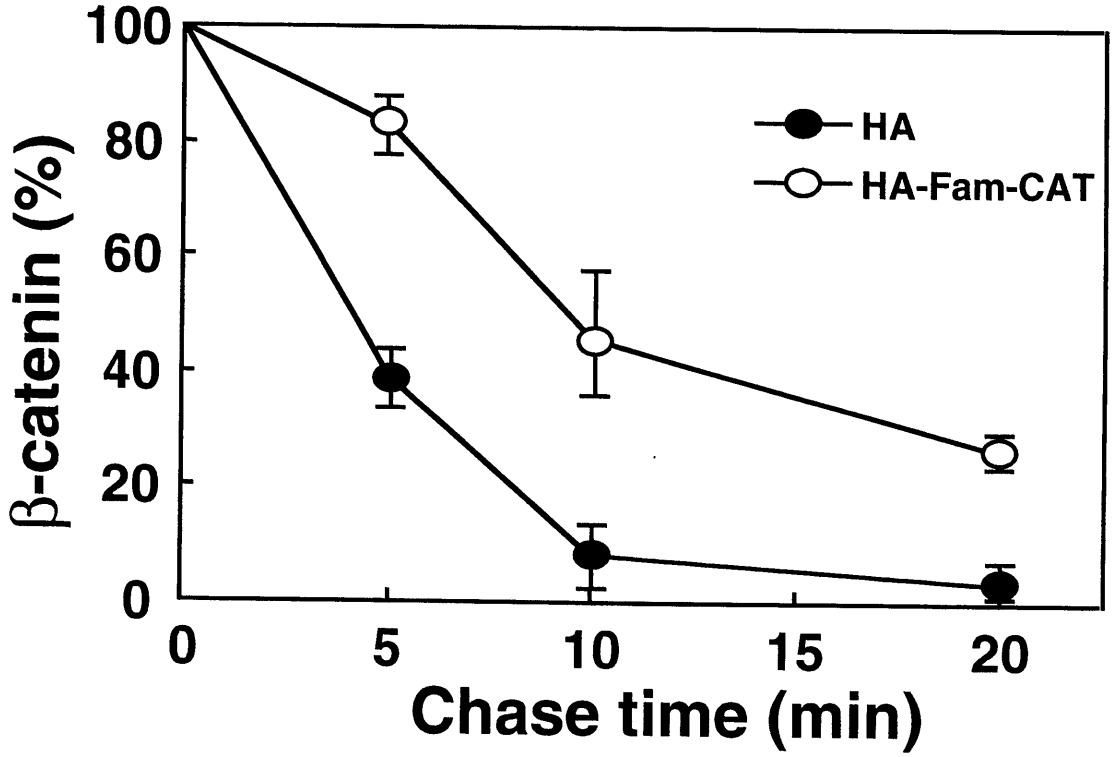

100
80
60
40
20
0
β-catenin (%)
0
5
10
15
20
Chase time (min)
HA
HA-Fam-CAT

Figure 13

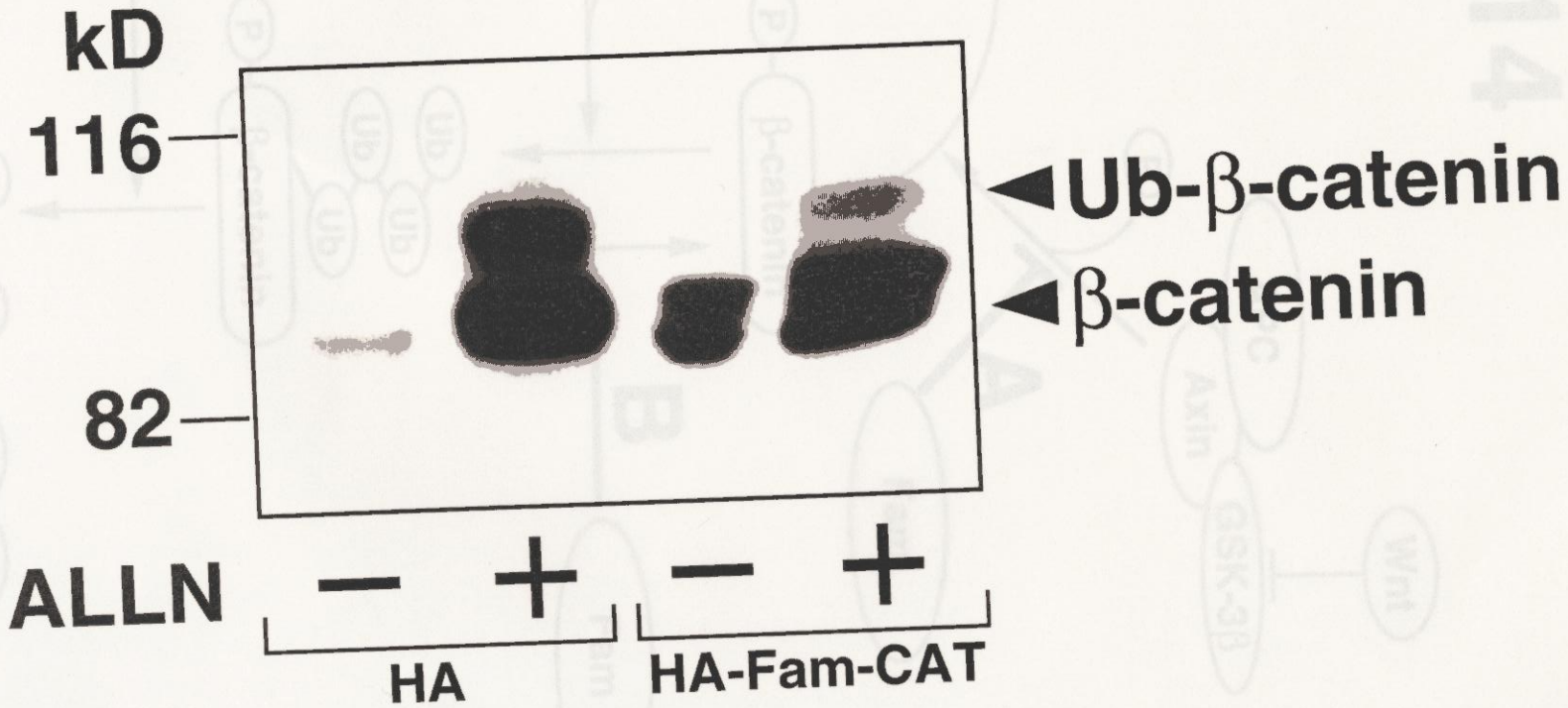
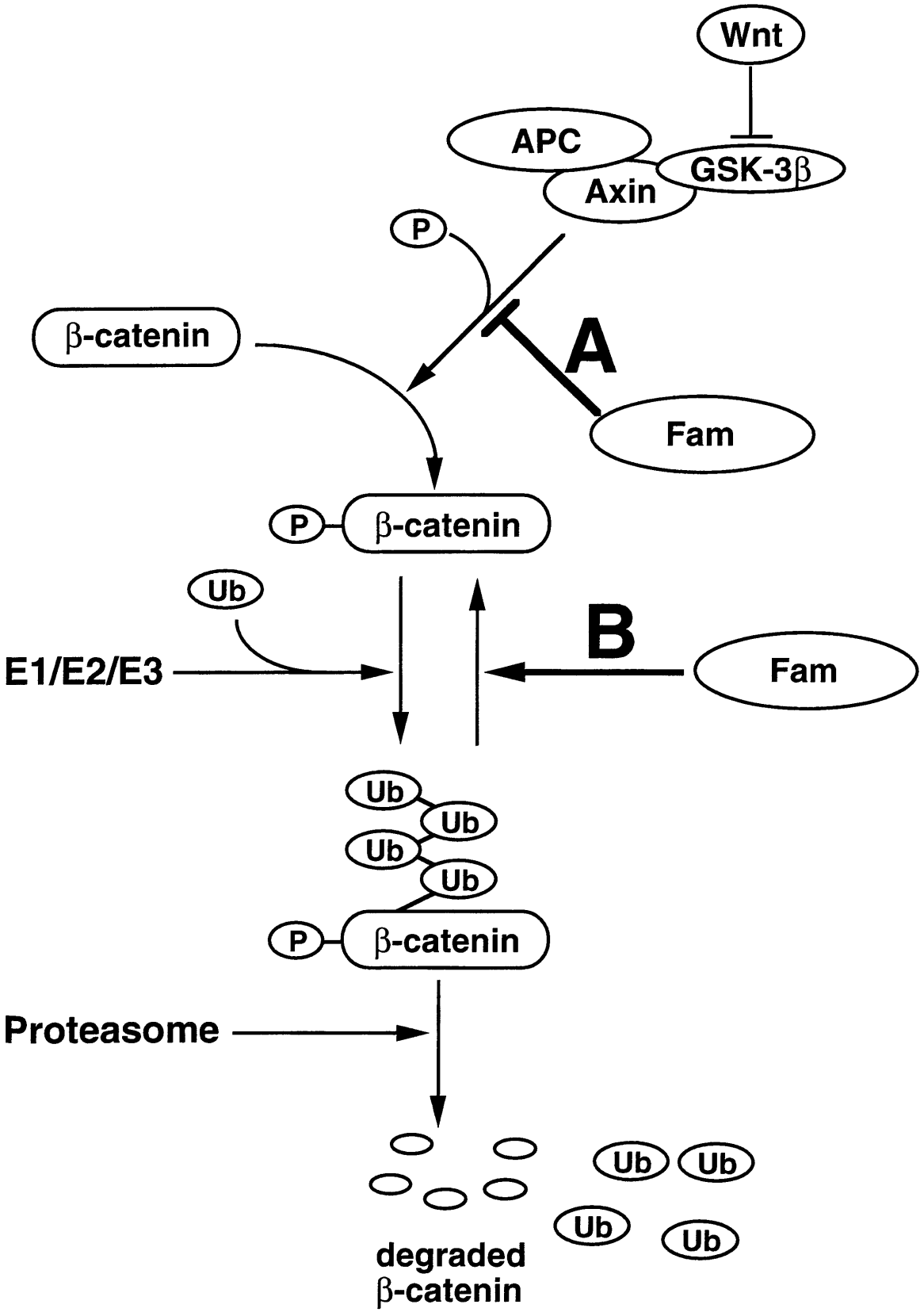


Figure 14


Wnt
APC
Axin
GSK-3β
P
β-catenin
A
Fam
P
β-catenin
Ub
E1/E2/E3
B
Fam
Ub
Ub
Ub
Ub
P
β-catenin
Proteasome
Ub
Ub
Ub
Ub
degraded
β-catenin