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Cellular FLIP Inhibits β-Catenin Ubiquitylation and Enhances Wnt Signaling

Mikihiko Naito,[1]†* Ryohei Katayama,[1]† Toshiyasu Ishioka,[1] Akiko Suga,[2] Kohei Takubo,[1] Masahiro Nanjo,[1] Chizuko Hashimoto,[1] Masanori Taira,[2,3] Shinji Takada,[4,5] Ritsuko Takada,[4,5] Masatoshi Kitagawa,[6] Shu-Ichi Matsuzawa,[7] John C. Reed,[7] and Takashi Tsuruo[1,8]

Institute of Molecular and Cellular Biosciences[1] and School of Science,[2] The University of Tokyo, Bunkyo-ku, and Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Toshima-ku,[8] Tokyo, CREST, Japan Science and Technology Corporation, Kawaguchi, Saitama,[3] Center for Integrative Bioscience, Okazaki National Research Institute, Okazaki, Aichi,[4] Kondoh Differentiation Signaling Project, Exploratory Research for Advanced Technology, Japan Science and Technology Corporation, Sakyo-ku, Kyoto,[5] and Hamamatsu University School of Medicine, Handayama, Hamamatsu,[6] Japan, and The Burnham Institute, La Jolla, California[7]

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Cellular FLIP (cFLIP) is a close homologue of caspase 8 without caspase activity that inhibits Fas signaling. The cFLIP protein is often expressed in human tumors and is believed to suppress antitumor immune responses involving the Fas system. Here, we report that a long form of cFLIP (cFLIP-L) inhibits β-catenin ubiquitylation and increases endogenous cytosolic β-catenin, which results in translocation of β-catenin into nuclei and induction of β-catenin-dependent gene expression in cFLIP-L-expressing cells. When cells stably expressing cFLIP-L were stimulated with Wnt3a, enhanced Wnt signaling was observed compared with the control cells. Conversely, depletion of endogenous cFLIP results in reduced Wnt signaling. Furthermore, cFLIP-L increases secondary-body axis formation when coinjected with suboptimal doses of β-catenin into early *Xenopus* embryos. Down-regulation of FADD by RNA-mediated interference abolishes the β-catenin-dependent gene expression induced by cFLIP-L. These results indicate that cFLIP-L, in cooperation with FADD, enhances canonical Wnt signaling by inhibiting proteasomal degradation of β-catenin, thus suggesting an additional mechanism involved with tumorgenesis, in addition to inhibiting Fas signaling.

Ligation of death receptors, such as Fas, triggers apoptosis in many types of cells (28), which is inhibited by cellular FLIP (cFLIP, also known as I-FLICE, FLAME-I, Casper, CASH, MRIT and Usurpin) (8, 11, 13, 16, 34, 40, 41, 44). The long form of cFLIP (cFLIP-L) is highly homologous to caspase 8, containing two death effector domains (DED) and a caspase-like domain at the amino and carboxy termini, respectively. cFLIP-L, however, does not have caspase activity due to the lack of a conserved cysteine residue in the caspase-like domain. Upon death receptor ligation, cFLIP-L is recruited to the death receptor complex, together with FADD and caspase 8, and inhibits apoptosis signaling. cFLIP-L is expressed in various cancers (2, 16, 27, 36, 43, 44), which suggests a role for cFLIP-L in protecting cancer cells from cellular immunity using the Fas system (5, 6, 25).

cFLIP, however, does not always inhibit apoptosis signaling but also mediates growth signals in some cases. Under conditions in which the proliferation of CD3-activated human T lymphocytes is increased by recombinant Fas ligand, cFLIP-L interacts with tumor necrosis factor receptor-associated factors 1 and 2, as well as the kinases RIP and Raf-1, resulting in the activation of the NF-κB and ERK signaling pathways (17). The ability of cFLIP-L to switch Fas-mediated glucose signaling from a death signal into a proliferation signal in human pancreatic β cells was also reported (24).

The Wnt signal transduction pathway plays a critical and evolutionarily conserved role during embryogenesis (3, 10, 46). The Wnt signal is mediated by β-catenin, a transcription factor that is normally degraded by the ubiquitin-proteasome system in cytosol. Phosphorylation of β-catenin by a large protein complex involving adenomatous polyposis coli (APC) protein, Axin, and glycogen synthase kinase 3β (GSK3β) initiates the ubiquitylation and proteasomal degradation of β-catenin (15, 49). Upon Wnt signaling, phosphorylation of β-catenin is inhibited, which results in the accumulation and translocation of β-catenin into nuclei, thereby inducing the expression of several genes, such as c-*myc* and the cyclin D gene. Mutations in APC, Axin, and β-catenin genes resulting in abolished β-catenin ubiquitylation are found in many human cancers (4, 9, 20, 26, 30, 32, 35, 37), indicating that inappropriate activation of Wnt signaling plays an important role in human cancers (31, 33).

In this paper, we report that cFLIP-L inhibits β-catenin ubiquitylation and enhances Wnt signaling, which suggests an additional mechanism involved in tumorgenesis, in addition to inhibiting apoptosis signaling.

* Corresponding author. Mailing address: Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Phone: 81-3-5841-7864. Fax: 81-3-5841-8487. E-mail: mnaito@iam.u-tokyo.ac.jp.

† M.N. and R.K. contributed equally to this work.

MATERIALS AND METHODS

Plasmids. Human cFLIP-L and a short splicing variant of cFLIP (cFLIP-S) were amplified by PCR from a Jurkat cDNA library and subcloned into pcDNA-based mammalian expression vectors (Invitrogen). For deletion mutant constructs, DNA sequences corresponding to different regions of cFLIP-L were

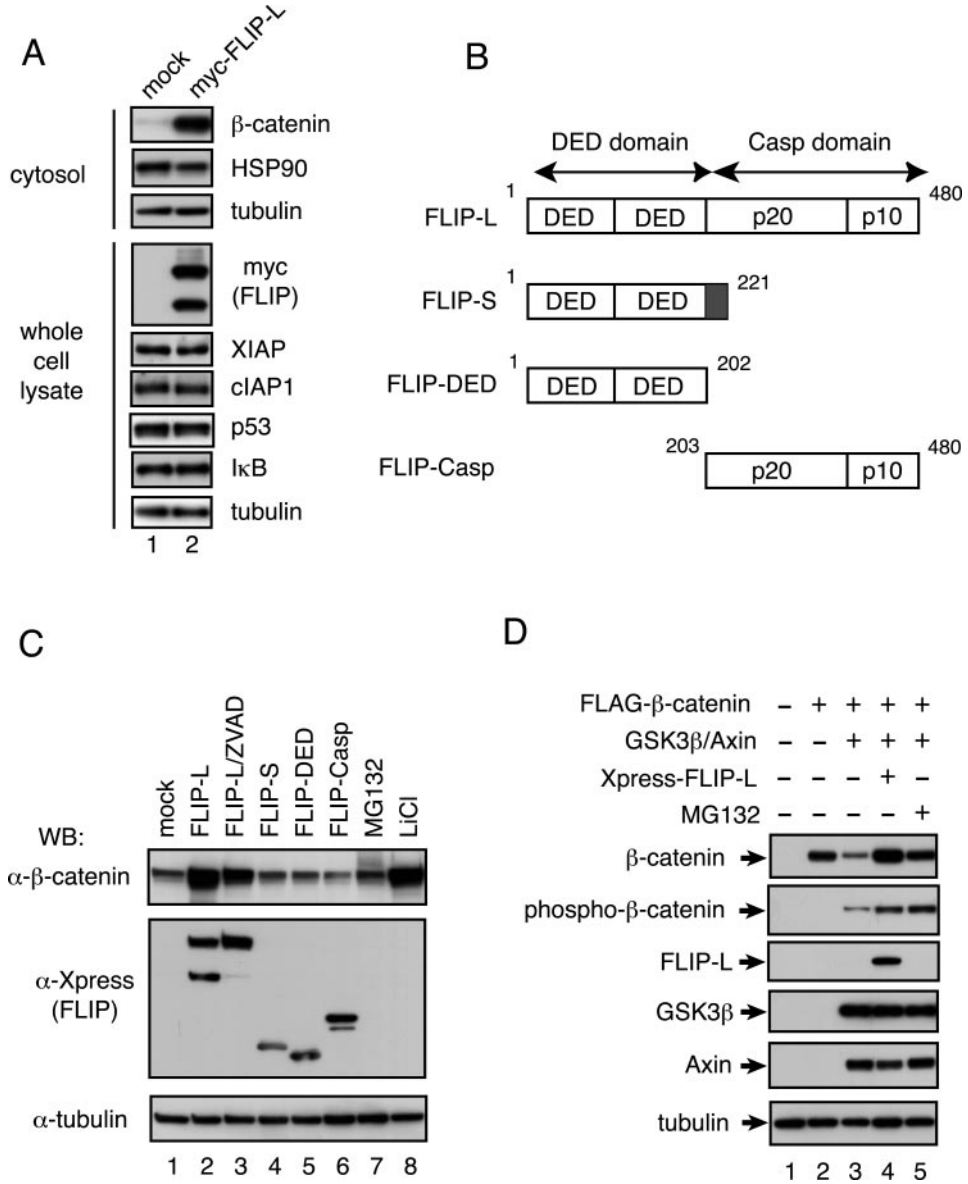

FIG. 1. cFLIP-L increases cytosolic β-catenin. (A) Increase in endogenous cytosolic β-catenin by expression of cFLIP-L. Whole-cell lysates and cytosolic fractions were prepared from 293T cells that had been transfected with 4.5 μg of Myc-tagged cFLIP-L in 60-mm-diameter dishes and were analyzed by Western blotting with the indicated antibodies. (B) Schemas of the cFLIP constructs used in this study. (C) cFLIP-L, but not cFLIP-S, increases endogenous cytosolic β-catenin. 293T cells were transfected with various FLIP constructs shown in panel B or treated with MG132 (20 μM; 6 h) and LiCl (30 mM; 12 h), and the cytosolic fractions were analyzed by Western blotting (WB) with the indicated antibodies. (D) cFLIP-L inhibits β-catenin degradation promoted by GSK3β-dependent phosphorylation. HT1080 cells in six-well plates were transfected with a total of 4 μg of plasmid DNAs encoding FLAG-tagged β-catenin (1 μg), GSK3β (1 μg)/Axin (1 μg), and cFLIP-L (1 μg), and the cytosolic fractions were analyzed by Western blotting. +, present; −, absent.

amplified by PCR from the above-mentioned constructs and subcloned into the respective expression vectors. All constructs generated from PCR products were sequenced.

Transfection, immunoprecipitation, and immunoblotting. HT1080 cells and 293T cells were transfected with various plasmid DNAs by lipofection (FuGENE [Roche]; Lipofectamine 2000 [Invitrogen]). In some cases, cells were treated with benzyloxycarbonyl-valinyl-alanyl-aspartate-fluoromethyl ketone (ZVAD) (50 μM) to inhibit apoptosis induced by cFLIP-L expression. The cells were lysed in 0.1% Triton X-100 or by repeated freezing-thawing in hypotonic buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, 0.1 mM EDTA) and centrifuged at 100,000 × *g* for 1 h to prepare the cytosolic fraction. We used the following antibodies for immunoprecipitation and immunoblot analysis: anti-FLIP (NF-6; generously provided by M. Peter); anti-β-catenin, anti-HSP90, and anti-FADD (Transduction Laboratory); anti-XIAP, anti-caspase 8, anti-Fas, and polyclonal anti-Myc (MBL); antihemagglutinin (anti-HA) and monoclonal anti-Myc (Roche); anti-cIAP1 (R&D); anti-p53 (Calbiochem); anti-IκB (Santa Cruz); antitubulin (Cosmobio); anti-phospho-β-catenin (Cell Signaling); anti-GFP (Clontech); anti-Xpress (Invitrogen); and anti-FLAG (Sigma).

Isolation of cFLIP stable transfectant clones. HT1080 cells were transfected with pcDNA3.1-His-cFLIP-L/S or empty vector. After 24 h, the cells were selected with 300 μg of G418/ml for 2 weeks, and the surviving colonies were cloned. The transfectants were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 μg of kanamycin/ml, and 150 μg of G418/ml at 37°C in a humidified atmosphere of 5% CO_2.

RT-PCR. Total RNAs were isolated from HT1080 cells and transformants with an RNA minikit (Qiagen). Reverse transcription (RT) was carried out with oligo(dT)16 primer at 42°C for 1 h, and fragments of β-catenin and GAPDH

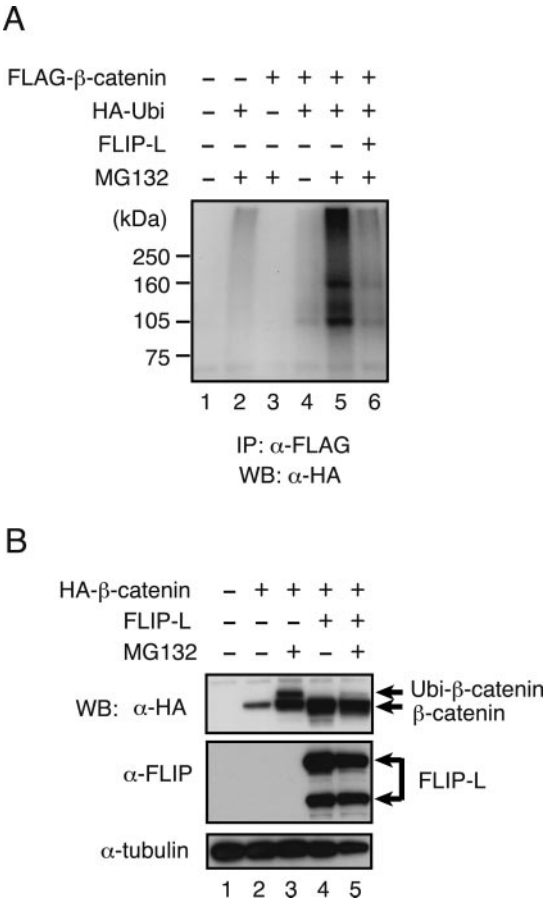

FIG. 2. Inhibition of β-catenin ubiquitylation by cFLIP-L. (A) 293T cells in 100-mm-diameter dishes were transfected with a total of 12 μg of plasmid DNA, as indicated (FLAG–β-catenin, 4 μg; HA-Ubi, 4 μg; cFLIP-L, 4 μg), and the cytosolic fractions were immunoprecipitated (IP) with anti-FLAG antibody, followed by Western blot (WB) analysis with horseradish peroxidase-conjugated anti-HA antibody. (B) 293T cells in six-well plates were transfected with 1 μg of HA–β-catenin and 1 μg of cFLIP-L for 36 h. In some wells, cells were treated with 10 μM MG-132 for 6 h before being harvested. Cytosolic fractions were analyzed with the indicated antibodies. +, present; −, absent.

(glyceraldehyde-3-phosphate dehydrogenase) were amplified by PCR using the following primer sets: β-catenin, 5′-CTC CCA AGT CCT GTA TGA GTG-3′ and 5′-CAA GGT CCC AGC GGT ACA ACG-3′; GAPDH, human GAPDH control amplimer set (Clontech).

Immunofluorescence microscopy. HT1080 cells that had been transfected with Myc-tagged cFLIP-L were fixed in 4% paraformaldehyde and treated with 0.1% Triton X-100–3% bovine serum albumin in phosphate-buffered saline for 30 min. The cells were then incubated with anti-Myc (rabbit polyclonal) and anti-β-catenin (mouse monoclonal) as primary antibodies and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G and rhodamine-conjugated anti-mouse immunoglobulin G as secondary antibodies. The cells were observed with an Olympus IX70 microscope equipped with a charge-coupled device camera.

Luciferase assay. Cells were transfected with a total of 2 μg of various combinations of plasmids: 125 ng of reporter plasmid (TOP-TK-Luc or FOP-TK-Luc; from H. Clevers), 12.5 ng of internal control (pRL-TK; Promega), 1 μg of cFLIP expression vector (pcDNA4-His), and empty pcDNA4-His vector as stuffer. The conditioned medium containing Wnt3a was prepared from L cells that had been transfected with the Wnt3a gene as described previously (39). Luciferase activities were measured 24 h after transfection using the Dual-Luciferase Reporter Assay System (Promega).

RNA interference. Oligonucleotides specific for human cFLIP (CCCTCACC TTGTTTCGGAC), mutated FLIP (CCCTCACCTTGTGTCGGAC), FADD (GAAGACCTGTGTGCAGCAT), mutated FADD (GAAGAACTGTGTGAA GCAT), and caspase 8 (GCAAGAACCCATCAAGGAT) were synthesized, and double-stranded RNA oligonucleotides were annealed in vitro before transfection using Lipofectamine 2000 (Gibco-BRL). Oligonucleotides designed to express short hairpin RNA (shRNA) specific for human cFLIP were inserted into the pSilencer3.1-H1neo vector (Ambion).

Embryo manipulation. cFLIP-L was subcloned into the pCS107 vector, and the plasmid was linearized with AseI for mRNA synthesis. mRNA was injected into the ventral equatorial region of each ventral blastomere at the four-cell stage as described previously (12), and the embryos were scored for axis duplication at stages 29 to 30 (29).

RESULTS

Elevation of cytosolic β-catenin levels by cFLIP-L. The levels of cFLIP are reportedly regulated by ubiquitylation and proteasome-dependent degradation (19). We tested the effect of cFLIP-L on the levels of proteins that are known to be subject to regulation by the ubiquitin-proteasome system. The expression of cFLIP-L increased endogenous cytosolic β-catenin, but not endogenous XIAP, cIAP1, p53 and IκB (Fig. 1A). The cFLIP-L protein consists of two DED and a caspase-like domain. In contrast, a short splicing variant, cFLIP-S, lacks the caspase-like domain and is expressed only in some cells. To examine the structure-function relationships of cFLIP-L-mediated elevation of β-catenin levels, we compared the effects of full-length cFLIP-L with those of various deletion mutants of cFLIP-L and cFLIP-S (Fig. 1B), testing their activities with respect to increasing endogenous cytosolic β-catenin. Full-length cFLIP-L, but not cFLIP-S or cFLIP deletions, increased endogenous cytosolic β-catenin (Fig. 1C). Coexpression of FLIP-DED and FLIP-Casp did not increase cytosolic β-catenin (data not shown). This result indicates that both the DED and the caspase-like domain of cFLIP-L in a single polypeptide are required for elevating endogenous cytosolic β-catenin.

β-Catenin is phosphorylated by GSK3β, which initiates the ubiquitylation and proteasomal degradation of β-catenin. Lithium chloride, an inhibitor of GSK3β, increased the level of cytosolic β-catenin (Fig. 1C, lane 8). Therefore, we next examined whether cFLIP-L inhibits β-catenin degradation initiated by GSK3β (Fig. 1D). For these experiments, epitope-tagged β-catenin was expressed in the cytosol of HT1080 cells (lane 2), and cotransfection with GSK3β/Axin resulted in the phosphorylation and reduction of cytosolic β-catenin (lane 3). cFLIP-L inhibited the GSK3β/Axin-induced reduction of β-catenin, while it did not inhibit phosphorylation of β-catenin by GSK3β (lane 4). *N*-Carbobenzoxyl-L-leucinyl-L-leucinyl-L-nor-leucinal (MG-132) blocked β-catenin reduction by the inhibition of proteasome (lane 5). These results indicate that cFLIP-L inhibits the degradation of β-catenin at a step downstream of GSK3β-mediated phosphorylation.

Inhibition of β-catenin ubiquitylation by cFLIP-L. To examine the effect of cFLIP-L on β-catenin ubiquitylation, cells were transfected with plasmids encoding HA-tagged ubiquitin and FLAG-tagged β-catenin. Cytosolic fractions were prepared after MG-132 treatment, and β-catenin was immunoprecipitated with anti-FLAG antibody. The resulting immune complexes were analyzed by Western blotting with anti-HA antibody to detect ubiquitylated β-catenin, which migrated as a smear of protein bands with slower mobility in gels (Fig. 2A, lane 5). The amounts of ubiquitylated β-catenin were greatly reduced by coexpression of cFLIP-L (lane 6). The inhibition of

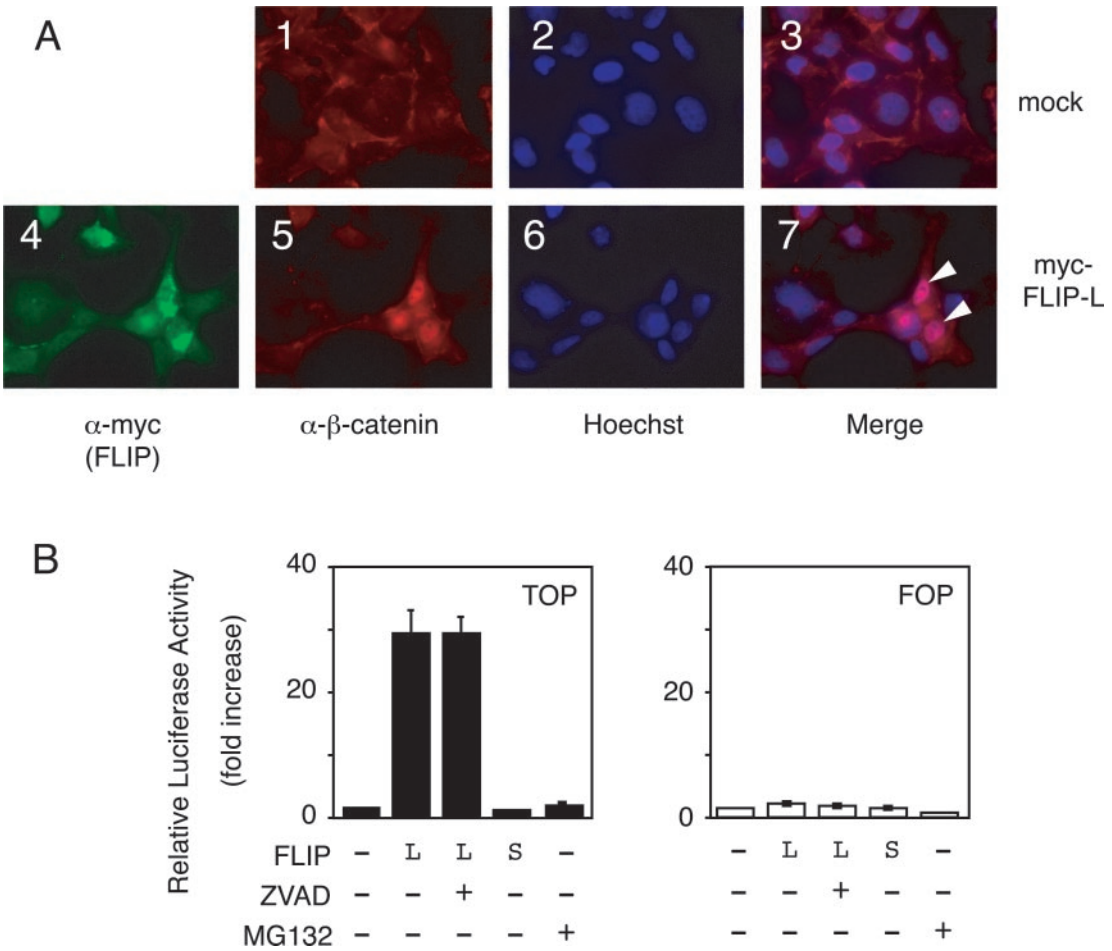

FIG. 3. cFLIP-L induces nuclear translocation of β-catenin and activates β-catenin-mediated gene expression. (A) Nuclear translocation of β-catenin by cFLIP-L expression. HT1080 cells were transfected with (images 4 to 7) or without (images 1 to 3) Myc-tagged cFLIP-L and stained with anti-Myc, anti-β-catenin, and Hoechst 33342. Merged images of β-catenin staining and Hoechst staining are shown. The open arrowheads in image 7 indicate the cells with nuclear β-catenin. (B) Activation of β-catenin-mediated gene expression by cFLIP-L expression. cFLIP plasmids were transfected into 293T cells with TOP-TK-Luc (left) or FOP-TK-Luc (right) as described in Materials and Methods. TOP-TK-Luc contains optimal and FOP-TK-Luc contains mutated TCF-binding sites placed upstream of a luciferase reporter gene. The luciferase activity was measured and expressed as the increase (*n*-fold) compared with the level observed in cells transfected without cFLIP. The data represent means (± standard deviations) of quadruplicate determinations. +, present; −, absent; L, cFLIP-L; S, cFLIP-S.

β-catenin ubiquitylation by cFLIP-L was further examined by Western blot analysis of the cells transfected with HA-tagged β-catenin (Fig. 2B). Probing with anti-HA antibody showed a monoubiquitylated β-catenin band in the cells treated with MG-132 (Fig. 2B, lane 3). The monoubiquitylated β-catenin band almost disappeared in the cells cotransfected with cFLIP-L (lane 5). These results indicate that cFLIP-L inhibits the ubiquitylation of β-catenin, thereby increasing cytosolic β-catenin.

Induction of β-catenin-mediated gene expression by cFLIP-L. In Wnt signaling, cytosolic β-catenin translocates into nuclei and activates β-catenin-dependent gene expression (31, 33). Therefore, we examined the effect of cFLIP-L on the translocation of β-catenin into nuclei. As shown in Fig. 3A, nuclear translocation of β-catenin was observed in the cFLIP-L-transfected cells (images 4 to 7), while a majority of β-catenin localized to the plasma membrane in control cells (images 1 to 3), consistent with the role of β-catenin as a cadherin-binding protein. We then examined the transactivation potential of β-catenin using a reporter gene construct, TOP-TK-Luc (22). Consistent with the appearance of the nuclear β-catenin in cFLIP-L-transfected cells (Fig. 3A), increased expression of the luciferase reporter was observed in cFLIP-L-expressing cells (Fig. 3B, left). cFLIP-L did not induce the expression of luciferase toward FOP-TK-Luc, in which the Tcf-binding sites were mutated (Fig. 3B, right), confirming the specificity of these results. Thus, cFLIP-L activates β-catenin-dependent gene expression in cells.

cFLIP-L physically interacts with FADD and caspase 8, and homozygous disruption of the FLIP, FADD, and caspase 8 genes in mice individually results in a failure of heart development at embryonic day 11.5 (45, 47, 48). To examine if FADD or caspase 8 is involved in the activation of β-catenin by cFLIP-L, we first treated cells with ZVAD, a broad-spectrum caspase inhibitor, and measured the levels of cytosolic β-catenin and β-catenin/TCF-dependent reporter gene expression. ZVAD effectively inhibited caspase activities, as shown by the reduction of cFLIP-L processing into a 45-kDa fragment (Fig. 1C, middle, lane 3). However, the accumulation of endogenous

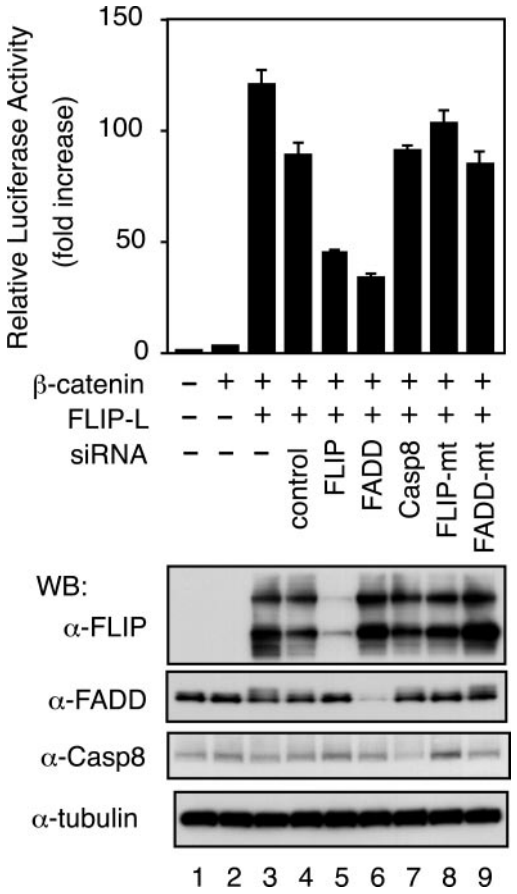

FIG. 4. Requirement for FADD in β-catenin-mediated gene expression induced by cFLIP-L. 293T cells were transfected with the indicated siRNAs (200 pmol), along with β-catenin (1 μg), cFLIP-L (1 μg), TOP-TK-Luc (125 ng) and pRL-TK (12.5 ng). FLIP-mt and FADD-mt represent the siRNAs that have mutations in the corresponding sequences. The luciferase activity was measured after 48 h of transfection and is expressed as the increase (*n*-fold) compared with the level observed in cells transfected with TOP-TK-Luc (top). Cell lysates were analyzed by Western blotting (WB) to confirm the reduction in the protein level (bottom). +, present; −, absent.

cytosolic β-catenin (Fig. 1C, top) and the expression of luciferase (Fig. 3B, left) were not suppressed by ZVAD in cFLIP-L-expressing cells. Similar results were obtained by expressing CrmA, a viral protein that potently inhibits caspase 8 (data not shown). Expression of the 45-kDa fragment of cFLIP-L and ZVAD treatment without cFLIP-L expression did not stabilize β-catenin or induce luciferase activity (data not shown). These results suggest that caspase activity is not required for the activation of β-catenin by cFLIP-L.

We next experimentally reduced the levels of the cFLIP-L, FADD, and caspase 8 proteins using small interfering RNA (siRNA) (Fig. 4, bottom, lanes 5 to 7). The luciferase activity was significantly reduced when FADD and cFLIP-L, but not caspase 8, were knocked down by siRNA (Fig. 4, top). Introduction of mutations into the siRNA for FADD and cFLIP-L abolished the reduction of the protein levels and luciferase activity (Fig. 4, bottom, lanes 8 and 9), confirming the specificity of these results. These findings suggest that FADD is required for β-catenin stabilization by cFLIP-L.

Ligation of Fas in the presence or absence of ZVAD did not induce β-catenin-mediated gene expression (data not shown), suggesting that Fas signaling does not play a role in Wnt signaling.

Enhancement of Wnt signaling by cFLIP-L. To study the role of cFLIP-L in β-catenin activation in a more physiological setting, we established cell lines stably expressing cFLIP-L and cFLIP-S (Fig. 5A). In contrast to the transient-expression experiments, in which a large amount of cFLIP-L was expressed and partially cleaved to a 45-kDa fragment by caspase 8, moderate expression of cFLIP-L without processing was observed in the stable transfectant clone (Fig. 5B). Consistent with previous reports (16, 18, 38), the FLIP transfectant clones were resistant to apoptosis induced by anti-Fas antibody (Fig. 5C, left) but not to that induced by an antitumor drug, etoposide (Fig. 5C, right). The cFLIP transfectant clones were treated with Wnt3a to examine Wnt signaling (Fig. 5D). In parental HT1080 cells, Wnt signaling was slightly stimulated by Wnt3a in a dose-dependent fashion. This Wnt signaling was significantly enhanced in the cFLIP-L transfectant clones but not in the cFLIP-S and vector transfectant clones.

To exclude the possibility that cFLIP-L affected β-catenin levels through a transcriptional as opposed to a posttranslational mechanism, we examined the level of β-catenin mRNA by semiquantitative RT-PCR. The cFLIP-L transfectant clones and the parental HT1080 cells express comparable levels of β-catenin mRNAs (Fig. 6A). Then, we transiently transfected the cFLIP-L stable transfectants with plasmids expressing epitope-tagged β-catenin from a cytomegalovirus promoter. When equal amounts of the β-catenin-encoding plasmid were transfected, the cFLIP-L transfectant clones accumulated more cytosolic β-catenin protein than did the parental HT1080 cells (Fig. 6B, lanes 1 to 7). Comparable levels of expression of green fluorescent protein (GFP) were found in the cFLIP-L transfectant clones and the parental HT1080 cells (lanes 8 to 11) serving as a control. In cycloheximide-treated cells, the turnover rate of cytosolic β-catenin was delayed in HT1080 cells transiently transfected with cFLIP-L (Fig. 6C, lanes 7 to 12, and D, FLIP-L/transient) and in the cFLIP-L stable transfectant clones (Fig. 6C, lanes 13 to 24, and D, L1 and L6), compared to the parental HT1080 cells (Fig. 6C, lanes 1 to 6, and D, HT1080). Taken together with other results, these results imply that cFLIP-L-mediated increases in β-catenin likely arise due to reduced degradation via the ubiquitin-proteasome system.

Reduced Wnt signaling in cFLIP-depleted cells. To study the role of physiologically expressed cFLIP in Wnt signal regulation, we experimentally reduced endogenous cFLIP in A549 cells that are responsive to Wnt3a. Wnt signaling was greatly suppressed in A549 cells (Fig. 7A), where the cFLIP protein level was downregulated by an shRNA (Fig. 7B). This result indicates that endogenous cFLIP modulates Wnt signaling.

Effects of cFLIP-L on *Xenopus* embryos. Wnt signaling plays a crucial role in body axis formation in the development of early embryos (7, 42, 46). To study the role of cFLIP-L in Wnt signaling in vivo, we injected cFLIP-L mRNA into the ventral equatorial region at the four-cell stage in *Xenopus* embryos. cFLIP-L mRNA injection induced secondary body axis formation in early embryos, although it was not as complete and frequent as observed for ventral expression of Xwnt8 or β-catenin

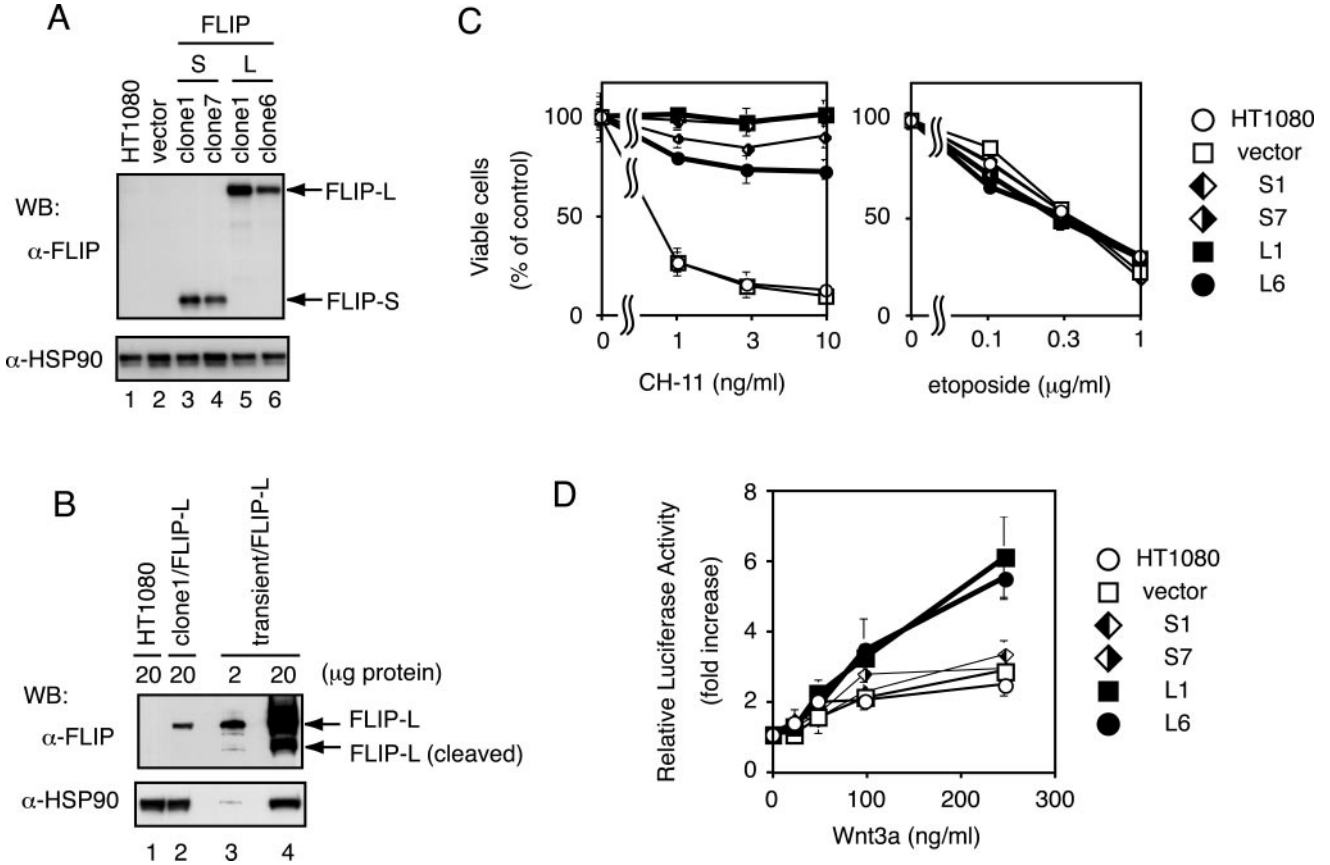

FIG. 5. cFLIP-L enhances Wnt signaling. (A) Expression of cFLIP-L and cFLIP-S in stable transfectant clones. HT1080 cells were transfected with cFLIP-L, cFLIP-S, or empty vector, and stable transfectant clones were isolated as described in Materials and Methods. Cell lysates prepared from the clones were analyzed by Western blotting (WB). Endogenous cFLIP-L was hardly visible under these conditions, but clone L1 expresses ~20-fold more cFLIP-L protein than do parental HT1080 cells. The blot with HSP90 shows the loading control. (B) Comparison of cFLIP-L expression levels. Whole-cell lysates were prepared from HT1080 cells (lane 1), cFLIP-L stable transfectant clone 1 (lane 2), and HT1080 cells that had been transiently transfected with 1 μg of cFLIP-L in six-well plates (lanes 3 and 4). The indicated amounts of protein were loaded onto the gel and analyzed by Western blotting with the indicated antibodies. (C) Resistance to Fas-mediated apoptosis in cFLIP transfectant clones. The cFLIP clones were treated with the indicated concentrations of anti-Fas antibody in the presence of 1 μg of cycloheximide/ml (left) and the indicated concentrations of etoposide (right) for 48 h. Viable cells were measured by MTS assay (Promega) and are expressed as percentages compared with untreated cells. (D) Enhanced Wnt signaling in cFLIP-L transfectant clones. The cFLIP clones and control cells were transfected with TOP-TK-Luc and pRL-TK and replated in 96-well plates after 12 h. After another 12 h, the cells were treated with conditioned medium containing the indicated concentrations of Wnt3a for 24 h. The luciferase activity was measured and is expressed as the increase (*n*-fold) compared with the level observed in cells treated without Wnt3a. The data represent means of quadruplicate determinations. The error bars indicate standard deviations.

(Fig. 8A and B). Injection of higher doses of cFLIP-L resulted in degeneration of the cells derived from the ventral marginal zone, consistent with the apoptosis induction by transient overexpression of cFLIP-L observed in some types of mammalian cells (8, 11, 16, 40). This may explain why cFLIP-L could not elicit complete axis formation, in contrast to Xwnt8 and β-catenin. When cFLIP-L was coinjected with suboptimal doses of β-catenin, cFLIP-L increased the frequency of body axis duplication (Fig. 8C) and significantly enhanced β-catenin-dependent gene expression in cells from animal caps (Fig. 8D). These results indicate that cFLIP-L can modulate Wnt signaling in vivo in the context of early *Xenopus* embryo development.

DISCUSSION

cFLIP is expressed in various human tumors (2, 16, 27, 36, 43, 44). The ability of cFLIP to attenuate Fas signaling suggests that cFLIP could render tumor cells more resistant to Fas-mediated apoptosis induced by immune cells, which could play a role in tumor progression (5, 6, 16, 25, 44). In this study, we demonstrated that transient expression of cFLIP-L increases cytosolic β-catenin by inhibiting ubiquitylation and induces nuclear translocation of β-catenin, leading to β-catenin-mediated gene expression in cells. In addition, we demonstrated enhanced Wnt signaling in the cells stably expressing cFLIP-L, which is attributed to the reduced degradation of β-catenin. Since aberrant β-catenin ubiquitylation resulting in inappropriate activation of Wnt signaling plays a crucial role in many tumors (31, 33), cFLIP-L could play an additional role in tumorigenesis by modulating Wnt signaling, in addition to the Fas signaling attenuation.

Cytosolic β-catenin is normally phosphorylated by a complex involving APC, Axin, and GSK3β, which initiates polyubiquitylation of β-catenin by the SCF^{Fbw1}-ubiquitin-ligase complex (3, 10, 15, 21, 33, 49). The polyubiquitylated β-catenin is sub-

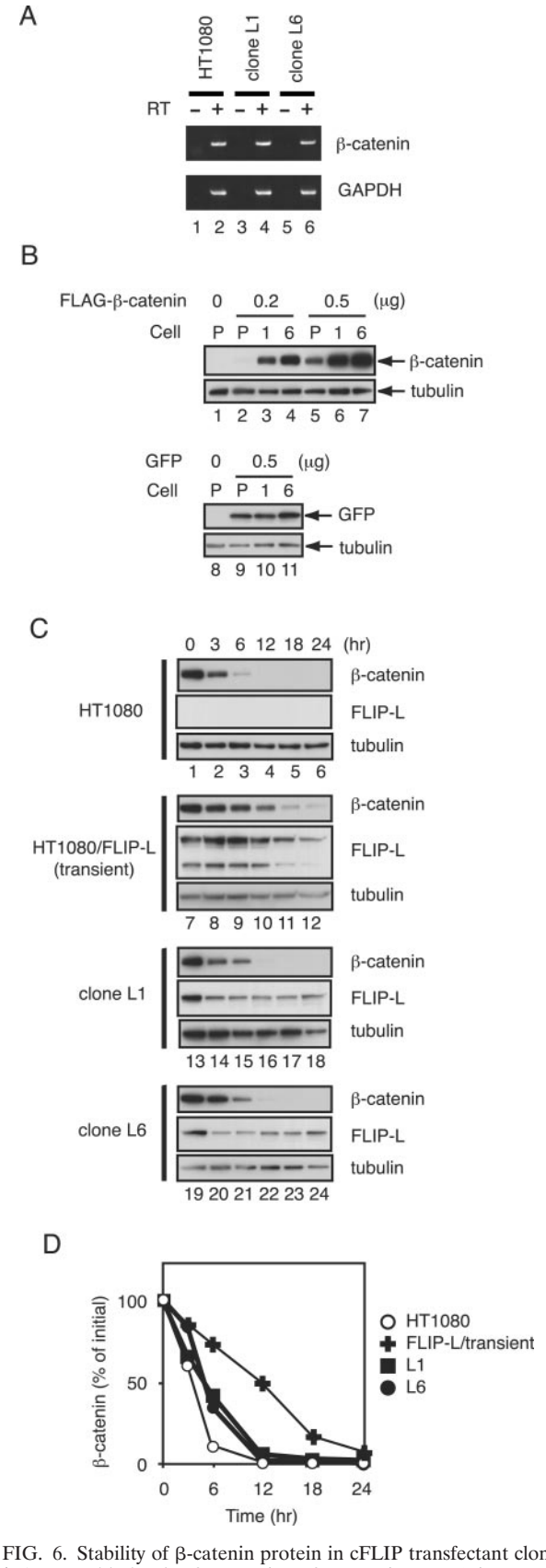

FIG. 6. Stability of β-catenin protein in cFLIP transfectant clones. (A) Comparable levels of expression of β-catenin mRNA in cFLIP-L transfectant clones. RT-PCR was carried out (+) with total RNAs prepared from parental HT1080 cells and cFLIP-L transfectant clones. (B) Increased accumulation of cytosolic β-catenin in cFLIP-L transfectant clones. The cFLIP-L clones (clone 1 and clone 6) and parental HT1080 cells (P) were transfected with the indicated doses of FLAG-tagged β-catenin or GFP, and the cytosolic fractions were analyzed by Western blotting with anti-FLAG (lanes 1 to 7) or anti-GFP (lanes 8 to 11). The blots with anti-tubulin show the loading control. (C) Delayed turnover of cytosolic β-catenin in cFLIP-L transfectant cells. The cFLIP-L clones and the parental HT1080 cells were transfected with FLAG-tagged β-catenin. After 24 h, the cells were treated with 40 μg of cycloheximide/ml for the indicated times, and the cytosolic fractions were prepared and analyzed by Western blotting with anti-FLAG. A parallel experiment was carried out with transient coexpression of cFLIP-L in HT1080 cells (lanes 7 to 12). (D) The β-catenin bands shown in panel C were measured and expressed as percentages compared with the corresponding protein level at time zero.

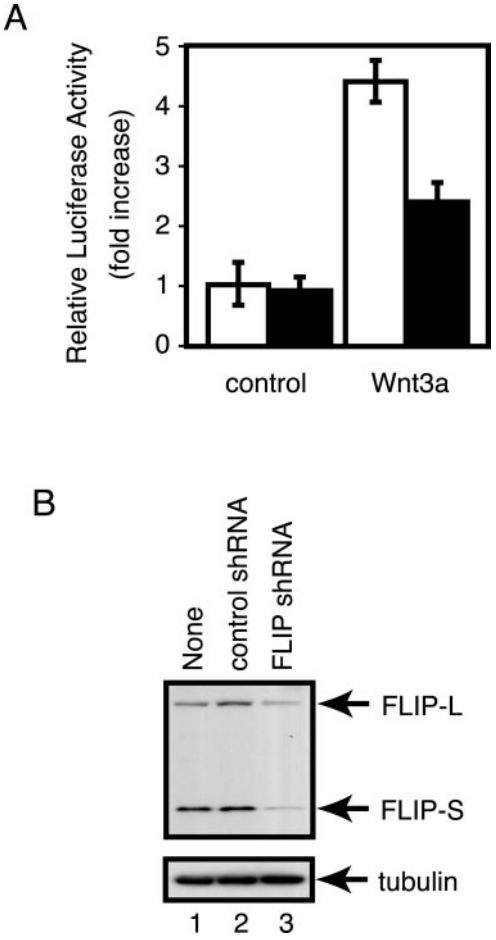

FIG. 7. Reduced Wnt signaling in cFLIP-depleted cells. (A) A549 cells in 60-mm-diameter dishes were transfected with a total of 2.4 μg of DNA containing 1.2 μg of control shRNA vector (open bars) or FLIP shRNA vector (solid bars), TOP-TK-Luc (400 ng), and pRL-TK (80 ng) for 12 h and replated in 48-well plates. After 24 h, the cells were treated with conditioned medium containing 500 ng of Wnt3a/ml for 12 h. The luciferase activity was measured and is expressed as the increase (*n*-fold) compared with the level observed in control cells treated without Wnt3a. The data represent means of triplicate determinations. The error bars indicate standard deviations. (B) Cell lysates from A549 cells that had been transfected with the indicated shRNA vectors were analyzed by Western blotting with anti-FLIP (top) and antitubulin (bottom).

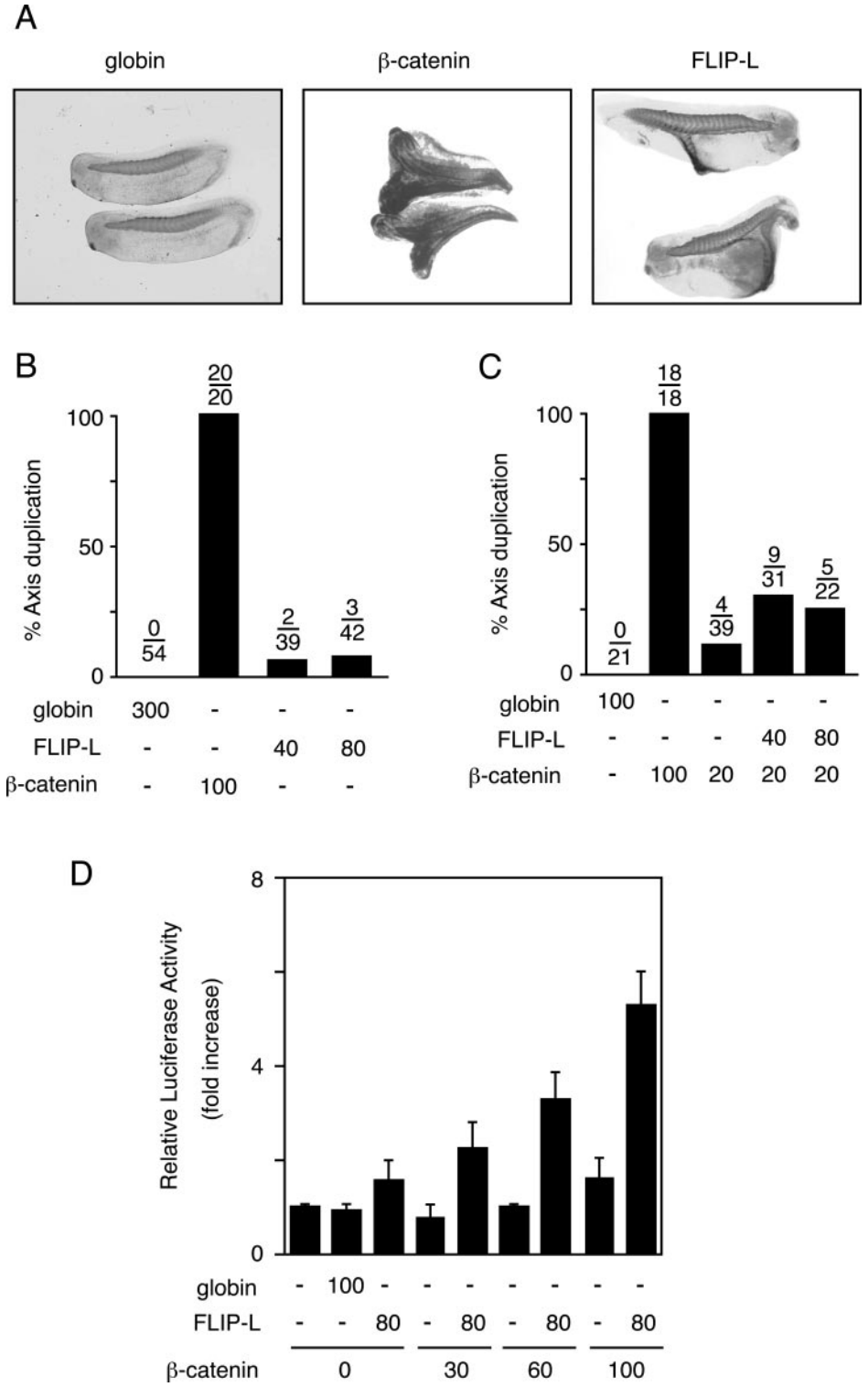

FIG. 8. Effects of cFLIP-L on *Xenopus* embryos. (A) Induction of axis duplication by cFLIP-L. Two blastomeres of four-cell stage embryos were injected in the ventral equatorial region with the indicated mRNAs (*Xenopus* β-globin, 300 ng; mouse β-catenin, 100 ng; cFLIP-L, 40 ng). Axis duplication was validated by ectopic muscle formation, shown by immunostaining using a muscle-specific 12/101 antibody. The embryos were transparentized by benzyl benzoate-benzyl alcohol (2:1). (B) Frequency of axis duplication by cFLIP-L. *Xenopus* embryos were injected with the indicated mRNAs (the number of nanograms per embryo is shown) as in panel A. The fraction of embryos with a duplicated axis is indicated above each bar. (C) cFLIP-L increases axis duplication induced by suboptimal levels of β-catenin. *Xenopus* embryos were injected with the indicated mRNAs (the number of nanograms per embryo is shown) as in panel A. The fraction of embryos with a duplicated axis is indicated above each bar. (D) cFLIP-L enhances β-catenin-mediated gene expression in *Xenopus* embryos. *Xenopus* embryos were injected with the indicated mRNAs (the number of nanograms per embryo is shown) as in panel A, along with TOP-TK-Luc and pRL-TK. The animal caps were dissected and cultured at 18°C for 24 h in modified Barth's solution containing 0.1% bovine serum albumin. The luciferase activity in the cell lysates from three animal caps was measured and expressed as the increase (*n*-fold) compared with the level observed in the cells injected with TOP-TK-Luc and pRL-TK. The data represent means of triplicate determinations. The bars indicate standard errors of the mean.

ject to proteasome-mediated proteolysis. Since cFLIP-L inhibits β-catenin ubiquitylation at a step downstream of phosphorylation by GSK3β, we investigated direct interaction of cFLIP-L with SCF^{Fbw1} complex components, including skp1, cullin1, Fbw1, and Rbx1. We also investigated the effects of cFLIP-L on the complex formation of SCF^{Fbw1} ligase and deubiquitylation of β-catenin. None of these experiments, however, showed significant activity that can explain the mechanism by which cFLIP-L inhibits β-catenin ubiquitylation (data not shown). At present, the precise mechanism is not clear, but cFLIP-L is the only isoform that inhibits β-catenin ubiquitylation and enhances Wnt signaling, because cFLIP-S did not increase endogenous cytosolic β-catenin (Fig. 1C) or activate β-catenin-dependent gene expression (Fig. 3B). Wnt3a-induced gene expression was not enhanced in the cFLIP-S transfectant clones (Fig. 5C).

cFLIP physically interacts with FADD and caspase 8 on their DED (8, 16, 40, 41). Gene-targeting studies demonstrated that cFLIP-, caspase 8-, and FADD-deficient mice, but not Fas-deficient mice, show symptoms of impaired heart development (1, 45, 47, 48). These results suggest that cFLIP, caspase 8, and FADD are likely to cooperate in cardiac development in the embryo, which is independent of Fas signaling regulation. Other studies reported that the Wnt/β-catenin pathway regulates the differentiation of precardiac cells and cardiac valve formation (14, 23). Thus, the cFLIP/FADD/caspase 8 and Wnt/β-catenin pathways are both essential for normal heart development, though the molecular mechanism connecting these pathways is not known. The present result using siRNA showed that FADD is required to induce β-catenin-mediated gene expression by cFLIP-L, suggesting the involvement of FADD in the enhancement of Wnt signaling by cFLIP-L. Caspase 8 may not be involved in this process, because neither inhibition of caspase activity nor reduction of the caspase 8 protein level affected β-catenin-dependent gene expression (Fig. 3B and 4). However, we could not rule out the possibility that the residual caspase 8 cooperates with cFLIP-L and FADD to regulate Wnt signaling. Further studies will clarify the mechanism by which these DED-containing proteins regulate ubiquitylation and Wnt signaling, which could be involved in tumorigenesis, heart development, and the regulation of immune responses.

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