Xenopus Skip Modulates Wnt/β-Catenin Signaling and Functions in Neural Crest Induction*

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The β-catenin–lymphoid enhancer factor (LEF) protein complex is the key mediator of canonical Wnt signaling and initiates target gene transcription upon ligand stimulation. In addition to β-catenin and LEF themselves, many other proteins have been identified as necessary cofactors. Here we report that the evolutionally conserved splicing factor and transcriptional co-regulator, SKIP/SNW/NcoA62, forms a ternary complex with LEF1 and HDAC1 and mediates the repression of target genes. Loss-of-function studies showed that SKIP is obligatory for Wnt signaling-induced target gene transactivation, suggesting an important role of SKIP in the canonical Wnt signaling. Consistent with its involvement in β-catenin signaling, the C-terminally truncated forms of SKIP are able to stabilize β-catenin and enhance Wnt signaling. In Xenopus embryos, both overexpression and knockdown of Skip lead to reduced neural crest induction, consistent with down-regulated Wnt signaling in both cases. Our results indicate that SKIP is a novel component of the β-catenin transcriptional complex.

Wnt/β-catenin signaling plays a pivotal role during vertebrate embryonic development as well as in tumorigenesis by promoting cell differentiation and proliferation (1–4). The key player of this signaling cascade is β-catenin, first identified as a core component of the cell adhesion complex and subsequently also found as a transcriptional co-activator (5–7). The cytosolic pool of β-catenin is constantly degraded through axin/APC/GSK3β/CK1δ-mediated phosphorylation and ubiquitination (4, 6, 8, 9). Upon Wnt stimulation, the cytosolic β-catenin is stabilized and translocates into the nucleus where it binds to high mobility group (HMG) box transcriptional factor TCF/LEF family members and initiates target gene expression. In the absence of Wnt signaling, the TCF/LEF proteins bind to the Wnt-responsive element of the target gene promoter in a sequence-specific manner and function as transcriptional repressors to prevent leakage of the target genes. This repressive activity of TCF/LEF is achieved by recruiting transcriptional repressors Groucho-HDAC and thus maintaining a closed chromatin conformation (10–13). Binding of β-catenin is believed to convert repression toward the activation state by displacing Groucho-HDAC with the transcriptional activator CBP (CREB-binding protein)/P300 (14–17). It also has been reported that β-catenin interacts with Hyrax, which links the β-catenin–LEF complex directly to the RNA polymerase II machinery (18, 19). However, the dynamic process of converting this complex from repression to activation is not fully understood.

One key event of Wnt signaling activation is the accumulation of nuclear β-catenin. In normal cells, an elevated β-catenin level is driven by extracellular Wnt ligand via activation of the cell surface receptor frizzled-LRP5/6 complex, whereas in certain cancer cells, it is caused by enhanced β-catenin stability due to, in most cases, APC or β-catenin mutations (20–23). Recently, ectopic β-catenin accumulation, which is crucial for tumor growth and metastasis, has been found in pancreatic cancer cells because of an elevated expression of ATDC (ataxin-telangiectasia group D-complementing gene), which interferes with the β-catenin degradation complex (24). This finding further exemplifies the intracellular promoting nature of cancerous β-catenin and suggests that misexpression of any protein capable of enhancing β-catenin stability may be oncogenic.

Skip is an evolutionally conserved 60–80-kDa nuclear protein, initially identified as Ski-interacting protein. The skip genes are present throughout the eukaryotic phyla, most likely with one gene per genome (25, 26). It has been shown that the yeast homologue prp45 is essential for cell viability, and in Caenorhabditis elegans, Ceskip is indispensable for development (27–29). This general requirement is likely due to its involvement in RNA splicing as well as RNA polymerase II-mediated transcription (30–32). However, accumulating evidence suggests that Skip also serves as a cofactor of several sequence-specific transcriptional factors, including Smad2/3, CFBl, vitamin D receptor (VDR), and MyoD, and thus is linked to signal transduction processes (33–36). Structurally, its SNW domain, the most conserved and characteristic region, is located in the
center of the Skip protein and mediates the interaction of Skip with many partners such as Ski, Smad2/3, and VDR (26, 36–38). The transcription regulating activity of Skip is probably best documented in Notch signaling, where it binds to NotchIC (intracellular domain of the Notch receptor) and is required for the replacement of SMRT/HDAC from the transcription factor CBF1 and the initiation of target gene expression by NotchIC. Further study has demonstrated that Skip, CBF1, and either NotchIC or SMRT form a ternary complex through binary interactions and has suggested a model in which NotchIC activates target promoters by competing with the SMRT-corepressor complex via interactions with both CBF1 and Skip (34, 39). The general scheme emerging is that Skip may also work as a scaffold protein in assembling a fully functional transcription initiation complex together with sequence-specific transcriptional factors.

In a functional screen, we identified a truncated Xenopus Skip as able to stabilize β-catenin and enhance canonical Wnt signaling. Analysis in mammalian cells demonstrated that both overexpression of full-length and knockdown of endogenous SKIP caused down-regulation of Wnt/β-catenin signaling, suggesting that SKIP is a necessary component of the Wnt/β-catenin pathway. Biochemical experiments showed that SKIP is associated with the LEF1-HDAC1 complex and involved in transcriptional regulation of the target genes. Furthermore, we report that in Xenopus embryos, Skip is involved in neural crest induction, a process in which Wnt/β-catenin signaling is clearly implicated.

**EXPERIMENTAL PROCEDURES**

Functional Screen and Constructs—A Xenopus tropicalis cDNA library was used in the functional screen (40). Pools of plasmid DNA from the library were co-transfected with mouse Wnt1, Wnt-responsive reporter TOPflash, and pRL-TK, as a loading reference, into HEK293T cells in 96-well plates using a FuGENE 6 transfection reagent (Roche Applied Science). Luciferase reporter assays were performed 40 h post-transfection using a Dual-Luciferase assay kit (Promega). TOPflash luciferase activity was normalized to that of Renilla. Plasmids per well were: SuperTOPflash, 15 ng; pRL-TK, 0.5 ng; Wnt1, 5 ng; β-catenin, 5 ng; SA-β-catenin, 5 ng; 107-2, 5 ng; and all other deletions, 5 ng. pCS2+ DNA was used to adjust the DNA amount to 150 ng/well.

siRNA—Two independent siRNAs against human SKIP mRNA were synthesized at GenePharma Co. Ltd. with the following sequences: SKI nRNA1, GCACGTGAGAGGGATGAAA; SKI nRNA2, CCCCATATGCGCAAGTCAT. siRNAs were transfected into HEK293T cells using Entranster™-R transfection reagent (Engreen Biosystem Co. Ltd.) with a final concentration of 100 nM. 24 h after siRNA transfection, plasmid DNA samples were transfected as usual.

Co-immunoprecipitation Assay, Cytosolic Fraction, and Western Blot—HEK293T cells were transfected in 6-well plates, and 48 h after transfection, cells per well were lysed in 300 μl of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 8.0, 1% Triton X-100) containing protease inhibitor mixture (Roche Applied Science) for 20 min on ice. After centrifugation at 14,000 rpm at 4 °C, 10% of the supernatant was kept as the loading control. The rest of the supernatant was incubated with FLAG-M2 beads (Sigma) at 4 °C for 4 h or with other antibodies at 4 °C for 4 h followed by incubation with protein A/G-Sepharose (Santa Cruz Biotechnology) overnight. The beads were then washed three times with lysis buffer at 4 °C for 5 min each time, and the bound proteins were eluted with SDS-loading buffer at 95 °C for 5 min. Total lysates and immunoprecipitates were subjected to SDS-PAGE and Western blot analysis. For two-step immunoprecipitation (IP), the first IP (IP1) was carried out with FLAG-M2 beads at 4 °C for 4 h, and immunoprecipitates were washed three times. The bound proteins were eluted with SDS-loading buffer at 4 °C for 5 min each time, and the bound proteins were eluted with SDS-loading buffer. Cytosolic fractions were prepared as described previously (41). For Western blot, protein samples were separated by 10 or 12% SDS-PAGE. Anti-FLAG (M2, Sigma), anti-Myc (Santa Cruz Biotechnology), anti-HA (Santa Cruz Biotechnology), anti-β-catenin (BD Biosciences), and anti-SKIP (Santa Cruz Biotechnology) antibodies were used and followed by incubation with anti-mouse or anti-rabbit IgG-horseradish peroxidases. Chemiluminescence detection (Pierce) was carried out according to the manufacturer’s instructions.

Chromatin Immunoprecipitation Assay (ChIP) and Real-time PCR—Chromatin immunoprecipitation assays were carried out as described previously (42). For real-time PCR, the Mx3000P™ real-time PCR system, Evagreen dye (Biotium), and 40 cycles of amplification were used. PCR primers for the c-myc Wnt-responsive element (WRE) were used accordingly (43).

Immunostaining Assay—HeLa cells were seeded in 6-well plates with glass coverslips. At 24 h after transfection, cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 10 min. Cells were washed twice in phosphate-buffered saline for 5 min and post-transfection using a Dual-Luciferase assay kit (Promega).
stained with 4,6-diamidino-2-phenylindole (Roche Applied Science). Finally, cells were mounted in ProLong Gold antifade reagent (Invitrogen). Images were recorded using the Olympus FluoviewTM FV1000 confocal microscope.

Embryos, Microinjection, and Explants—Adult Xenopus laevis frogs were obtained from Nasco. Female frogs were induced to ovulate by being injected with 300–500 IU of human chorionic gonadotropin and incubation at 18–22 °C for about 12 h; they were squeezed manually to yield eggs. Testes were removed from male frogs and minced in amphibian Ringer’s solution (6.6 g/liter NaCl, 0.15 g/liter KCl, 0.15 g/liter CaCl2, 0.05 g/liter NaHCO3). Eggs were mixed thoroughly with sperm suspension (one-quarter testis in 0.3 ml of frog Ringer’s solution) using a blue micropipette tip. 3 min later, the Petri dish was filled with 0.1× Barth’s buffer (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.82 mM MgSO4·7 H2O, 0.33 mM Ca(NO3)2·4 H2O, 0.41 mM CaCl2, 10 mM HEPES, pH 7.4). After 30 min, the fertilized eggs were dejellied with 2% Barth’s buffer. To explant animal caps, blastula-stage embryos were transferred back to 0.1× Barth’s buffer in a Petri dish. For micro-injection, embryos were transferred to 0.3× Barth’s buffer in a Petri dish with a 2% agarose bottom and microinjected at the 2/4-cell stage using the Harvard microinjection apparatus. lacZ RNA was co-injected as a lineage tracer. After injection, the embryos were transferred back to 0.1× Barth’s buffer and allowed to develop at room temperature. To explant animal caps, blastula-stage embryos were transferred into 2% agarose dishes containing 0.5× Barth’s buffer. The vitelline envelope was removed manually, and a small square of animal pole tissue was dissected with hair knives. The explants were cultured in 0.5× Barth’s buffer until the indicated stage and harvested for further analysis.

RNA Synthesis and in Situ Hybridization—To in vitro synthesis capped mRNAs, the template plasmids were linearized with NotI and transcribed with SP6 polymerase according to the manufacturer’s instructions (MEGAscript kit, Ambion). For DIG RNA probes, the plasmids were linearized and transcribed with T7, T3, or SP6 RNA polymerase (Promega). The whole mount in situ hybridizations were performed following the standard protocol (44).

Morpholino Antisense Oligonucleotides—The 5′-nucleotide sequences of two pseudoalleles for the X. laevis skip gene were obtained from the expressed sequence tag data base. According to these 5′-UTR sequences, two antisense morpholino oligonucleotides targeting two pseudoalleles, respectively, around the ATG start codon were designed: skip-MO1, GCGATTTTCTCTGTCGATCTCTTCC; 5MM MO, GCGATTTACTCTCTCGAACTCTACC; skip-MO2, CAACGCCATTCTCGCTGAGTTTTC.

Reverse Transcription (RT)-PCR—RT-PCR was carried out as described (45). The gene-specific primers used were H4 (46); Xbra (47); NCAM (48); slug (49); human AXIN2 (43); X. laevis skip (forward, GACAGAGACAGCTCTGGACAAA; reverse, CCGTGGCAGCTTCGAGTTGA); human β-catenin (forward, CAAGCCCAAGAGTTACACAAGAAACGG; reverse, CCATCACAATGATAGTCCAGACC).

RESULTS

Skip Inhibits Wnt Signaling and Neural Crest Induction—In a Wnt-responsive reporter-based functional screen in HEK293T cells (40), we identified a cDNA encoding a C-terminal truncated Skip protein as a potent enhancer of canonical Wnt signaling (data not shown). Sequencing analysis indicated an artificial cDNA clone from the misannealing of oligo(dT) to an internal A-rich region of the skip mRNA during cDNA library construction. The truncated Skip contains amino acids 1–314 covering the N-domain and most of the SNW portion (supplemental Fig. 1). The full-length skip cDNA was obtained, and to our surprise, it functioned as a potent inhibitor of β-catenin and Wnt1-induced reporter gene expression in transfected HEK293T cells (Fig. 1, A and B). In addition to signaling by transfected wild type β-catenin, signaling by LiCl, which stabilizes endogenous β-catenin by inhibiting GSK3β, and by S37A-β-catenin, a stabilized mutant, were both significantly down-regulated by Skip (Fig. 1, B and C). Furthermore, we found that Skip inhibited both the mRNA and the protein level of β-catenin (Fig. 1D). The inhibitory activity was not Xenopus Skip specific, as human SKIP was also able to inhibit β-catenin signaling at very low doses (supplemental Fig. 2A). Moreover, the inhibition was not due to massive overexpression. As shown in supplemental Fig. 2B, we detected the total Skip protein level in transfected cells and found that as little as a double amount of Skip, compared with the endogenous amount, inhibited β-catenin signaling in HEK293T cells. The inhibition of Wnt signaling was specific because: 1) the signaling of the TOPflash reporter, which harbors mutant TCF binding sites, was not affected (data not shown); and 2) the wild type Skip enhanced, rather than inhibited, TGF-β signaling (supplemental Fig. 3) as reported previously (36). Together, these results suggest that Skip is able to inhibit canonical Wnt signaling downstream of β-catenin.

As neither the expression nor function of Xenopus skip had been reported, we therefore performed RT-PCR and found that the skip gene is expressed maternally and its expression level persists until the late neurula stage (Fig. 2A). In situ hybridization results (Fig. 2B and supplemental Fig. 4) indicated that the transcripts were located mainly in the animal blastomeres before middle blastula transition. In gastrulae, skip mRNA is detected in the ectoderm and the involuting mesoderm surrounding the forming blastopore. skip expression becomes dominant in the neural ectoderm at late gastrula stage, and a stronger signal is observed at the anterior and anterolateral border. In neurulae, skip is expressed in the head region and the neural tube and weakly in the presomitic mesoderm. At the tail bud stage, skip expression is found in the neural tube, eyes, branchial arches, and subdomains of the brain as well as in the otic vesicle. Considering that the skip gene is widely expressed in early Xenopus embryos, we performed a TOPflash reporter assay and found that skip mRNA injection inhibited β-catenin signaling (Fig. 2C), similar to the results in HEK293T cells. To explore the physiological role of skip during Xenopus embryogenesis, in vitro synthesized skip mRNA was microinjected into 4-cell-stage embryos. The injected embryos were largely normal until the tail bud stage (data not shown). However, in situ hybridization indicated that in the injected side, the expression
cause Skip modulates TGF-β with Smad2/3 and CBF1, respectively (34, 36), we examined the overexpression of Skip. Skip, GFP, and dehydrogenase (Fig. 2A), Sox3 (Fig. 2D), or the forebrain marker Otx2 (data not shown). To confirm a direct role of Skip in neural crest induction, we performed animal cap assays in which slug expression was not significantly changed. Thus, these results support our conclusion that overexpression of Skip blocks Wnt/β-catenin signaling.

**Skip Forms a Ternary Complex with LEF1 and HDAC1**—Because Skip modulates TGF-β and Notch signals by interacting with Smad2/3 and CBF1, respectively (34, 36), we examined the interactions of Skip with multiple TCF/LEF family members in HEK293T cells by co-IP experiments. As shown in Fig. 3A, Skip formed complexes with all TCF/LEF proteins tested. In the absence of Wnt stimulation, the TCF/LEF family proteins function as transcriptional repressors by recruiting the Groucho-TLE-HDAC complex. We therefore tested whether Skip represses TCF/LEF target gene expression by recruiting HDAC, a known mechanism of Skip in mediating transcriptional repression (50). As shown in Fig. 3B, a robust interaction between Skip and HDAC1 was observed. Because each two of these three proteins are able to form bilateral interactions, we sought to further address whether these three proteins exist in the same complex using the two-step IP assay. The lysates of transfected cells were first immunoprecipitated by anti-FLAG antibody to pull down FLAG-Skip and then eluted with FLAG peptide. Subsequently, the eluate was further precipitated by anti-HA to immunoprecipitate HA-HDAC1. As shown in Fig. 3C, lane 2, after the two-step IP, LEF1 was recovered only when all three proteins were co-expressed but not when HDAC1 was absent. This result strongly suggested that LEF1-Skip-HDAC1 formed a ternary complex. Because Skip interacts with both LEF1 and HDAC1, we addressed its role in the induction of neural crest marker slug was clearly down-regulated (52.9%, n = 34), accompanied by a slight inhibition of midbrain-hindbrain boundary marker engrailed2 (en2) (57.7%, n = 26) (Fig. 2D). Skip overexpression did not affect the pan-neural marker Sox3 (Fig. 2D) or the forebrain marker Otx2 (data not shown). To confirm a direct role of Skip in neural crest induction, we performed animal cap assays in which slug expression was induced by Wnt signaling in neuralized animal cap cells. As shown in Fig. 2E, overexpression of Skip completely inhibited slug expression induced by Noggin/Wnt cooperation, consistent with the in situ hybridization result in whole embryos. In the same experiments, NCAM (Fig. 2E), Sox3, and en2 (data not shown) expression was not significantly changed. Thus, these results support our conclusion that overexpression of Skip blocks Wnt/β-catenin signaling.

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level (Fig. 4A). Furthermore, β-catenin- as well as Wnt1-induced reporter gene expression was dramatically reduced when SKIP was silenced (Fig. 4B and C). The requirement is specific, as the signal was significantly rescued by co-transfection of a minimal amount of Xenopus skip expression plasmid (Fig. 4D).

To confirm that Skip functions downstream of β-catenin, we examined the Wnt-induced cytosolic β-catenin accumulation in SKIP siRNA-transfected cells and found no difference from that of control siRNA-transfected cells (Fig. 4E), further supporting a role of Skip in the nucleus, downstream of β-catenin.

Next, we asked whether skip is required for Wnt-induced target gene expression in HEK293T cells and examined the expression of the direct Wnt target gene AXIN2. As shown in Fig. 4F, LiCl-induced AXIN2 expression was clearly down-reg...
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The skip gene were injected into 4-cell-stage embryos. Both MOs worked efficiently against the corresponding mRNA, blocking protein synthesis (Fig. 5A). As further evidence of specificity, whereas injection of each MO alone caused a weak phenotype, their co-injection elicited an enhanced phenotype (data not shown). skip-MO-injected embryos were largely normal at early stages (data not shown), but at the late neurula and tail bud stages, the skip-MO-injected embryos clearly had fewer pigmented cells and showed an abnormal head structure with small eyes, a less developed forebrain, and a ventrally bent trunk (Fig. 5, B and C). The observed phenotypes are specific because the standard control MO (Std MO) as well as the 5-mismatch MO (5MM)-injected embryos were completely normal, and the abnormalities induced by skip-MO were fully rescued by co-injected skip mRNA (Fig. 5B and data not shown). Because loss of pigment cells indicates a failure of neural crest formation, and because Wnt/β-catenin signaling plays a key role during neural crest induction (51, 52), we examined the expression of several neural plate marker genes in early neurula stage embryos by in situ hybridization. In skip-morphants, expression of the neural crest marker slug was drastically inhibited on the injected side compared with the control side (80.1%, n = 156) (Fig. 5D and supplemental Fig. 5B). Expanded Sox3 expression (69.0%, n = 58) and slightly reduced en2 expression (52.4%, n = 42) were also observed in skip-morphants (Fig. 5D). We also examined slug expression induced by Noggin/Wnt8 with or without MO co-injection in Xenopus animal caps and found that the induction of slug was down-regulated in skip-MO-injected caps (Fig. 5E). Taken together, these results indicate that skip is required for neural crest development in Xenopus embryos, consistent with a requirement for Wnt signaling in vivo.

Domain Analysis of Skip in Modulating Wnt Signaling—Skip protein can be subdivided into three portions with a centrally localized SNW domain, which binds to most Skip-interacting partners, a C-terminal domain, which mediates spliceosome interaction and dimerization, and an N-terminal domain, which is essential for survival in yeast (26, 28). The last six amino acids of the Skip protein have been recognized as a nuclear localization signal (NLS) (53). To explore the function of each domain in affecting Wnt signaling, we created several

### TABLE 1

| Skip Location  | Wnt/β-catenin Interaction with LEF1 | Interaction with HDAC1 |
|----------------|-----------------------------------|-----------------------|
| 1–535 N        | +                                 | +                     |
| 1–529 N/C      | Not affected                      | +                     |
| 1–341 C        | +                                 | +                     |
| 1–173 C        | Not affected                      | –                     |
| 174–341 C      | +                                 | –                     |
| 342–535 N      | +                                 | +                     |
| 174–535 N      | –                                 | +                     |
| 1–341 N        | +                                 | +                     |

* * *
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In agreement with a previous report (53), wild type Skip was exclusively nuclear when transfected into HeLa cells, and deletion of the NLS redistributed part of the protein to the cytosol (1–529) (Fig. 6B). This result differs slightly from that of the previous report where deletion of NLS from human SKIP causes exclusive cytosolic distribution (53); the reason for this discrepancy is unknown. Nevertheless, we found that further deletion of the entire C-domain caused much stronger cytosolic localization (comparing Skip-(1–529) with Skip-(1–341)), indicating that in addition to the NLS, the C-domain also contributes to the nuclear translocation/retention (Fig. 6B). In agreement with this conclusion, Skip-(1–173) and -(174–341) are cytosolic, whereas the other deletions (Skip-(342–535), -(174–535), and -(1–341N)), all of which contain the NLS, are indeed nuclear proteins (Fig. 6B).

In the Wnt-responsive reporter assay, wild type Skip was the strongest inhibitory protein against Wnt, particularly against β-catenin signaling (Fig. 6C). Simply deleting the NLS completely abolished inhibition (compare Skip-(1–529) and -(1–529) in Fig. 6C). Comparisons of Skip-(1–535) with -(1–341) and Skip-(174–535) with -(174–341) clearly indicated that the C terminus (including NLS) is obligatory for inhibition. The absolute requirement of the C terminus in mediating both nuclear localization and inhibition of Wnt signaling suggested again that Skip inhibits β-catenin signaling in the nucleus. The function of the N terminus and the SNW domain is likely to mediate the LEF1-HDAC1 interaction because the SNW domain alone was sufficient for LEF1 binding (Fig. 6D) and the N-domain or SNW domain alone was sufficient for HDAC binding (Fig. 6E). In support of this conclusion, Skip-(174–535) was also found to be inhibitory (Fig. 6C), as it contained the full C terminus as well as the SNW domain, which was sufficient to recruit LEF-HDAC (Fig. 6, D and E). The result, that Skip-(342–535) was inhibitory to β-catenin signaling (Fig. 6C), is puzzling; however, this truncation seemed able to reduce the β-catenin protein level for some unknown reason (see Fig. 7A), which is consistent with the fact that it inhibited neither the basal nor the Wnt1 signaling (Fig. 6C). We concluded that the C terminus of the Skip protein functions to target Skip protein to the proper nuclear localization, the N-terminal and SNW domains mediate LEF1-HDAC1 recruitment, and both together fulfill the inhibitory activity (Table 1).

C-terminally Trimmed Skip Enhances Wnt/β-Catenin Signaling—In contrast to the inhibitory full-length Skip, Skip-(1–341), Skip-(174–534), and Skip-(1–341N) were found to be potent activators for Wnt/β-catenin signaling (Fig. 6C). What are common to them are the existence of an SNW domain and the simultaneous lack of a C-terminal portion, whereas the NLS is irrelevant (Fig. 6, A and C). Correlating well with the activated signaling, the β-catenin protein levels were significantly up-regulated when co-expressed with these constructs (Fig. 7A). The up-regulation happened downstream of the transcription, because the level of β-catenin mRNA was unaffected. The up-regulation was also specific to β-catenin because the level of GFP was constant (Fig. 7B). Human SKIP-(1–341) had the same effects on β-catenin, so the phenomena had nothing to do with species (supplemental Fig. 6, A and B). The amount of transfected SKIP-(1–341) protein was slightly higher than that of the endogenous SKIP but not massively so (supplemental Fig. 6B). Indeed, monitoring protein stability after cycloheximide treatment demonstrated that β-catenin was dramatically stabilized in the presence of Skip-(1–341) (Fig. 7C). The fact that Skip-(1–341)N, a nuclear localized Skip deletion, was also able to stabilize β-catenin (Fig. 7A) and enhance Wnt signaling (Fig. 6C) is somewhat unexpected and suggests that β-catenin stability also might be regulated in the nucleus. We also verified whether endogenous β-catenin can
be modulated and found that, indeed, both the signal and the β-catenin accumulation induced by LiCl and Wnt1 were further enhanced by Skip-(1–341) (supplemental Fig. 6, C and D).

We went on to test whether Skip-(1–341) could promote Wnt signaling in *Xenopus* embryos by injecting *in vitro* synthesized *skip*(1–341) mRNA into 4-cell-stage embryos. Overexpression of Skip-(1–341) induced no more than 10% partial secondary axis and very mild Wnt target gene expression in an animal cap assay (*siamois* and *Xnr3*; data not shown). However, Skip-(1–341) was indeed able to enhance β-catenin protein stability as well as it induced reporter gene expression in *Xenopus* embryos (data not shown). More importantly, when *skip*(1–341) mRNA was injected animaly, the injected embryos developed until the tail bud stage without anterior head structures, mimicking the posteriorizing activity of Wnt signaling during neuralization (Fig. 7D).

These results suggested that, consistent with the observations in mammalian cells, Skip-(1–341) is able to potentiate Wnt/β-catenin signaling in *Xenopus* embryos. It is unclear why its signaling activity is apparently weaker in cleavage embryos; it may be due to different cellular contexts between early and late *Xenopus* embryos.

The enhancement of β-catenin stability by truncated Skip suggested that Skip might interact with β-catenin. We there-
fore tested this possibility by co-IP experiments and observed a weak interaction between full-length Skip and S37A-β-catenin, a stabilized mutant (Fig. 8A). Interestingly, this interaction was strengthened when Skip truncations were applied, especially Skip-(174–535) (Fig. 8A), suggesting that different conformations of Skip protein might interact differentially with β-catenin. The possible interaction between Skip and β-catenin was further supported by the observation that in HeLa cells they were colocalized in a punctate manner in the nucleus (Fig. 8B).

The β-catenin-Skip interaction and nuclear colocalization suggested the possibility that β-catenin and Skip act together on the promoter of target genes.

**DISCUSSION**

*Skip May Work as a Scaffold in β-Catenin-TCF-mediated Transcriptional Regulation—* Our major finding is that in mammalian cells and Xenopus embryos, both under- and overexpression of Skip resulted in down-regulation of canonical Wnt signaling. Our molecular analysis explains this puzzling result. Upon overexpression, Skip was associated with LEF-HDAC
FIGURE 7. C-terminally truncated Skip enhances Wnt signaling. A, some of the Skip deletions cause β-catenin accumulation. Myc-β-catenin was co-transfected with Skip deletions in 293T cells, and cell lysates were analyzed by Western blot (IB, immuno blot). B, Skip-(1–341) promotes β-catenin accumulation without affecting its mRNA level or co-transfected GFP. Skip-(1–341), GFP, and β-catenin were transfected in 293T cells as indicated. Total cell suspension was divided into two parts; one was analyzed by Western blot (WB) and the other by RT-PCR. Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as loading controls. C, β-catenin degradation is drastically blocked in the presence of Skip-(1–341). 100 μg/ml cycloheximide (CHX) was added 40 h after transfection; cells were then collected every 2 h, and the total lysates were analyzed by Western blot. D, Skip-(1–341) induces posteriorization of Xenopus embryos. 4-Cell-stage embryos were injected animal caps, preprolactin was used as the injection control.

FIGURE 8. Skip colocalizes and weakly interacts with β-catenin. A, β-catenin is co-immunoprecipitated with Skip and its deletions. 293T cells were transfected as indicated, and total cell lysates were immunoprecipitated by anti-Myc antibody and analyzed by Western blot (IB, immunoblot). HC, heavy chain of immunoglobulin; LC, light chain of immunoglobulin. B, full-length Skip colocalizes with β-catenin in the nucleus. HeLa cells were co-transfected with EGFP-Skip and red fluorescent protein-β-catenin. The green color stands for Skip and red for β-catenin. The nucleus stained by 4,6-diamidino-2-phenylindole (DAPI) is shown in blue.

and was capable of enhancing the formation of the repressive complex (Fig. 3, C and D). Moreover, our ChIP assay results clearly indicated that the overexpressed Skip was anchored to the Wnt-responsive element of the target gene, c-myc (Fig. 3E). Therefore, it is reasonable to conclude that Skip is able to inhibit canonical Wnt signaling by repressing β-catenin-mediated target gene transcription. Consistent with this conclusion, our results from domain analysis also indicated that a C terminus that targets to the proper nuclear localization and an N-terminal or SNW domain that mediates LEF1-HDAC1 recruitment are both required for the inhibitory activity of Skip protein.

On the other hand, when the Skip level was reduced, Wnt signaling was not able to fully activate target gene expression in either mammalian cells or Xenopus embryos. Our results do not rule out the possibility that Skip functions downstream of transcriptional initiation, e.g. via elongation and splicing. However, these findings are more supportive of a specific role for Skip in β-catenin-mediated transcriptional initiation because: 1) Skip was able to interact with LEF1 and β-catenin (Figs. 3A and 8A); 2) the general transcription of housekeeping genes such as GAPDH, TUBULIN, and Xenopus H4 were not impaired when Skip was silenced; 3) in Xenopus animal caps, skip-MO affected only Wnt but not bone morphogenetic protein (BMP) signaling (see Fig. 5E; NCAM expression would have changed if BMP signaling was affected); and 4) endogenous SKIP protein was anchored to the WRE of the Wnt target gene, c-myc (Ref. 32 and data not shown). It seems that the SKIP levels need to be finely tuned for proper Wnt signaling because a very low and well titrated dose of SKIP was needed to rescue proper Wnt signaling when endogenous SKIP was down-regulated (Fig. 4D).

It has been well established that TCF/LEF proteins constantly bind to the promoters of Wnt target genes to control their transcription (4). In the absence of a Wnt signal, they recruit a complex of co-repressors to keep the locus completely off (14, 15). Upon Wnt stimulation, β-catenin enters the nucleus and, via interaction with TCF/LEF, replaces co-repressors LEF1-HDAC1 recruitment are both required for the inhibitory activity of Skip protein.
Skip Modulates Wnt Signaling

sors with co-activators to initiate gene expression (17). Because of its dual role during this conversion, TCF/LEF proteins have been considered bifunctional molecules. Thus far, the mechanism of this conversion process has not been fully understood. Our results suggest that SKIP is likely to be one more regulatory component necessary for β-catenin-LEF-mediated target gene expression. Biochemical analysis revealed that Skip was able to interact not only with TCF/LEF proteins but also with β-catenin, supporting a potential function for Skip during β-catenin-induced conversion of transcriptional status. Although the interaction detected in this study is a little weak, binding partner(s) and posttranslational modification(s) might contribute to conformational switches, thus affecting β-catenin-Skip interaction in the cells.

The role of Skip in regulating Wnt target gene transcription may be related to its role during Notch signaling, where Skip interacts with both NotchIC and CBF1 and is involved in NotchIC-mediated conversion from transcriptional repression toward the activation of target genes (34). Therefore, our results have added Skip-LEF-HDAC as one more Skip-mediated tripartite complex with three bipartite interactions, in addition to Skip-CBF1-NotchIC, Skip-CBF1-SMRT, Skip-VDR-RXR, and Skip-pRb-E7 (33, 34, 54, 55). How Skip is involved dynamically in these conversion processes of Wnt signaling is not fully understood. However, as proposed in the case of Notch and VDR signaling, Skip may regulate a transition step between the assemblies from repressing to activating complexes.

Skip Mediates Wnt/β-Catenin Signaling in Neural Crest Induction—Neural crest cells are a population of multipotent progenitor cells that later on will give rise to melanocytes and much of the peripheral nervous system and craniofacial skeleton. In Xenopus embryos, these cells are induced at the border of the neural plate and non-neural ectoderm during the gastrula and neurula stages. Although there has been evidence that bone morphogenetic protein (BMP), fibroblast growth factor (FGF), Notch, and retinoic acid signals are involved in neural crest induction, it has become more and more evident that Wnt/β-catenin signaling plays a key role during this multistep induc- 

Our results have further strengthened the findings that β-catenin induces neural crest marker in a cell-autonomous manner (49) and the promoters of Xenopus slug and human SOX9 contain TCF/LEF-binding elements that are important for neural crest-specific expression (62–64). Here we show that the knockdown of skip in Xenopus embryos caused reduced slug expression and significant loss of neural crest derivatives, including pigmented melanocytes. The phenotype is quite similar to that when c-myc was down-regulated (65), another essential gene for neural crest induction. This similarity raises an interesting question for future study, which is whether c-myc expression is under the control of Wnt/β-catenin signaling during neural crest induction. The finding that the injection of skip-MO in neuralized animal caps also inhibited slug induction by Wnt signaling without affecting neuralization strongly suggests that Skip is involved directly in Wnt signaling. Considering the requirement for Wnt/β-catenin signaling in mammalian cells as well as the ability of Skip to interact with β-catenin and LEF, we concluded that Skip is implicated in neural crest induction via the mediation of Wnt signaling.

C-terminally Truncated Skip May Enhance Wnt/β-Catenin Signaling—The stability of β-catenin is crucial for its signaling activity, and its regulation is the key to Wnt signaling transduction; however, its stability is not solely controlled by Wnt ligands. The best example is that in most colon cancer patients, cancerous β-catenin is accumulated because the cells harbor mutations in APC or β-catenin itself, either of which leads to nonphosphorylated and stabilized β-catenin (20–22). Many proteins have been identified which, via different mechanisms, affect β-catenin stability, e.g. ATDC (24) and Pin1 (66), and all are implicated in human diseases. Here we report that the SKIP protein, once it is C-terminally truncated, may turn into a β-catenin-stabilizing factor. How the truncated SKIP stabilizes β-catenin is unknown; however, our results indicated that some artificial C-terminal truncations are able to stabilize β-catenin; and the NLS of SKIP protein is localized in the C terminus, which is very crucial for its normal function, and a small truncation in this region would cause cytosolic distribution. The loss-of-function results did not support a role for wild type SKIP in affecting β-catenin stability. However, we think this observation has at least two implications: 1) it provides supportive evidence that SKIP has something to do with β-catenin, e.g. interaction with β-catenin and/or its stability-regulating factors; and 2) it is very suggestive of the possibility that under pathological conditions, truncated SKIP protein might indeed cause β-catenin accumulation.

In addition, we have noted that in some of the cell lines, for example HeLa cells, an endogenous shorter form of SKIP protein, about 50 kDa, was detectable (data not shown). It will be interesting to identify the protease(s) that regulates proteolysis (at the mRNA level, we found no splicing isoforms corresponding to this shorter form of protein, and we speculate that it is a proteolytic product) and to search for clinical samples of cytosolically localized SKIP. If our hypothesis is correct, β-catenin should be up-regulated in these samples.

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