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The Calpain System Is Involved in the Constitutive Regulation of β-Catenin Signaling Functions*

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β-Catenin is a multifunctional protein serving both as a structural element in cell adhesion and as a signaling component in the Wnt pathway, regulating embryogenesis and tumorigenesis. The signaling fraction of β-catenin is tightly controlled by the adenomatous polyposis coli-axin-glycogen synthase kinase 3β complex, which targets it for proteasomal degradation. It has been recently shown that Ca^{2+} release from internal stores results in nuclear export and calpain-mediated degradation of β-catenin in the cytoplasm. Here we have highlighted the critical relevance of constitutive calpain pathway in the control of β-catenin levels and functions, showing that small interference RNA knock down of endogenous calpain *per se* (*i.e.* in the absence of external stimuli) induces an increase in the free transcriptional competent pool of endogenous β-catenin. We further characterized the role of the known calpain inhibitors, Gas2 and Calpastatin, demonstrating that they can also control levels, function, and localization of β-catenin through endogenous calpain regulation. Finally we present Gas2 dominant negative (Gas2DN) as a new tool for regulating calpain activity, providing evidence that it counteracts the described effects of both Gas2 and Calpastatin on β-catenin and that it works via calpain independently of the classical glycogen synthase kinase 3β and proteasome pathway. Moreover, we provide *in vitro* biochemical evidence showing that Gas2DN can increase the activity of calpain and that *in vivo* it can induce degradation of stabilized/mutated β-catenin. In fact, in a context where the classical proteasome pathway is impaired, as in colon cancer cells, Gas2DN biological effects accounted for a significant reduction in proliferation and anchorage-independent growth of colon cancer.

β-Catenin plays a dual role both as a major structural element of cell-cell adherent junctions and as a pivotal signaling molecule in the Wnt pathway, transmitting transcriptional cues into the nucleus (1–3). In the absence of Wnt signaling, the cytoplasmic levels of β-catenin are kept low through interaction with a protein complex that can phosphorylate β-catenin and target it to ubiquitin-mediated proteasomal degradation (4). Activation of Wnt signaling leads to inactivation of glycogen synthase kinase 3β (GSK3β),[1] a kinase responsible for phosphorylation of β-catenin, resulting in accumulation of cytoplasmic β-catenin (5). Enhanced cytoplasmic levels of β-catenin allow its translocation to the nucleus where it cooperates with a member of the T cell factor (TCF)/lymphocyte-enhancer binding factor (LEF) family of transcription factors to activate expression of target genes (6). The three regulatory genes in this pathway most frequently mutated in human cancers are APC, β-catenin, and axin. Their mutations result in the accumulation of nonphosphorylated β-catenin, thereby constitutively activating gene transcription and promoting carcinogenesis (7). Wnt signaling operates via cell surface receptors to stimulate also the activation of Gq pathway and the subsequent increase in intracellular calcium (8). Li and Iyengar (9) highlighted the consequences of activation of Gq signaling pathway showing that calcium release from internal stores results in μ-calpain-dependent degradation of β-catenin.

Calpains are a large family of calcium-dependent intracellular proteases whose precise and limited cleavage of specific proteins, in concert with their endogenous inhibitor Calpastatin, is thought to play an integral regulatory aspect in various signaling pathways from cytoskeletal remodeling to cell cycle regulation and apoptosis (reviewed in Ref. 10). In addition to Calpastatin, we have recently characterized Gas2 as an important calpain inhibitor (11).

Gas2 is a component of the microfilament system and colocalizes with actin fibers (12); it binds the calmodulin-like region of m-calpain (domain III and IV of large subunit) with its amino-terminal domain and with its carboxyl-terminal region exerts an inhibitory function (11). The isolated amino-terminal region has been demonstrated to act as a dominant negative form of Gas2 (Gas2DN), being able to bind, but not to inhibit, calpain activity and to rescue *in vivo* the effects of Gas2 on calpain function.

Here we underline the contribution of the calpain system to β-catenin degradation, showing that m-calpain, as regulated by the endogenous inhibitors Calpastatin and Gas2, can modulate β-catenin levels and activity in the absence of external stimuli. More importantly, we focused our attention on the properties of

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[1] The abbreviations used are: GSK3β, glycogen synthase kinase 3β; TCF, T cell factor; LEF, lymphocyte-enhancer binding factor; GFP, green fluorescent protein; siRNA, small interference RNA; BrdUrd, bromodeoxyuridine; HA, hemagglutinin; Gas2DN, Gas2 dominant negative; IVT, *in vitro* translated; E1, ubiquitin-activating enzyme; CH domain, Calponin homology domain; wt, wild type; m-calpain, milli-calpain.

Gas2DN, which can abrogate both Gas2 and Calpastatin effects, providing biochemical evidence that it can stimulate calpain activity. The Gas2DN-mediated calpain activation was utilized in colon cancer cells, where β-catenin degradation via proteasome is impaired. We show that in this cellular context, the calpain system controls deregulated β-catenin and that the biological effects of Gas2DN account for a significant reduction in the proliferation and anchorage-independent growth of colon cancer cells, therefore representing a potentially critical way to control deregulated β-catenin.

MATERIALS AND METHODS

Cell Lines, Transfections, and Reporter Assay—Cells were cultured in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C and 5% CO_2. Transfections were performed using FuGENE 6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. Luciferase assays were performed with the dual luciferase kit (DLR) from Promega.

U2OS are human osteosarcoma cells; HCT-116 are human colon carcinoma cells harboring β-catenin mutation on exon 3 of β-catenin with three base deletions at codon 45. LS-174 are human colon carcinoma cells harboring β-catenin mutation, whereas HT-29 are human colon carcinoma cells harboring APC deletion. MEF GSK3β−/− are murine embryo fibroblasts null for GSK3β, provided by Dr. J. Woodgett (Ontario Cancer Institute); H1299 are lung carcinoma cells. The TS-20 cell line is a temperature-sensitive mutant derived from the mouse cell line BALB/c 3T3 obtained from Dr. Gianni Del Sal (Laboratorio Nazionale del Consorzio Interuniversitario per le Biotecnologie, Trieste, Italy).

Plasmids—pEGFPC1-Gas2DN (12), pCMVHA-tagged β-catenin cloned from pMT2-VSV-tagged β-catenin (13), pGDSV7S Gas2wt (12), and the Calpastatin plasmid (PM194) have been described previously (14). TOP Luc plasmid was provided by H. Clever. His_6-tagged Gas2wt, His_6-tagged Gas2DN, and His_6-tagged Calpastatin were generated as described below under "Calpain Activity Assay."

Western Blot and Antibodies—Western blot analysis was performed according to standard procedures using the following primary antibodies: anti-β-catenin (C19220; Transduction Laboratories), anti-Gas2 antibody (12), anti-Calpastatin (monoclonal antibody-C-270), anti-HA (Roche Applied Science), and anti-m-calpain polyclonal antibody (C0728) and anti-actin polyclonal antibody (A2066) from Sigma. Anti-GFP (Invitrogen):polyclonal GFP antiserum (Invitrogen).

Stable Cell Lines—To obtain GFP-Gas2DN/HCT116 and GFP/HCT-116 cells, selection was performed for 2 weeks in the presence of 1 mg/ml G418. G418-resistant colonies were clonally expanded, and expression of Gas2DN and GFP was analyzed by Western blot.

Calpain Cleavage Assay in Vitro and in Vivo—In *vitro* cleavage assay was performed as previously described (11). The *in vivo* protein assays were performed on HCT-116. Cell lysates were prepared freshly at 4 °C in ice-cold Nonidet P-40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.4% Nonidet P-40, 1 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 1 mM EGTA) and rotated for 30 min at 4 °C. Cell debris was pelleted by centrifugation (15 min, 13,000 RPM, 4 °C), and the supernatant was used in β-catenin cleavage assay. 2 mM $CaCl_2$ was added for endogenous m-calpain activation, and different aliquots of the samples were collected at the indicated time points. 8 mM EGTA was added to one of the samples to completely inhibit calcium-dependent calpain reaction.

Inhibition of m-calpain Expression by RNAi—Small interfering RNA duplexes, si-m-calpain, were purchased from Dharmacon: siCONT 5′-AACCUUUUUUUUUGGGGAAAA-3′, si-m-calpain 5′-GUACCUCAACCAGGACUAC-3′, and siGENOME SMARTpool Upgrade MU-009979–00-0020 human CAPNS1 for calpain small subunit. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's suggestions.

Immunofluorescence and BrdUrd Incorporation Assay—After transfection with the various plasmids, cells were fixed with paraformaldehyde (3% in phosphate-buffered saline) and then processed for immunofluorescence with 12CA5 anti-HA antibody (Roche Applied Science). For BrdUrd incorporation assay, 24 h after transfection cells were pulsed with 50 μM BrdUrd for 4 h and fixed with paraformaldehyde. To reveal incorporated BrdUrd, coverslips were treated with 50 mM NaOH for 30 s and immediately washed with phosphate-buffered saline. BrdUrd was revealed by anti-BrdUrd monoclonal antibody (GH Healthcare) followed by incubation with rhodamine isothiocyanate (RITC)-conjugated anti-mouse IgG2a antibody (Southern Biotechnology). Nuclei were counterstained with Hoechst 33342.

Soft Agar Assay—For each cell pool, 30,000 cells were suspended in 2 ml of Dulbecco's modified Eagle's medium + 10% fetal bovine serum and warmed to 37 °C. 200 μl of prewarmed (52 °C) 5% agarose/phosphate-buffered saline solution was mixed with the cell suspension and then layered into 60-mm dishes that were previously coated with 2 ml of 1% agarose in Dulbecco's modified Eagle's medium. The agar was allowed to solidify at room temperature for 20 min before the addition of 2 ml of growth medium to each well. After 14 days, the colonies were counted.

Subfractionation Experiments—Subfractionation experiments were performed as indicated by the manufacturer (ProteoExtract subcellular proteome extraction kit 539790; Calbiochem).

Calpain Activity Assay—Recombinant His_6-tagged Gas2 (pQE11Gas2wt), His_6-tagged Gas2DN (pETM11Gas2DN), and His_6-tagged Calpastatin (pETM11Calpastatin) were used to evaluate calpain activity in biochemical studies. To generate His_6-tagged Gas2wt protein, full-length Gas2wt was subcloned from pGDSV7SGas2wt (12) into pQE11 vector using BamHI and HindIII sites. For the construction of His_6-tagged Gas2DN and Calpastatin fusion proteins, specific oligonucleotides upstream and downstream containing, respectively, NcoI and BamHI or NcoI and XhoI sites were used to generate polymerase chain reaction fragments of Gas2DN or Calpastatin that were cloned in pETM11 vector. One unit of calpain activity was defined as the amount of the enzyme causing the production of 1 μmol acid-soluble NH_2 revealed with fluorescamine and using acid-denatured globin as substrate (15). Rat skeletal muscle m-calpain activity was monitored after incubating for 3 min with His_6-tagged recombinant proteins Gas2, Gas2DN, and Calpastatin in a calpain assay buffer containing 50 mM sodium borate buffer, pH 7.5, 50 μM $CaCl_2$, and rat skeletal muscle m-calpain. Activity was measured 10 min after the addition of 1 mM calcium and 2 mg/ml denatured globin as previously described (15). Gas2 and Calpastatin, known calpain inhibitors, were used as control of calpain activity in the same assay.

RESULTS

Constitutive Activity of Calpain in Vivo Regulates β-Catenin—To address the role of constitutive activity of calpain toward β-catenin, we designed small interference RNA (siRNA) to selectively knock down calpain levels. Because the two ubiquitous calpain isoforms, m- and μ-calpain, are functional only when associated with the common regulatory small subunit, we decided to design siRNA to selectively knock down the small regulatory subunit to block both calpain isoforms and a siRNA to specifically knock down m-calpain to address the relevance of this isoform in this context. The ability of the specific siRNAs (siSmall or si m-calpain) to down-regulate calpain protein levels was tested in U2OS cells, where expression of endogenous small subunit of calpain or of endogenous m-calpain was, respectively, almost completely abolished already at 24 h after specific silencing transfection (Fig. 1*A*). A scrambled siRNA (*siControl*) was used as control. To evaluate the effects of calpain siRNAs toward the activity of β-catenin, U2OS cells were transfected as indicated in Fig. 1*B*. The TOPflash construct is a β-catenin-sensitive promoter widely used as an indicator for binding of β-catenin to hTCF-4. The resulting complex is responsible for activating transcription of the TOPflash reporter (16). Data were normalized by cotransfection with a pCMV-*Renilla* luciferase vector, which controls for the variability in transfection efficiency. Luciferase assays were performed 48 h after transfection, and Western blot analyses were carried out using the same lysates to monitor steady-state levels of endogenous or overexpressed β-catenin. As shown in Fig. 1*B*, down-regulation of calpain levels as induced either by siSmall or si m-calpain resulted in the stabilization and activation of both endogenous and overexpressed β-catenin. These results strengthen the argument for a role of the m-calpain isoform for the observed effects. As a negative control for the transcriptional competence of β-catenin, we used FOPflash reporter, which contains mutant Tcf-4 binding site, and this was not

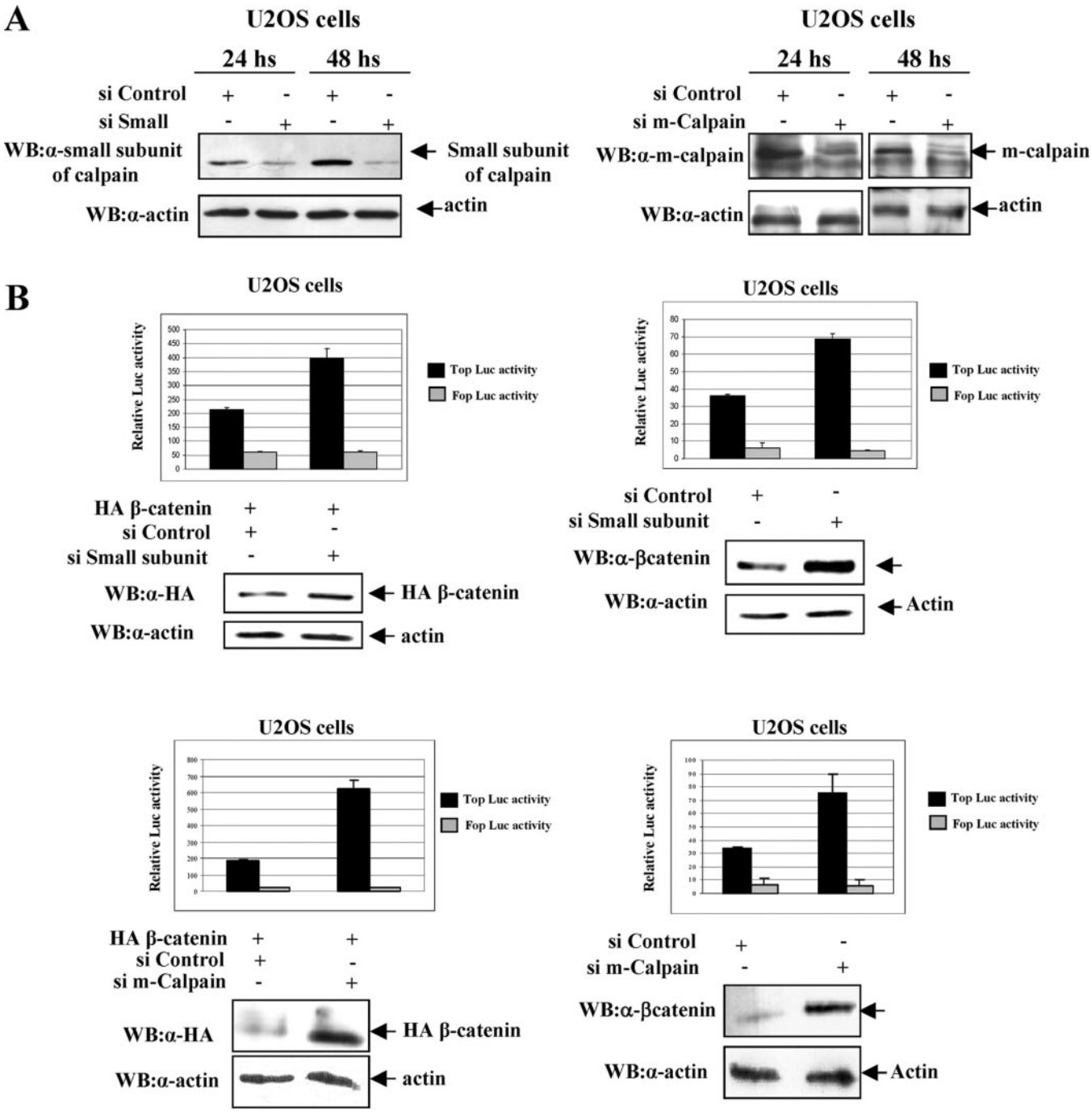

FIG. 1. **Constitutive activity of calpain toward β-catenin.** *A*, U2OS cells were transfected with siControl and siSmall (*left panel*) or siControl and si m-calpain (*right panel*). 24 and 48 h later, lysates were analyzed by Western blot using anti-small subunit of calpain (*upper panel*) and anti-actin (*lower panel*) antibodies in the first case and anti-m-calpain (*upper panel*) and anti-actin (*lower panel*) in the second case. *B*, U2OS cells were transfected as indicated. 24 h later, Western blot analysis was performed using anti-HA antibody (*left panels*) and an anti-β-catenin (*right panels*). Loading control was tested using anti-actin antibody (*lower panels*). Luc activity was determined in the same lysates by calculations of Top-Luc and Fop-Luc (as negative control). The data represent means ± S.D. from three independent experiments. *C*, U2OS cells were transfected with siControl and si-m-calpain. 36 h later cell fractionation was performed and cytoplasmic (*C*), membrane (*M*), and nuclear (*N*) fractions were analyzed. Western blot was performed using anti-β-catenin antibody or anti-c-Fos and anti-actin to check for fractionation efficiency. *D*, the same experiments as in *panel C* were performed in H1299 cells. *E*, confocal microscope image of U2OS cells transfected with HA-tagged β-catenin together with siControl (*a*) or si-m-calpain (*b*) and indicating change of β-catenin localization. Panels show two different corresponding fields of cells stained for β-catenin (*red*).

affected by β-catenin and siControl or siSmall/si m-calpain (Fig. 1*B*).

These observations suggest that, by regulating calpain and particularly the m-calpain isoform, the free cytoplasmic levels of β-catenin could be altered, inducing its translocation into the nucleus where it can function as a transcription factor. To confirm our hypothesis, we transfected U2OS cells with siControl and si m-calpain. 36 h after siRNA transfection, cell fractionation was performed and cytoplasmic (*C*), membrane (*M*), and nuclear (*N*) fractions were separately analyzed. Western blot analysis indicated that the increased levels of β-catenin after m-calpain down-regulation are significantly accumulated

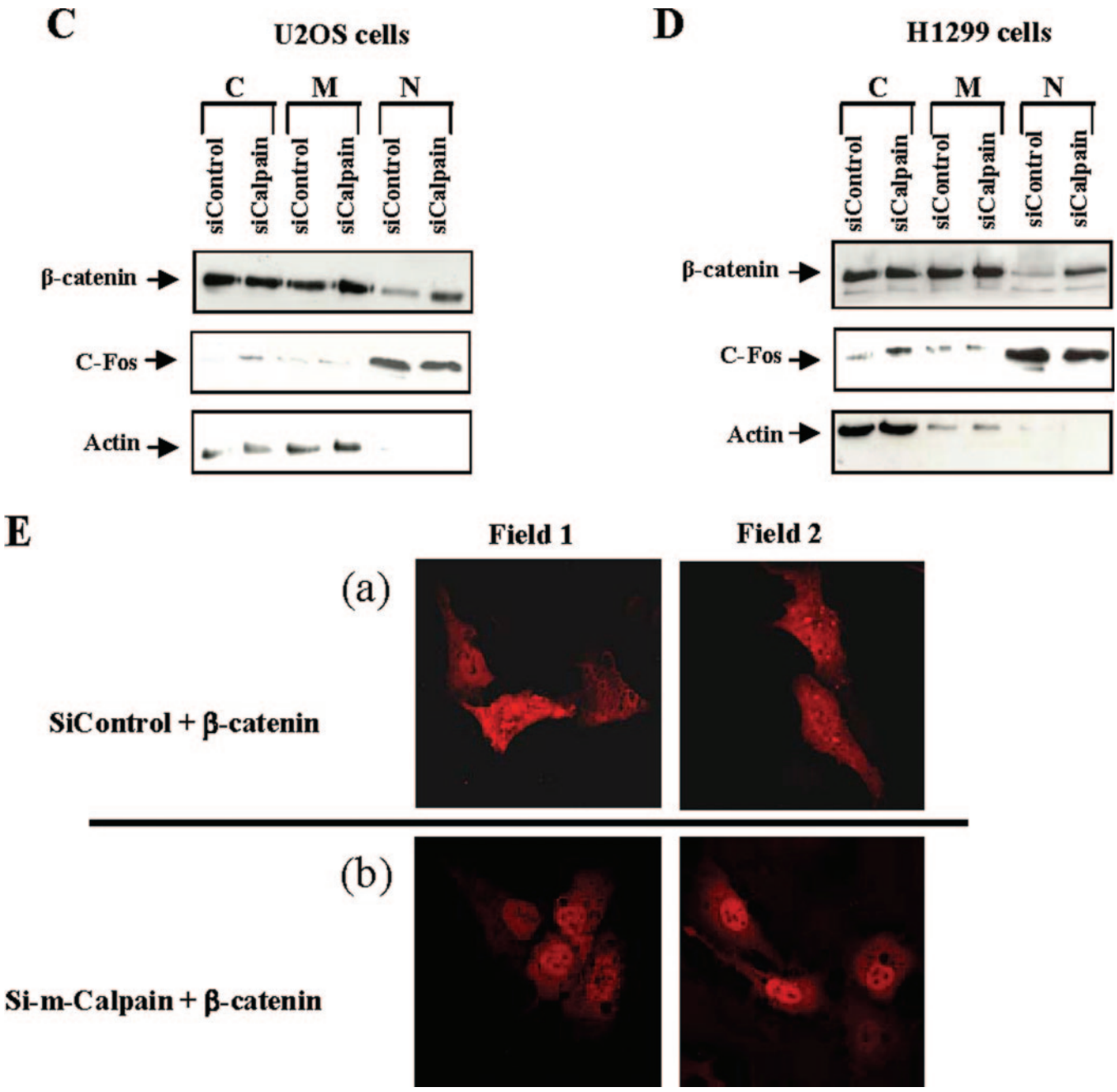

FIG. 1—*continued*

in the nucleus (Fig. 1*C*). The same results were obtained in the H1299 cell line (Fig. 1*D*) and by using siSmall (data not shown). Cellular localization of overexpressed β-catenin after si m-Calpain treatment was also tested by immunocytochemistry, and a supportive change in the nuclear localization was observed by knocking down calpain expression (Fig. 1*E*). Altogether, these results demonstrate that the calpain proteolytic system can alter the transcriptionally active fraction of β-catenin, thus representing a potential mechanism to regulate β-catenin in parallel to the proteasome.

Gas2 and Calpastatin, the Two Endogenous Inhibitors of Calpain, Regulate the Levels and Function of β-Catenin—To better characterize the constitutive m-calpain function toward β-catenin, we decided to analyze the effects of both its endogenous inhibitors, Gas2 (11) and Calpastatin (10). As a first approach, we tested whether overexpression of Gas2 and Calpastatin might enhance the steady-state levels of β-catenin *in vivo*. Cotransfection of HA-tagged β-catenin with Gas2 (Fig. 2*A*, *left panel*) and Calpastatin (*right panel*) caused a substantial accumulation of β-catenin, indicating that calpain regulation by its inhibitors could function as a constitutive system to regulate β-catenin.

As a next step we evaluated the transcriptional competence of stabilized β-catenin by determining the effect of Gas2 and Calpastatin overexpression on the ability of β-catenin to stimulate transcription from TCF-responsive elements. The relative luciferase activity (normalized by cotransfection with pCMV-*Renilla* luciferase) was significantly stimulated by overexpression of Gas2. A similar response was obtained with transfection of Calpastatin (Fig. 2*B*), suggesting that both Gas2 and Calpastatin overexpression can render β-catenin transcriptionally competent. As a control, we looked for the ability of Gas2 and Calpastatin to cause β-catenin translocation into the nucleus (Fig. 2*C*) where it can function as a transcription

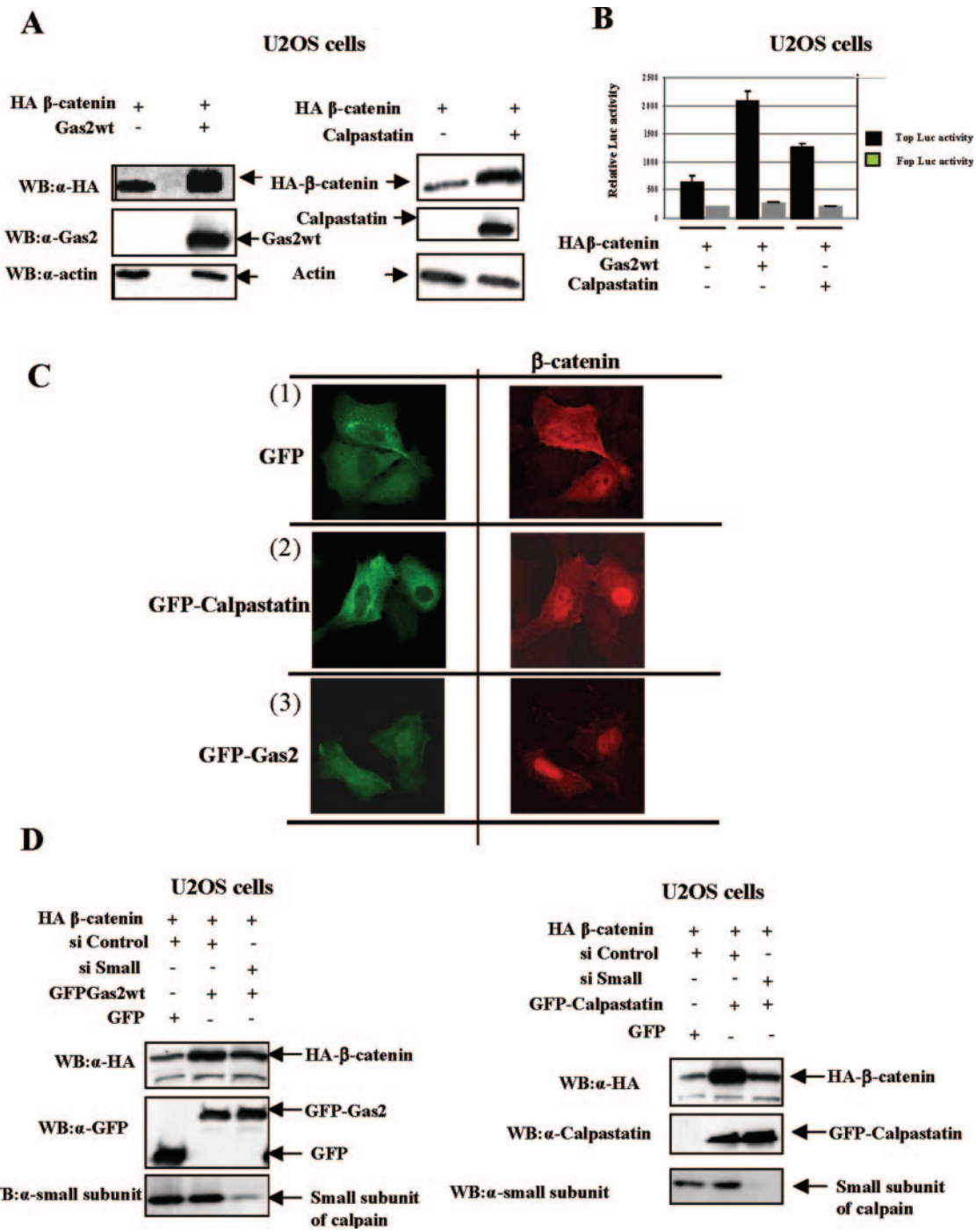

FIG. 2. **Gas2 and Calpastatin regulate levels and function of β-catenin.** *A*, U2OS cells were transiently transfected as indicated. Western blot analysis was performed using anti-HA (*upper panel*), anti-Gas2 or anti-Calpastatin (*middle panel*), and anti-actin (*lower panel*) antibodies. *B*, U2OS cells were cotransfected with indicated plasmids. Luc activity was evaluated 48 h later. Data represent means ± S.D. from three independent experiments. *C*, confocal microscope images of U2OS cells transfected with HA-tagged β-catenin together with GFP (*1*), GFP-Calpastatin (*2*), and GFP-Gas2 (*3*). Panels show corresponding fields of cells stained for β-catenin (*red*) and expressing GFP-tagged proteins (*green*). *D*, U2OS cells were transfected with HA-β-catenin together with a combination of vectors and siRNA oligos as indicated. Cells were harvested 48 h after transfection and analyzed by Western blot analysis using the indicated antibodies. The nonspecific band with *arrows* was considered as a loading control.

factor. We therefore transfected β-catenin into U2OS together with GFP-tagged Gas2, GFP-tagged Calpastatin, and GFP protein alone as negative control. 24 h after transfection, immunofluorescence analyses were performed to visualize β-catenin localization. As can be seen in Fig. 2*C*, the presence of both GFP-tagged Gas2 and GFP-tagged Calpastatin was able to relocalize the cytoplasmic β-catenin into the nucleus. Finally, to confirm that endogenous calpain is critical for the effect of Gas2 and Calpastatin on β-catenin stability, HA-tagged β-catenin levels were monitored after transfection in U2OS cells together with Gas2 or Calpastatin and SiControl or siSmall. Calpain silencing abrogated both Gas2 and Calpastatin effects on β-catenin stabilization, thus indicating the critical role for calpain in the Gas2 and Calpastatin regulation of β-catenin levels (Fig. 2*D*). Altogether, these data suggest that Gas2 and Calpastatin, as endogenous calpain inhibitors, play important roles in controlling β-catenin levels, localization, and transcriptional activity.

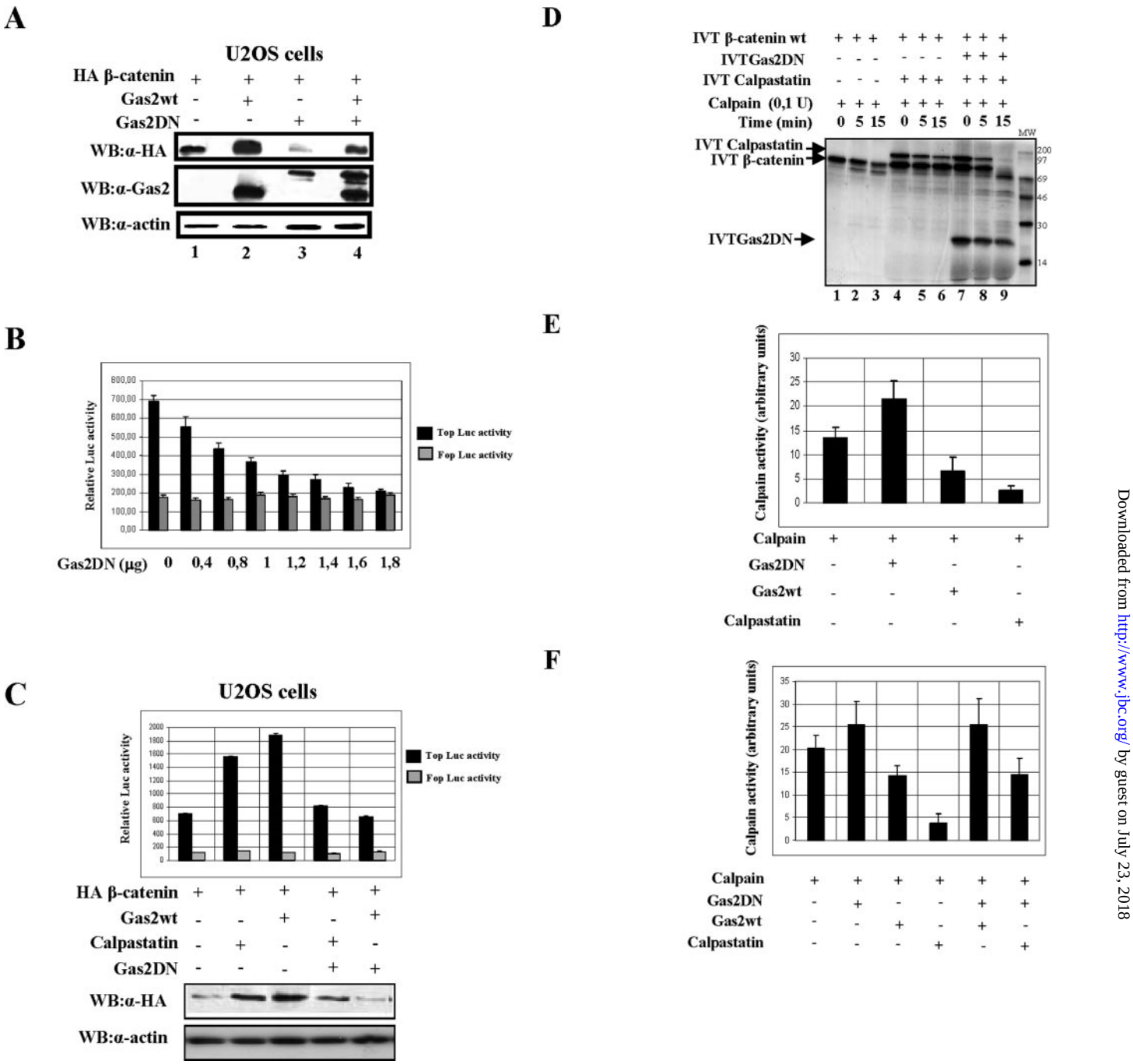

FIG. 3. **Gas2DN can counteract both Gas2 and Calpastatin effects.** *A*, U2OS cells were transfected as indicated. Protein levels were monitored by using anti-HA (*upper panel*), anti-Gas2 (*middle panel*), and anti-actin (*lower panel*) as loading control antibodies. *B*, U2OS cells were transfected with Top-Luc reporter together with increasing amounts of Gas2DN. Luc activity was evaluated 48 h later. Data represent means ± S.D. from at least three independent experiments. *C*, U2OS cells were transiently transfected with the combination of plasmids as indicated. Luciferase and Western blot analysis were performed with the same lysates. *D*, IVT β-catenin was incubated with m-calpain (*lanes 1-3*), m-calpain and IVT Calpastatin (*lanes 4-6*), or m-calpain, IVT Calpastatin, and IVT Gas2DN (*lanes 7-9*). Samples were collected at the indicated time points, analyzed by SDS-PAGE, and evaluated by autoradiography. *Arrows* indicate full-length proteins. Being itself a calpain substrate, the Calpastatin band is also degraded during the time although it is fully functional as a calpain inhibitor after its cleavage (40, 41). *E*, biochemical assay to test the effect of recombinant Gas2, Calpastatin, and Gas2DN on m-calpain activity. Assay was performed as described under "Materials and Methods." *F*, biochemical effect of Gas2DN on Gas2 and Calpastatin ability to inhibit calpain activity. Assay was performed as described under "Materials and Methods."

Gas2DN Blocks Both Calpastatin and Gas2 Regulatory Arms on Calpain Activity—We have previously characterized Gas2DN as a dominant negative form of Gas2, demonstrating that it can still bind m-calpain without showing any inhibitory function (11). We therefore decided to analyze the effects of Gas2DN on the levels and activity of transfected β-catenin.

Gas2DN was transfected into U2OS cells together with β-catenin alone or β-catenin and Gas2. As can be seen in Fig. 3*A*, its overexpression reduced the levels of β-catenin and of the Gas2-dependent β-catenin stabilization.

As a next step we evaluated the transcriptional competence of endogenous β-catenin by determining the effects of Gas2DN overexpression on the ability of β-catenin to stimulate transcription from TCF-responsive elements. We observed that Gas2DN significantly decreased β-catenin transcriptional activity in a dose-dependent manner (Fig. 3*B*). Altogether, the

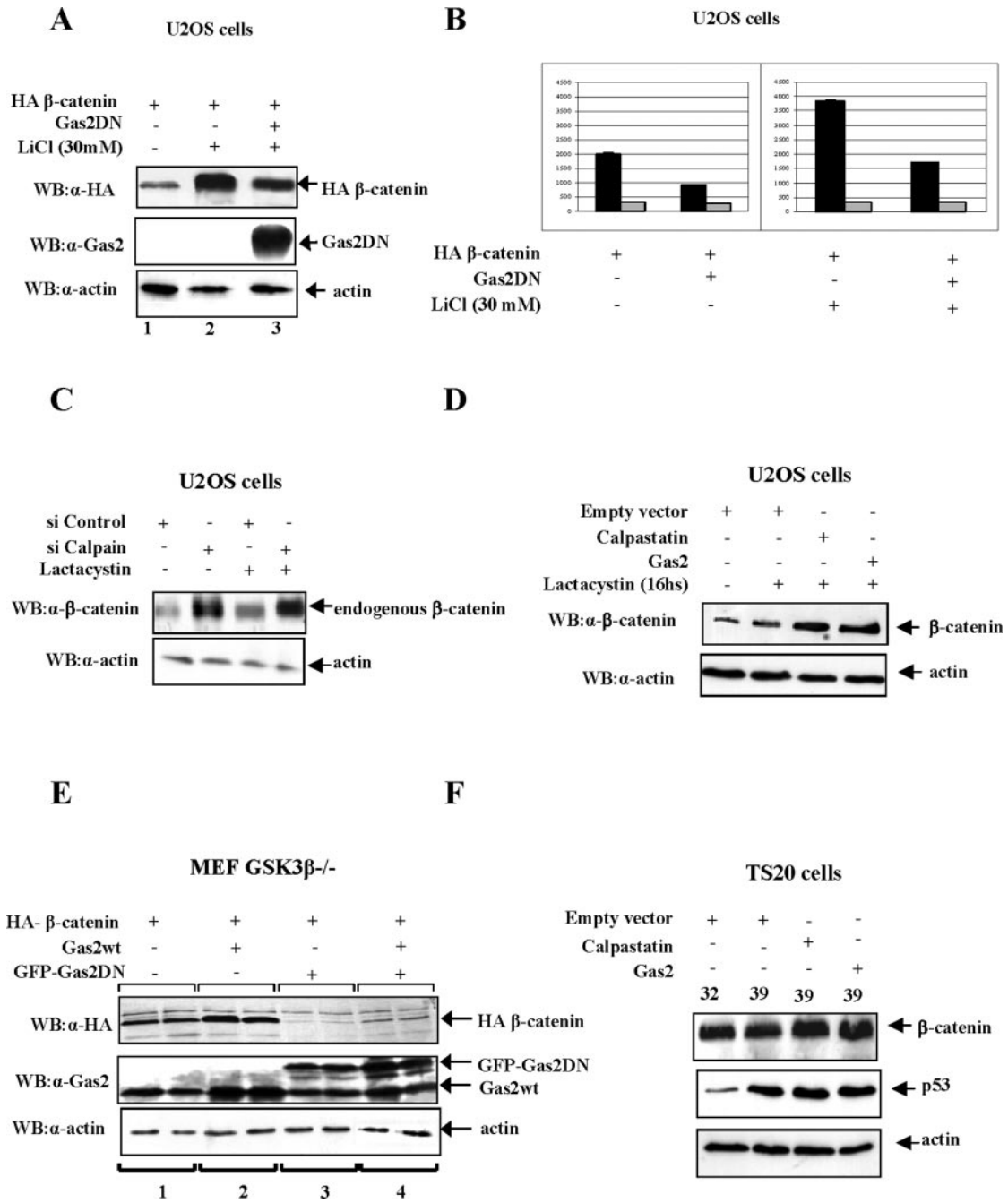

FIG. 4. **Gas2DN induces β-catenin degradation independently of GSK3β, and calpain regulates β-catenin functions independently of the proteasome.** *A*, U2OS cells were transfected with indicated plasmids and then treated with an aqueous vehicle or LiCl 30 mM for 18 h as indicated. Cells lysates were analyzed by Western blot using anti-HA (*upper panel*), anti-Gas2 (*middle panel*), and anti-actin (*lower panel*) antibodies. *B*, U2OS cells were transfected with the indicated plasmids and then treated with an aqueous vehicle or LiCl 30 mM as indicated. Luc activity was evaluated 48 h later. Data represent mean ± S.D. from three independent experiments. *C*, U2OS cells were transfected with siControl or si m-calpain and treated or not with lactacystin for the indicated times. Lysates of the cells were then analyzed using anti-β-catenin antibody and anti-actin as a loading control. *D*, U2OS cells were transfected with the indicated plasmids and treated with lactacystin for 16 h. Lysates of the cells were then analyzed as in *panel C*. *E*, GSK3β−/− mouse embryo fibroblasts were transfected as indicated. Protein levels were monitored by using anti-HA (*upper panel*), anti-Gas2 (*middle panel*), and anti-actin as loading control (*lower panel*) antibodies. *F*, TS20 cells were transfected as indicated. Cells were then grown at non-permissive temperature for 12 h. Cell lysates were subsequently analyzed by Western blot, and the endogenous levels of β-catenin, p53, and actin as loading control are indicated.

data indicating the relevance of m-calpain pathway in β-catenin regulation, together with the evidence that Gas2DN is able to down-regulate β-catenin levels and function (Fig. 3, *A* and *B*), prompted us to investigate whether Gas2DN interference with m-calpain activity could counteract not only Gas2 but also Calpastatin function, thus serving as a general reagent to control calpain pathway. As shown in Fig. 3*C*, in U2OS cells HA-β-catenin transcriptional activity as stimulated by Gas2 or Calpastatin coexpression was repressed in both cases by Gas2DN, and as expected HA-β-catenin levels, due to the presence of Gas2 or Calpastatin, were clearly reduced when Gas2DN was cotransfected. We therefore conclude that Gas2DN can also rescue Calpastatin effects on β-catenin stabilization. We decided to perform an *in vitro* assay to analyze whether the ability of Calpastatin to protect calpain-dependent β-catenin degradation could be abrogated by Gas2DN. As

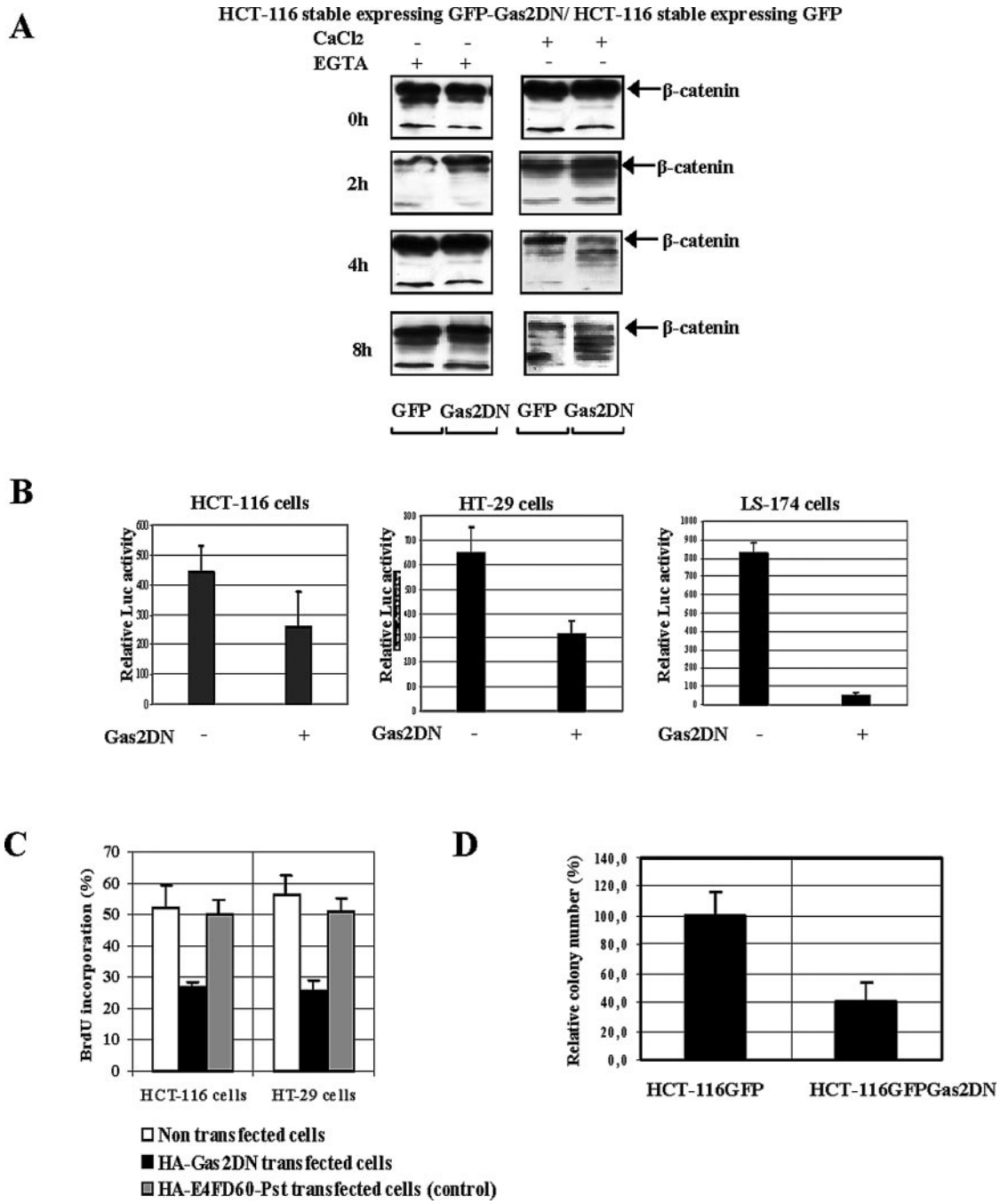

FIG. 5. **Gas2DN affects β-catenin activity and growth rate in colon carcinoma cells.** *A*, proteolytic cleavage of endogenous β-catenin by endogenous m-calpain was followed in GFP/HCT-116 or GFP-Gas2DN/HCT-116 cells. Aliquots of the samples were collected at the indicated time points in the presence of $CaCl_2$ (2 mM) or EGTA (8 mM). β-Catenin proteolysis in each sample was monitored by Western blot. *B*, HCT-116, HT-29, and LS-174 were transfected as indicated. Luciferase activity was measured 48 h after transfections. Graphs are the mean of at least three different experiments. *C*, HCT-116 and HT-29 cells were transfected with equal amounts of HA-Gas2DN or a control protein (HA-E4DΔ60-Pst). 24 h after transfection cells were pulsed with 50 μM BrdUrd for 4 h and then fixed and stained with anti-BrdUrd and anti-HA antibodies. Results are the mean of at least four independent experiments and were obtained by scoring more than 500 cells for each transfected construct in every cell line. Percentage of S-phase inhibition = [(percentage of cells in S-phase in the background − percentage of cells in S-phase in the transfected cells)/(percentage of cells in S-phase in the background)] × 100. *D*, 3×10^4 GFP/HCT-116 or GFP-Gas2DN/HCT-116 cells were plated as described under "Material and Methods." The colony number obtained with GFP stable expressing cells was set as 100%. Experiments were repeated five times with consistent and repeatable results.

shown in Fig. 3*D*, whereas Calpastatin can efficiently block β-catenin degradation (compare *lanes 4-6* with *lanes 1-3*), Gas2DN can effectively recover the inhibitory effect of Calpastatin on m-calpain (*lanes 7-9*). The same amount of *in vitro* translated (IVT) glyceraldehyde-3-phosphate dehydrogenase failed to show a similar result (data not shown). To provide final evidence of the calpain activator properties of Gas2DN, its effect on calpain activity was tested in a biochemical *in vitro* assay measuring calpain activity. Rat skeletal muscle m-calpain activity was measured as described under "Materials and Methods" and was monitored in the presence of both the recombinant inhibitors Gas2wt and Calpastatin and in the presence of Gas2DN. Whereas the function of calpain, as can be observed in Fig. 3*E*, was efficiently blocked by its inhibitors Gas2 and Calpastatin, the presence of recombinant Gas2DN induced an increase in calpain activity, suggesting a potential

activator role for this protein. With the same biochemical approach we confirmed *in vitro* the ability of Gas2DN to counteract the inhibitory effects of both Gas2 and Calpastatin (Fig. 3*F*). We therefore conclude that Gas2DN can disengage endogenous m-calpain from both its inhibitors, Gas2 and Calpastatin, possibly because both inhibitors share the same binding region on the calmodulin-like domain of m-calpain, as already described (11, 17).

Calpain Regulates β-Catenin Functions Independently of GSK3β Activity and of the Proteasome—After showing Gas2/Calpastatin/Gas2DN as tools to regulate β-catenin levels and function via calpain, we investigated whether β-catenin could be regulated in the absence of GSK3β, a critical component of the multimolecular complex responsible for targeting β-catenin to the proteasome (18). As a first approach, the Gas2DN effect as a general calpain activator was analyzed on stabilized β-catenin in U2OS cells treated with LiCl, a known inhibitor of GSK3β activity (19). Under these conditions, Gas2DN overexpression was still able to reduce β-catenin levels (Fig. 4*A*) as well as to decrease its transcriptional competence (Fig. 4*B*), indicating that its effect on β-catenin is a GSK3β-independent process. However, because β-catenin can also be degraded in a proteasome-dependent, but GSK3β-independent, mechanism (20, 21), we investigated whether calpain is truly acting in a proteasome-independent manner by using lactacystin as a proteasome-specific inhibitor. To set the conditions we followed the well characterized p53 protein stability as an independent marker of proteasome inhibition. In U2OS cells we found that p53 reaches its highest levels 8 h after lactacystin treatment and maintains such levels for 24 h (data not shown), suggesting that during this time the proteasome system can be considered significantly blocked. In the same context, transfections of U2OS cells with siControl or si m-calpain confirmed that m-calpain knock down causes the increased levels of β-catenin even when the proteasome is blocked (Fig. 4*C*). The same effects were observed when lactacystin-treated cells were transfected with Gas2 and Calpastatin (Fig. 4*D*). Altogether, these data indicate that calpain acts in a GSK3β- and proteasome-independent manner to regulate β-catenin levels and functions. To confirm this hypothesis, we chose two alternative cell lines, respectively knocked out for GSK3β (mouse embryo fibroblast GSK3β−/−) and containing a thermolabile ubiquitin-activating enzyme (E1) that is inactivated at 39 °C, thus preventing ubiquitination (TS20 cells). In GSK3β−/− mouse embryo fibroblasts, Gas2wt was still able to stabilize β-catenin (Fig. 4*E*), and, more importantly, Gas2DN maintained its ability to reduce β-catenin levels either alone or when cotransfected with Gas2. Finally, as shown in Fig. 4*F*, transfection of TS20 cells at the non-permissive temperature (39 °C) with Gas2 or Calpastatin resulted in the accumulation of endogenous β-catenin, confirming that ubiquitination of β-catenin does not seem to be required for its regulation by calpain system. We can therefore conclude that calpain modulates β-catenin levels and activity independently of both GSK3β and proteasome function, thus indicating that the calpain pathway could play an alternative role when canonical proteasome-mediated β-catenin degradation is impaired.

Gas2DN Modulates the Activity of β-Catenin in Colon Carcinoma Cells, Reducing Their Proliferation and Anchorage-independent Growth—Colon cancer cells represent an interesting model to study the relevance of calpain pathway because in this context canonical proteasome-mediated β-catenin degradation is strictly impaired. Therefore we decided to test whether Gas2DN is able to induce endogenous β-catenin degradation in HCT-116 colon carcinoma cells, which harbor mutations in β-catenin that prevent proteasome-dependent turnover. We generated a cell line stably expressing GFP-Gas2DN or GFP (GFP-Gas2DN/HCT-116 and GFP/HCT-116). Lysates of these cells were then separately incubated with $CaCl_2$ to activate m-calpain or EGTA to block its activity. Aliquots were taken at defined time points (0, 4, and 8 h) to follow the kinetics of β-catenin degradation. As can be observed in Fig. 5*A*, *lanes 3* and *4*, addition of calcium, but not EGTA, to the cell lysates promoted β-catenin degradation. Notably, GFP-Gas2DN/HCT-116 cells show degradation of β-catenin starting earlier and becoming significantly more pronounced compared with GFP/HCT-116 (Fig. 5*A*, *lanes 3* and *4*). In all cases β-catenin cleavage was inhibited by EGTA (*lanes 1* and *2*).

These results indicate that endogenous m-calpain can degrade endogenous β-catenin in cells that are defective in regulating its levels through the classical proteasomal pathway. Even more interesting, it suggests the possibility that Gas2DN can stimulate such degradation.

We then tested the effect of Gas2DN in various colon carcinoma cell lines, where proteasome degradation is blocked because of mutations of β-catenin itself (HCT-116 and LS-174) or of its regulator APC (HT-29). As shown in Fig. 5*B*, overexpression of Gas2DN induced a significant reduction in β-catenin activity in all tested cells. These results suggest that overexpression of Gas2DN can regulate the activity of a form of β-catenin that is not under the control of proteasome-dependent degradation.

Because accumulated evidence demonstrates that β-catenin alters cell cycle progression (22), we tested whether the negative effect of Gas2DN on β-catenin was also manifested in the proliferation rate of colorectal cancer cells. Transient overexpression of Gas2DN in HCT-116 and HT-29 cells showed a significant inhibition of S-phase entry as assessed by BrdUrd incorporation assay (Fig. 5*C*).

Finally, because a characteristic feature of transformed cells is their ability to grow in an anchorage-independent way and β-catenin is involved in this process, we compared the ability of GFP-Gas2DN/HCT-116 or GFP/HCT-116 cells to form colonies in semisolid medium. Significant reduction in the ability to form colonies for Gas2DN-expressing cells was observed with respect to GFP-expressing cells (Fig. 5*D*). Altogether, these results demonstrate that Gas2DN modulates the function of deregulated β-catenin and reduces both proliferation and anchorage-independent growth in a context of deregulated β-catenin.

DISCUSSION

The intracellular signaling pathway of Wnt is most conserved during evolution and regulates cellular proliferation, morphology, motility, cell fate, and organ development. Aberrant Wnt signaling pathway is an early event in 90% of colorectal cancers that results in β-catenin accumulation. It can occur through inefficient phosphorylation of β-catenin by GSK3β or because of mutations on APC, β-catenin, and/or axin. As a result of a defective degradation via proteasome, accumulated β-catenin translocates into the nucleus and becomes transcriptionally active, enhancing expression of genes regulating cell proliferation and survival. Our results indicate a constitutive calpain-dependent mechanism regulating β-catenin stability and function and suggest Gas2DN as a reagent to activate calpain pathway, emerging as an effective way to reverse oncogenic β-catenin/LEF/TCF signals that occur when β-catenin degradation via the proteasome is defective (Fig. 6).

In this study, we have shown the physiological and constitutive involvement of calpain in the regulation of signaling β-catenin, which can translocate into the nucleus where it functions as a transcription factor. The physiological involvement of calpain in the regulation of β-catenin was demonstrated by

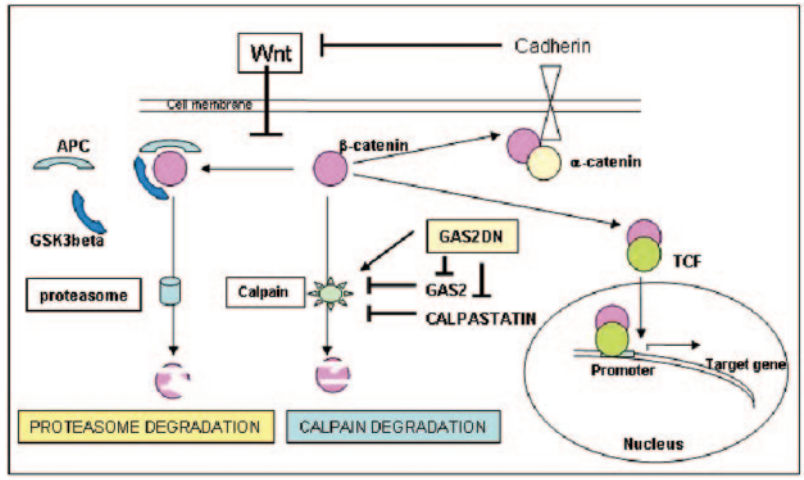

FIG. 6. **A working model for the regulatory network involving Gas2, Calpastatin, Gas2DN, calpain, and β-catenin.** Control over calpain activity by overexpression of its inhibitors Gas2 and Calpastatin is crucial to up-regulate β-catenin levels, to relocate β-catenin to the nucleus, and therefore to increase its signaling properties through a pathway that does not require the proteasome function. Gas2DN, disengaging calpain from both its inhibitors, stimulates endogenous calpain activity *versus* β-catenin and therefore can be used to down-regulate β-catenin signaling potential in the context of colon cancer, where accumulated β-catenin cannot be degraded through the classical proteasome pathway.

siRNA-mediated knock down of calpain that resulted in a significant increase of both the levels and transcriptional activity of β-catenin and in its clear translocation into the nucleus (Fig. 1). Control over calpain activity through the overexpression of Gas2 and Calpastatin was shown to be crucial for β-catenin regulation (Fig. 2). To better characterize the effects of both inhibitors, we studied the effects of Gas2DN, which was initially defined as Gas2wt-dominant negative form, being able to still bind calpain but not to inhibit its activity. We provided evidence that Gas2DN can be considered a novel and specific molecule to modulate calpain activity because it can efficiently counteract the inhibitory effect of both Gas2 and Calpastatin *in vivo*. The recombinant Gas2DN protein can also be used to stimulate endogenous calpain activity in a biochemical *in vitro* assay (Fig. 3). These results suggest that Gas2DN can disengage endogenous calpain from both its inhibitors, Gas2 and Calpastatin, possibly because Gas2 and Calpastatin share the binding region on the calmodulin-like domain of calpain. In fact, we have previously demonstrated that the amino-terminal region of Gas2 (which comprises Gas2DN) binds subdomains III and IV of m-calpain and that the Gas2 CT region is required to exert the inhibitory function (11). The inhibitory domains of Calpastatin are defined by three highly conserved regions, A, B, and C. Whereas subdomain B is responsible for inhibition, regions A and C are responsible for binding calpain in a strictly Ca^{2+}-dependent manner to subdomains III and IV, where Gas2 also binds (23, 24). Binding of regions A and C of Calpastatin shifts the conformational equilibrium of calpain toward the active form, but activation of calpain is not manifested because its active site is blocked by subdomain B. Regions A and C, on the other hand, lack this inhibitory potential and have been suggested as potential activators for m-calpain (24). Similarly, Gas2DN, lacking the carboxyl domain of Gas2 that is responsible for the calpain inhibitory function, could be involved in calpain activation.

In all tested cases, regulation of calpain proteolytic activity through overexpression of Gas2DN was shown to be crucial to regulate both β-catenin protein levels and its transcriptional activity in the absence of GSK3β or under conditions where GSK3β is blocked through the addition of LiCl. The use of LiCl has been reported to similarly block GSK3α (25), thus excluding any role for both GSK3 isoforms (Fig. 4).

These results indicate that the calpain pathway works independently of the β-catenin phosphorylation state as regulated by GSK3 activity. Moreover, because it has been demonstrated that β-catenin can also be degraded in a GSK3β-independent but proteasome-dependent manner (20, 21), we performed a set of experiments to exclude any involvement of the proteasome in the calpain-mediated degradation of β-catenin. We provide evidence that when the proteasome pathway is impaired by using a specific proteasome inhibitor the alternative calpain-dependent β-catenin degradation is still active. The same conclusions were obtained using a mutant cell line with a thermolabile E1 ubiquitin-activating enzyme, demonstrating that calpain function toward β-catenin can occur in the absence of a functional proteasome pathway. Altogether, these observations suggest that under physiological conditions the calpain system can cooperate with the proteasomal pathways to regulate β-catenin degradation and could therefore represent a mechanism to recapture non-phosphorylated and accumulated cytoplasmic β-catenin, targeting it to an alternative degradation when the most established proteasome pathway becomes altered during tumorigenesis, such as in a colon carcinoma context. Our findings are in apparent contrast with a prior study on prostate tumor cells, in which it has been reported that calpain cleavage, by removing the NH_2-terminal regulatory domain of the β-catenin protein, leads to the accumulation of a stable 75-kDa deletion able to increase TCF/LEF-dependent transcription (26). However, in the context of colon cancer cells we observed that calpain-dependent cleavage of endogenous β-catenin causes a complex pattern of smaller degradation products (Fig. 5*A* and data not shown), suggesting either that this unique 75-kDa proteolytic cleavage product is not formed in colon cancer or that it is unstable in these cells. The different degradation patterns observed in colon cancer cells and prostate cells could be due to the different complexes formed by β-catenin in the two cell types, limiting further degradation of the 75-kDa product in the case of prostate cells by calpains, or to amino acid difference of β-catenin at the cleavage sites. The molecular basis of this difference represents an interesting subject for future investigations.

Considering the relevance of β-catenin function in colon cancer cells, we showed that colon cancer cells stably expressing Gas2DN are suppressed both in proliferation and anchorage-independent growth (Fig. 5). These results underline the relevance of calpain activity toward β-catenin regulation. It is worth noting the recent evidence obtained with microarray analysis on colon carcinoma cells associating increased expression of Calpastatin mRNA (27) with increased metastatic potential. This could represent an interesting system to definitely link β-catenin and the calpain system, possibly suggesting a potential mechanism to alternatively regulate β-catenin degradation along with the metastatic phenotype.

In conclusion, we demonstrated that accumulated β-catenin that cannot be degraded through the proteasome can be targeted to the alternative calpain-dependent degradation, thus

blocking its oncogenic activity. In this respect, Gas2DN emerged as a tool to modulate the protease activity. Interestingly Gas2DN (amino acids 1–171) contains one Calponin homology domain (CH domain) (amino acids 37–153). This domain has been found in a variety of proteins ranging from actin cross-linking to signaling elements and has been proposed to function with a quite striking variability (28). In general, proteins containing a single CH domain act as regulatory proteins (29). It will be interesting to clarify whether and how the single CH domain could be linked to modulation of calpain activity. In this view, it can be hypothesized that the CH domain could function in the recruitment of calpain to a specific subcellular compartment for localized activation, therefore providing in such a microenvironment the requested calpain conformational changes necessary to reduce the calcium requirement for the activation. Our findings reveal a mode of control over calpain that is extensively implicated in diverse cellular and developmental contexts and open interesting avenues on the mechanisms regulating its activation, thus complementing the described requirement for Ca^{2+} signaling (30–32). Further biochemical *in vitro* and *in vivo* studies on the involvement of the CH domain contained in Gas2 with respect to calpain activity could finally provide new tools for therapeutic intervention in the control of the Wnt/β-catenin signaling pathway and, specifically, for the treatment of colon cancer.

In addition to its function in the conventional Wnt signaling pathway, β-catenin also binds tightly to the cytoplasmic domain of type I cadherins and plays an essential role in structural organization and function of cadherins by linking cadherins through α-catenin to the actin cytoskeleton (33, 34). Progress in understanding β-catenin signaling, as well as cadherin-mediated cell adhesion, generally followed separate lines of investigation. However, it is clear that there are many connections between these pathways; alteration in cell fate, adhesion, and migration are, for example, characteristics of cancer in which cells ignore normal regulatory cues from their environment. Unchecked Wnt signaling (4) and/or the loss of cell-cell adhesion (35–37) are involved in cancer induction and progression. Moreover, recent studies have shown that both Wnt signaling and cadherin-mediated cell-cell adhesion are important in the organization and maintenance of stem cells (38). The critical component of these pathways is β-catenin, and the key events are the regulation of β-catenin stability and availability. In this view, calpain could really represent an important bridge to switch the signaling of nuclear β-catenin that affects gene expression to a signaling toward the cytoskeleton and cell polarity in tissues. In fact, whereas inhibition of calpain activity through its endogenous inhibitors causes β-catenin translocation to the nucleus where it becomes transcriptionally competent (Fig. 1), stimulation of calpain activity induces β-catenin exit from the nucleus (9) (Fig. 3), where it lacks its transcriptional ability (Fig. 3) and could be strictly involved in cytoplasmic signaling. Cell migration requires dynamic interaction among a cell, its substratum, and the actin cytoskeleton. It is worth noting that calpain is an attractive candidate to be a regulator of cell migration and membrane protrusion through its capacity to regulate focal adhesion dynamics and rear retraction (39, 40). In this context it will be very interesting to connect the regulation of β-catenin by the calpain system to the described role of calpain activity with respect to E-cadherin degradation (41, 42) to contribute to the integration of the complex and still puzzling interplay of cell motility, cytoskeleton rearrangement, and cell polarity required during tissue morphogenesis. In this perspective tight and localized control on calpain activity could represent a critical link for the different β-catenin actions both during development and in a cancer context.

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The Calpain System Is Involved in the Constitutive Regulation of β-Catenin Signaling Functions

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