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NARF, an Nemo-like Kinase (NLK)-associated Ring Finger Protein Regulates the Ubiquitylation and Degradation of T Cell Factor/Lymphoid Enhancer Factor (TCF/LEF)*[S]

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β-Catenin is a key player in the Wnt signaling pathway, and interacts with cofactor T cell factor/lymphoid enhancer factor (TCF/LEF) to generate a transcription activator complex that activates Wnt-induced genes. We previously reported that Nemo-like kinase (NLK) negatively regulates Wnt signaling via phosphorylation of TCF/LEF. To further evaluate the physiological roles of NLK, we performed yeast two-hybrid screening to identify NLK-interacting proteins. From this screen, we isolated a novel RING finger protein that we term NARF (NLK associated RING finger protein). Here, we show that NARF induces the ubiquitylation of TCF/LEF *in vitro* and *in vivo*, and functions as an E3 ubiquitin-ligase that specifically cooperates with the E2 conjugating enzyme E2-25K. We found that NLK augmented NARF binding and ubiquitylation of TCF/LEF, and this required NLK kinase activity. The ubiquitylated TCF/LEF was subsequently degraded by the proteasome. Furthermore, NARF inhibited formation of the secondary axis induced by the ectopic expression of β-catenin in *Xenopus* embryos. Collectively, our findings raise the possibility that NARF functions as a novel ubiquitin-ligase to suppress the Wnt-β-catenin signaling.

The Wnt family of signaling proteins constitutes a large group of highly conserved secreted glycoproteins (1). Wnt proteins are pleiotropic factors that play crucial roles in multiple embryonic developmental processes and also play a role in tumorigenesis (1, 2). Wnt proteins initiate signal transduction via their extracellular surface receptor complex, which is composed of Frizzled proteins (Fz) and lipoprotein receptor-related proteins 5 and 6 (LRP-5/6). In the absence of Wnt stimulation, cytoplasmic β-catenin is maintained at low levels by the continuous process of ubiquitin-proteasome-mediated degradation involving a scaffold complex of axin, adenomatous polyposis coli, (APC) and active glycogen synthasekinase-3β (GSK-3β). In the canonical pathway of β-catenin signal transduction, Wnt signaling relieves this process of proteasome-mediated degradation, and β-catenin consequently accumulates in the cytoplasm. β-Catenin then translocates into the nucleus and forms a transcriptional unit with the HMG box class T cell factor/lymphoid enhancer factor (TCF/LEF)[3] to activate expression of its target genes.

Nemo-like kinase (NLK) was originally isolated as a murine orthologue of the *Drosophila* Nemo by RT-PCR from embryonic mouse brain mRNA using degenerate primers designed for the conserved kinase domains I, VI, VII, and IX of the extracellular-signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK) family (3). The amino acid sequence of the NLK kinase domain shows 39–47% identity to both ERK/MAPK and cyclin-directed kinase 2. The ERK/MAPK family kinases contain a characteristic conserved phosphorylation motif, Thr-*X*-Tyr, in their kinase domain VIII that is required for activation. However, the corresponding sequence in NLK is Thr-Gln-Glu, which is quite similar to the sequence Thr-His-Glu found in some cyclin-directed kinases. The threonine residue in this motif is suggested to be a major site for autophosphorylation of NLK. Thus, NLK may not be a target for an ERK/MAPK activator such as MEK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase) (3).

In our current studies, we demonstrate that NLK is involved in the suppression of the Wnt/β-catenin signaling pathway.

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[3] The abbreviations used are: TCF, T cell factor; LEF, lymphoid enhancer factor; NARF, Nemo-like kinase-associated RING finger protein; NLK, Nemo-like kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; LS-MS/MS, liquid chromatography coupled to tandem mass spectrometry; RT, reverse transcriptase; GST, glutathione *S*-transferase; HA, hemagglutinin; siRNA, small interfering RNA; Ub, ubiquitin.

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NLK inactivates a transcriptional unit composed of β-catenin/TCF/LEF via phosphorylation of TCF/LEF, resulting in the inhibition of binding to its target gene sequences (4, 5). NLK functions downstream of transforming growth factor-β-activated kinase 1 (TAK1), a member of the MAPK kinase kinase family (MPAKKK) (4, 6–8), Wnt1 (9), and Wnt5a (10). Our recent data indicate that, in addition to TCF/LEF, NLK associates with and modulates the activities of other transcriptional factors including Sox11 (11), HMG2L1 (12), and STAT3 (6). This suggests that NLK may contribute to various signaling pathways via its ability to interact with a diverse collection of transcription factors.

Alteration in protein function by covalent post-translational modification, including phosphorylation, acetylation, ubiquitylation, sumoylation, neddylation, glycosylation, and ribosylation, is commonly observed in the cell (13–15). Ubiquitylation and phosphorylation are two major types of protein modification and are observed in all eukaryotes. Ubiquitin is a highly conserved 76-amino acid globular protein that can be ligated to proteins and functions to mark them for destruction by the 26 S proteasome (16). In general, ubiquitylation proceeds via a sequential multienzyme reaction directed by E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme (also known as Ubc), and E3 ubiquitin-ligase. E1 initiates the first step in the ubiquitylation by forming a thiol-ester bond directly with the carboxyl-terminal glycine of ubiquitin in an ATP-dependent reaction. Subsequently, the activated ubiquitin on E1 is transferred to the cysteine within the active site of an E2 by transesterification. In the last step, E3 associates with ubiquitin-charged E2 and the target protein, and facilitates the formation of an isopeptide linkage between the carboxyl-terminal glycine of ubiquitin and the ϵ-amino group of an internal lysine residue on the target proteins, or on the extended polyubiquitin chains attached to the protein (17).

Distinctive types of E3 ligases specifically recognize proteins targeted for ubiquitylation. The number of E3 ubiquitin-ligase candidates has vastly expanded to over 100, and these are classified into four major groups based on several motifs: the HECT (homologous to E6-AP carboxyl terminus) domain, the RING (really interesting new gene) finger domain, the U-box, and the PHD (plant homeo-domain) or LAP (leukemia-associate protein) finger domains (15). The RING finger domain in E3 ubiquitin-ligases consists of the conserved sequence motif: C-X_2-C-$X_{(9-39)}$-C-$X_{(1-3)}$-H-$X_{(2-3)}$-C/H-X_2-C-$X_{(4-48)}$-C-X_2-C, and functions to coordinate two zinc ions in a unique "cross-brace" arrangement (18). The family of RING finger proteins can be divided into the single subunit type E3 and the multisubunit type E3. Single subunit type E3 proteins contain the RING finger domain and the substrate recognition site on the same polypeptide. Examples of this type are Mdm2, which ubiquitylates p53 (19, 20), c-Cbl, which ubiquitylates growth factor receptors (21–23), and the inhibitors of apoptosis (24, 25). Multisubunit type E3 proteins contain minimally a small RING finger protein, a member of the cullin family of proteins, and an associated substrate recognition subunit. Well characterized E3 proteins of this type are SCF (25–28) and anaphase-promoting complex (29–31). The SCF ubiquitin-ligase complex contains Skp1, Cul1 (also known as Cdc53), a small RING finger protein Rbx1 (Hrt1/Roc1), and an F-box protein, which provides substrate recognition. Among the substrates ubiquitylated by the SCF complex are β-catenin (32, 33), IκBα (34), and G_1 cyclin (35).

In the present study, we identified a novel RING finger protein that associates with NLK, and termed it NARF (NLK-associating RING finger protein). Biochemical analyses showed that NARF associates with the carboxyl terminus of NLK and provides E3 ubiquitin-ligase activity that specifically cooperates with the E2 ubiquitin-conjugating enzyme E2-25K (Hip-2/UbcH1). We found that NARF targets TCF/LEF proteins for ubiquitylation, and that NLK kinase activity augments this ubiquitylation. NARF-ubiquitylated TCF/LEF is subsequently degraded by the proteasome, resulting in the down-regulation of TCF/LEF-dependent transcriptional activity. In addition to these observations, we found that microinjection of NARF mRNA inhibited secondary axis formation induced by the expression of β-catenin in *Xenopus* embryos. These results reveal a new mechanism for regulating Wnt-β-catenin signaling involving the ubiquitylation and degradation of TCF/LEF proteins by NARF.

EXPERIMENTAL PROCEDURES

Plasmids—The RING finger domain mutants of *Xenopus* NARF at C17A/C53A (xNARF-CA), human NARF at C18A/C54A (hNARF-CA), and a catalytically inactive mutant of *Xenopus* NLK at K89R (xNLK-KN) were generated by site-directed mutagenesis. For generating GST fusion proteins, *Xenopus* NARF wild-type (xNARF-WT) or RING finger domain mutant (xNARF-CA) cDNAs were subcloned into pGEX-4T-1 (Amersham Biosciences). To produce amino-terminal FLAG-tagged or T7-tagged recombinant proteins, cDNAs xNARF-WT, xNARF-CA, human NARF (hNARF-WT), hNARF-CA, xNLK-WT, xNLK-KN, and mouse Wnt1 were subcloned into pCS2 (36). These pCS2-derived plasmids were used to express recombinant proteins in mammalian cells and as templates for *in vitro* transcription to produce RNA for microinjection into *Xenopus* embryos. Amino-terminal hemagglutinin (HA)-tagged or T7-tagged each of xNARF-WT, E2-25K, human TCF4, and human LEF1 were prepared using a mammalian expression vector. FLAG-NLK-ΔN and FLAG-NLK-ΔC were made by deletion of amino acids 1–201 and 202–447 of *Xenopus* NLK, respectively.

Antibodies and Chemicals—The monoclonal antibodies against T7 (Novagen), FLAG (M2, Sigma), HA (16B12, Babco), GST (26H1, Cell Signaling), ubiquitin (P4G7, Covance), TCF4 (6H5-3, Upstate), and β-actin (AC-15, Sigma) were used for immunoprecipitation and/or Western blotting analysis. Anti-NARF rabbit polyclonal antibody was raised against a peptide corresponding to amino acids 234–245 of human NARF, and then affinity purified using antigen-conjugated affinity Sepharose. The proteasome inhibitor MG132 was purchased from Calbiochem.

Yeast Two-hybrid Screening and cDNA Cloning—The yeast two-hybrid screening was performed as previously described (12). A *Xenopus laevis* oocyte MATCHMAKER cDNA library (Clontech) was screened using pGBDU-xNLK-C, which

encodes amino acids 202–447 of *Xenopus* NLK as bait. 3×10^6 clones from a *Xenopus* oocyte cDNA library were screened.

Immunoprecipitation and Western Blotting Analysis—293 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. 293 cells (5×10^5 cells) in 60-mm diameter plates were transfected with the indicated plasmids by the calcium phosphate precipitation method. The total amount of plasmids was adjusted to 2.5 μg using the empty expression vector. After 24 h post-transfection, cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM dithiothreitol. Lysates were precleared with protein G-Sepharose beads (Amersham Biosciences) and immunoprecipitated with the appropriate antibodies. To detect the ubiquitylation of TCF/LEF, 293 cells were incubated with 10 μM MG132 for 4 h before harvest and lysed in RIPA buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM dithiothreitol. Lysates were treated with 1% SDS at 95 °C for 10 min, and diluted 10-fold before use in immunoprecipitation. For Western blotting analysis, whole cell lysates or immunoprecipitates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were probed with the appropriate antibody, and proteins of interest were visualized with horseradish peroxidase-conjugated mouse or rabbit IgG using Western Lightning™ Chemiluminescence Reagent *Plus* (PerkinElmer Life Science).

In Vitro Ubiquitylation Assay—Bacterially expressed GST-NARF-WT and GST-NARF-CA were purified with glutathione-Sepharose 4B beads (Amersham Biosciences). For *in vitro* ubiquitylation assays, reactions were performed in 30-μl reaction volumes with the following components as indicated: 0.1 μg of GST-NARF, 0.1 μg of rabbit E1 (Boston Biochem), 0.1 μg of E2-25K (Boston Biochem), 5 μg of ubiquitin (Sigma), 50 mM Tris-HCl, pH 7.4, 0.2 mM ATP, 0.5 mM $MgCl_2$, 0.1 mM dithiothreitol, 1 mM creatine phosphate, 15 units of creatine phosphokinase, and 3 μM ubiquitin aldehyde (Boston Biochem). Reactions were incubated at 30 °C for 2 h and terminated by adding 2× SDS sample buffer and boiled to dissociate proteins. The ubiquitylated proteins were resolved by SDS-PAGE and detected by Western blotting analysis with either anti-ubiquitin antibody (P4G7, Covance) or anti-GST antibody (26H1, Cell Signaling). To detect the ubiquitylation of TCF/LEF *in vitro*, ^{35}S-labeled TCF/LEF was prepared with a TNT-coupled reticulocyte lysate system (Promega) and used as a substrate for the *in vitro* ubiquitylation assay. The ubiquitylated [^{35}S]TCF/LEF was separated by SDS-PAGE and visualized by autoradiography.

Pulse-Chase Assay—293 cells (5×10^5 cells) were transfected with the expression plasmids encoding T7-TCF4, FLAG-NARF, and FLAG-NLK. After 24 h post-transfection, cells were pre-starved for methionine and cysteine for 1 h and labeled for 1 h with 100 μCi/ml Pro-mix L-[^{35}S] *in vitro* cell labeling mixture (Amersham Biosciences). Cells were washed twice and incubated with Dulbecco's modified Eagle's medium containing L-methionine and L-cysteine for the indicated chase periods. At each indicated time point, cells were lysed in RIPA buffer and [^{35}S]T7-tagged TCF4 was immunoprecipitated with anti-T7 antibody (Novagen). Precipitated immune complexes were resolved by SDS-PAGE and ^{35}S-labeled TCF4 was visualized by autoradiography.

RNA Interference—We designed small interference RNAs (siRNAs) against human NARF (sense 5′-GAGAGAGAG-CAUGUCCUGA-3′) mRNA along with its corresponding antisense RNA oligonucleotides with two thymidine residues (dTdT) at the 3′ end of the sequence (Dharmacon). The control siRNA (*siControl*, Dharmacon) is targeted against luciferase. These siRNAs were transfected into 293 cells using Lipofectamine 2000 (Invitrogen). After 24 h post-transfection, cells were used for the experiment.

Semiquantitative Multiplex RT-PCR—Semiquantitative multiplex RT-PCR was performed by the procedure described previously, with some modifications (49). Multiplex PCR was carried out with specific primer sets for human *DKK-1* (sense, 5′-AGG-CGTGCAAATCTGTCTCG-3′; antisense, 5′-TGCATTTGG-ATAGCTGGTTTAGTG-3′) and Quantum RNA classic II 18 S internal standards (Ambion). Aliquots of the PCR products were electrophoresed, visualized with SYBR Green I (Molecular Probes, Inc.), and analyzed by computerized densitometric scanning of the images using Light Capture (ATTO).

Reporter Assay—293 cells (2×10^5 cells) in 35-mm diameter plates were transfected with the indicated plasmids by the calcium phosphate precipitate method. The total amount of plasmids for each transfection was equalized among transfections using empty vector. Cells were harvested after 24 h post-transfection and assayed for luciferase activity.

Secondary Axis Formation—Mature oocytes were collected from *X. laevis* ovaries and *in vitro* fertilization was performed as previously described (50). The jelly layer covering the embryos was removed using 3% cysteine. Capped mRNAs for the indicated proteins were prepared using mMESSAGE mMACHINE SP6 kit (Ambion). Synthesized mRNAs were microinjected into two ventral blastomeres at the 4-cell stage and the ectopic axis formations were counted at tadpole stage.

Protein Identification by LC-MS/MS Analysis—The NARF-associated complexes were digested with *Axhromobacter* protease I, and the resulting peptides were analyzed using a nanoscale LC-MS/MS system as described previously (37).

RESULTS

Identification of NARF as an NLK-associating Protein—To identify protein partners that regulate the signaling of NLK, yeast two-hybrid screening was carried out using the carboxyl terminus encoding amino acids 202–447 of *Xenopus* NLK as bait (11). One of the positive clones was found to encode a novel *Xenopus* sequence containing a characteristic RING finger domain. We termed this protein NARF (accession number DQ011285). Sequence analysis of the full-length NARF cDNA revealed that NARF contains a RING finger domain in the amino terminus and is a *Xenopus* orthologue of a previously uncharacterized human RING finger protein RNF138 (NM 016271), sharing 47% identity (Fig. 1*A*).

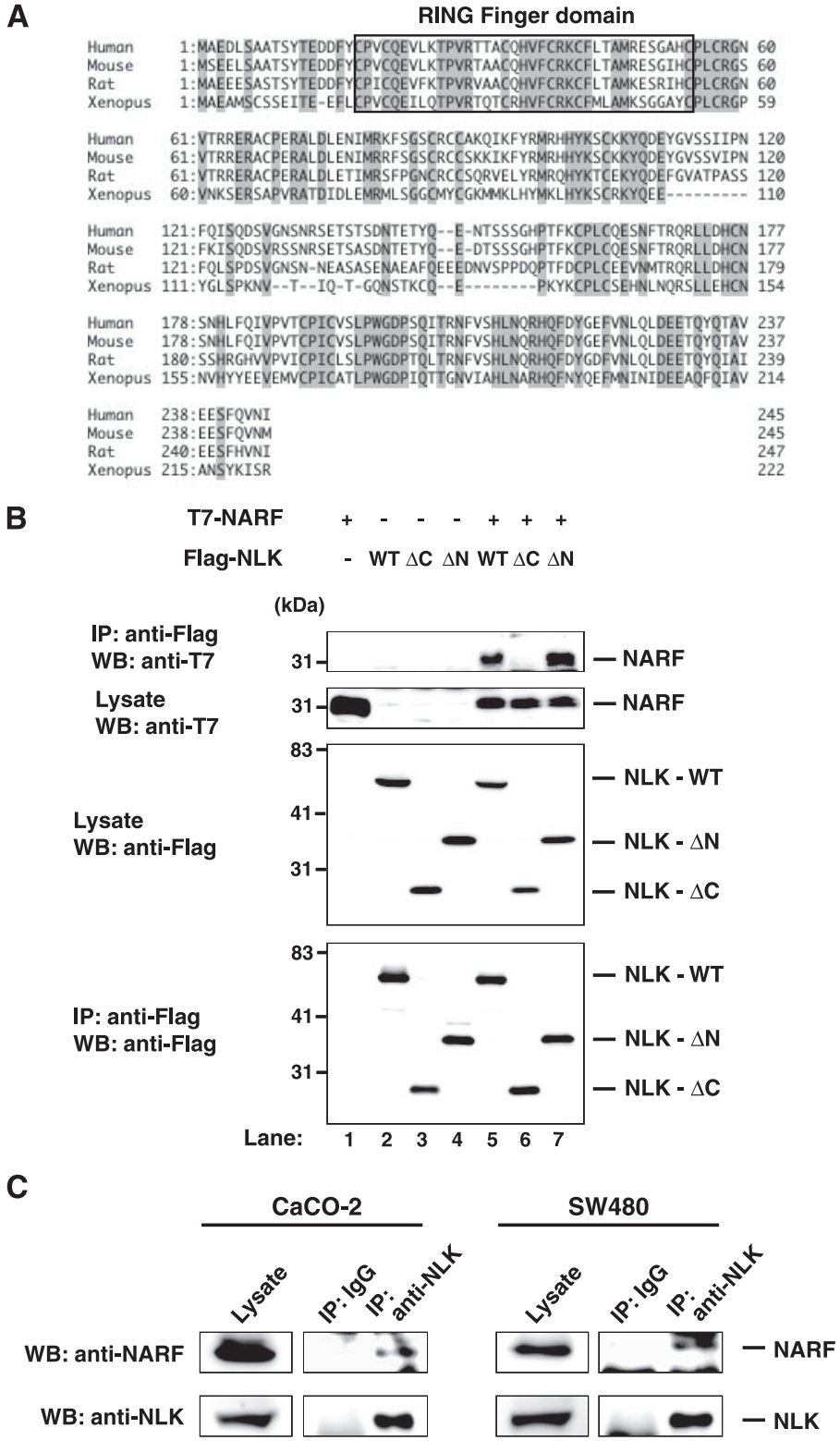

FIGURE 1. **Primary structure of *X. laevis* NARF and association between NARF and NLK.** *A*, *Xenopus* NARF (*xNARF*, accession number DQ011285) and its orthologue from human RING finger protein 138, isoform 1 (*hRNF138*, isoform 1, accession number NP 057355), mouse RNF138, isoform 1 (*mRNF138*, isoform 1, accession number NP 997506), and rat RIKEN cDNA (accession number XP 228926) are shown aligned. Identical residues are *shaded* and the RING finger motif is *boxed*. *B*, 293 cells were transfected with the indicated T7-NARF and FLAG-NLK expression plasmids. *WT*, wild-type NLK; Δ*N*, amino terminus-truncated NLK; Δ*C*, carboxyl terminus-truncated NLK. Immunoprecipitation (*IP*) was carried out with anti-FLAG antibody from whole cell lysates. Co-immunoprecipitation of T7-NARF with FLAG-NLK was visualized by Western blot (*WB*) analysis with anti-T7 antibody (*top panel*). Expression levels of each of the introduced recombinant proteins were apparently equivalent, as judged from Western blotting analysis using whole cell extracts with either anti-T7 antibody for T7-tagged NARF (*second panel*) or anti-FLAG antibody for FLAG-tagged NLKs (*third panel*). Immunoprecipitated FLAG-NLKs were also equivalent (*bottom panel*). *C*, cell lysates were prepared from CaCO-2 and SW480 cells and used for immunoprecipitation with anti-NLK rabbit polyclonal antibody. Rabbit normal IgG was used as control for immunoprecipitation. Co-immunoprecipitation of NARF was visualized by Western blotting with anti-NARF antibody.

The interaction between NARF and NLK observed in the yeast system was next examined in mammalian cells. T7-tagged NARF (T7-NARF) and FLAG-tagged full-length NLK (FLAG-NLK-WT) were co-expressed in 293 cells, and cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. As shown in Fig. 1*B*, T7-NARF was co-immunoprecipitated with FLAG-NLK-WT and FLAG-NLK-ΔN (Fig. 1*B*, *lanes 5* and *7*), but not with FLAG-NLK-ΔC (Fig. 1*B*, *lane 6*). These results suggest that NARF specifically associates with the carboxyl-terminal region of NLK in mammalian cells.

To prove the existence of the endogenous NLK and NARF complex, extracts isolated from CaCO-2, SW480, and C2C12 cells were subjected to co-immunoprecipitation analysis using anti-NLK and NARF antibodies. Endogenous NARF was co-immunoprecipitated in these samples (Fig. 1*C* and data not shown). We conclude that NLK and NARF can form a complex in cells.

NARF Exhibits Auto-ubiquitylating Activity Associated with E2-25K—Recent data indicate that numerous RING finger proteins can function as E3 ubiquitin-ligases. To determine whether NARF may interact with a partner protein(s) in the ubiquitylation system, we attempted to identify protein(s) that physiologically interact with NARF in mammalian cells. FLAG-tagged NARF (FLAG-NARF) was expressed in 293 cells, and NARF and associated proteins were recovered from cell extracts by immunoprecipitation with anti-FLAG antibody. The precipitated proteins were eluted with a FLAG peptide and digested with Lys-C endopeptidase. The cleaved fragments were directly analyzed and sequenced by nanoflow LC-MS/MS. A data base search of the peptide sequences obtained identified one of the E2 ubiquitin-conjugating enzymes, E2-25K, as a NARF-interacting protein (Table 1). The interaction between NARF and E2-25K was confirmed with transiently expressed HA-tagged E2-25K (HA-E2-25K) and FLAG-NARF in 293 cells (Fig. 2*A*, *lane 3*). To define whether NARF mediates ubiquitylation as an E3 ubiquitin-ligase, we performed an *in vitro* auto-ubiquitylation assay for NARF using E2-25K as an E2 conjugating enzyme. We generated a GST fusion with wild-type NARF (GST-NARF-WT), and incubated this with purified E1 ubiquitin-activating enzyme, E2-25K, and ubiquitin. Western blotting analysis showed that NARF was poly-ubiquitylated, as indicated by the appearance of a broad ladder detected with either anti-ubiquitin (Ub) antibody (Fig. 2*B*, *lanes 5–8*) or anti-GST antibody (Fig. 2*B*, *lanes 21–24*). GST-NARF-WT ubiquitylation levels increased in a time-dependent manner (Fig. 2*B*, *lanes 5–8* and *21–24*), and required the presence of E2-25K (Fig. 2*B*, *lanes 1–4* and

TABLE 1
Identification of human E2-25K by LC-MSMS
Sequence coverage was 30%.

Sequence determined	Peptide	Charge	Residues
	m/z		
ANIAVQRIK	527.8	2	2–10
IWHPNISSVTGAICLDILK	713.1	3	28–46
IPETYPFNPPK	651.8	2	62–72
IENLCAMGFDRNAVIVALSSK	770.1	3	115–135

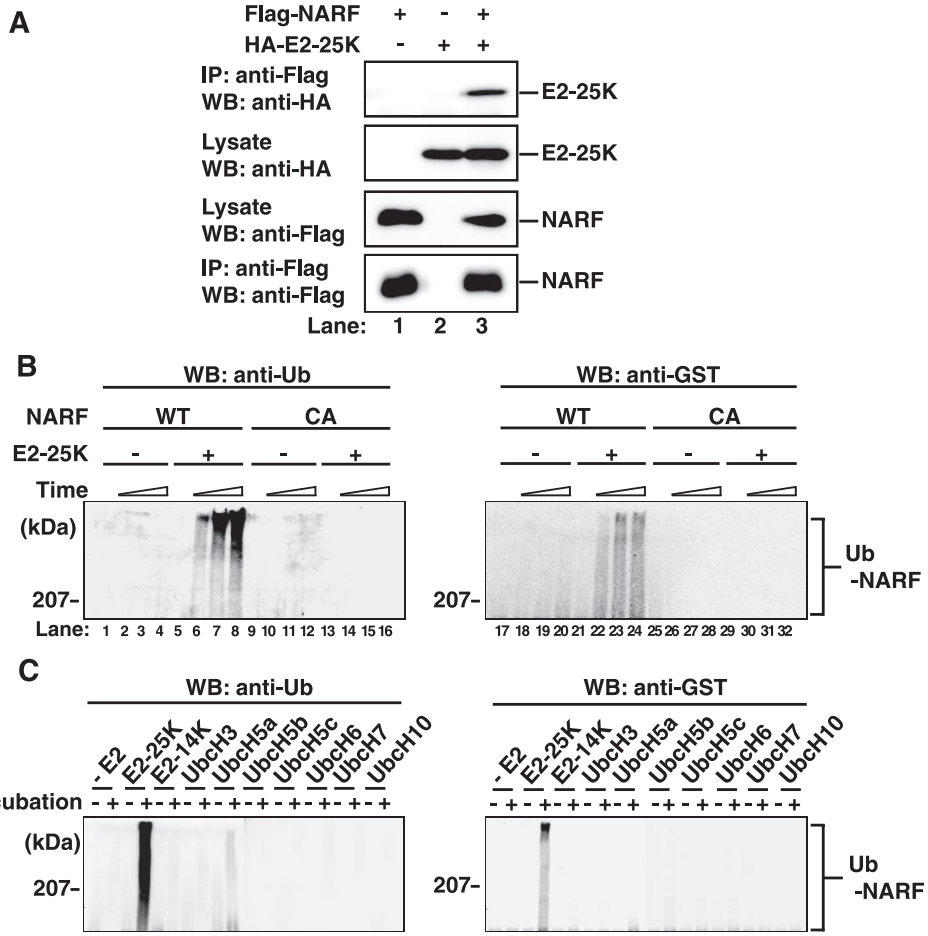

FIGURE 2. **NARF exhibits E3 ubiquitin-ligase activity in cooperation with the ubiquitin conjugating enzyme, E2-25K.** *A*, E2 ubiquitin-conjugating enzyme E2-25K was identified as a NARF-associating protein by LC-MS/MS analysis. Interaction between NARF and E2-25K was confirmed in 293 cells transiently expressing FLAG-NARF and HA-E2-25K. Whole cell lysates were subjected to immunoprecipitation (*IP*) with anti-FLAG antibody, followed by Western blotting analysis (*WB*) with anti-HA antibody to detect HA-E2-25K co-immunoprecipitated with FLAG-NARF (*top panel*). *B*, *in vitro* ubiquitylation assay was performed with bacterially expressed GST-NARF wild-type (*WT*) or RING finger domain mutant (*CA*). GST-NARF was incubated with purified rabbit E1, bovine ubiquitin, and human E2-25K at 30 °C for 0, 30, 60, or 90 min. The reaction mixtures were resolved by SDS-PAGE, and Western blotting analysis with both anti-Ub antibody (*left panel*) and anti-GST antibody (*right panel*) detected the state of GST-NARF-WT poly-ubiquitylation. *C*, *in vitro* ubiquitylation assay of GST-NARF was performed with a panel of E2-conjugating enzymes including E2-25K, E2-14K, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, and UbcH10. The reaction mixtures were incubated for 0 (−) or 120 min (+). Poly-ubiquitylated GST-NARF was detected by Western blotting analysis with anti-Ub antibody (*left panel*) and anti-GST antibody (*right panel*).

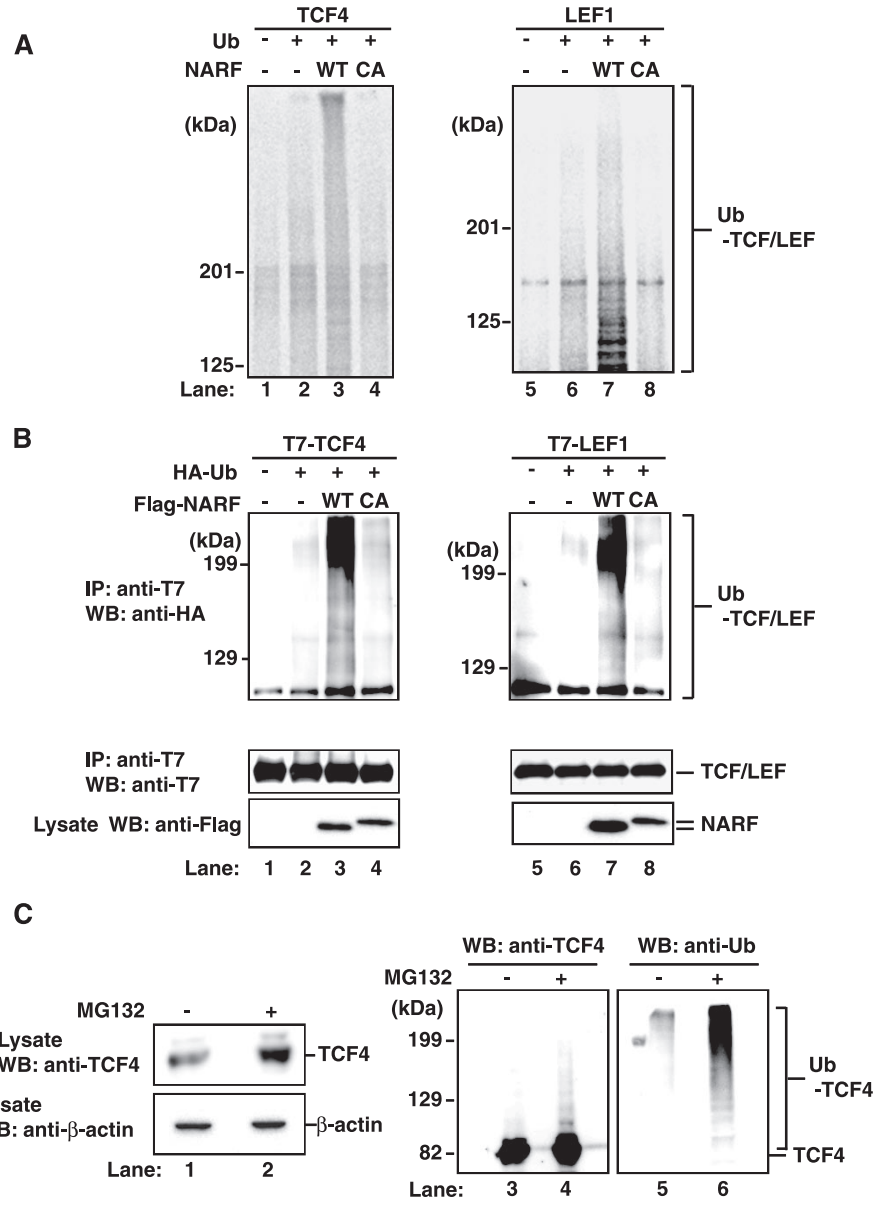

FIGURE 3. **NARF ubiquitylates the transcription factors TCF/LEF.** *A*, ^{35}S-labeled recombinant TCF4 or LEF1 was prepared by an *in vitro* transcription/translation system, and used as a substrate for an *in vitro* ubiquitylation assay with GST-NARF wild-type (GST-NARF-WT) or RING finger domain mutant (GST-NARF-CA). Poly-ubiquitylated ^{35}S-labeled TCF4 and LEF1 were detected by autoradiography (*lanes 3* and *7*). *B*, T7-TCF4 or T7-LEF1 were co-expressed with HA-ubiquitin (*HA-Ub*) and FLAG-NARF in 293 cells. T7-TCF4 or LEF1 were immunoprecipitated by anti-T7 antibody from whole cell lysates, and the poly-ubiquitylated states of each were detected by Western blot (*WB*) analysis with anti-HA antibody (*top panels*, *lanes 3* and *7*). Western blot analysis with anti-T7 antibody (*middle panels*) or anti-FLAG antibody (*bottom panels*) using whole cell lysates confirmed that there were equivalent levels of expressed recombinant proteins in each experiment. *C*, 293 cells were incubated with 10 μM of the proteasome inhibitor MG132 for 4 h, and whole cell lysates were analyzed by Western blot analysis with anti-TCF4 antibody (*upper left panel*) or anti-β-actin antibody (*lower left panel*). Endogenous TCF4 was immunoprecipitated (*IP*) with anti-TCF4 antibody, and analyzed with both anti-TCF4 antibody and anti-Ub antibody (*two right panels*).

17–20). It is noteworthy that no GST-NARF-WT ubiquitylation was observed when the two conserved cysteine residues at 17 and 53 within the RING finger domain were replaced with alanine (GST-NARF-CA, Fig. 2*B*, *lanes 13–16* and *29–32*). To clarify whether E2-25K is an authentic E2-conjugating enzyme for NARF in the ubiquitylation process, we assayed GST-NARF-WT auto-ubiquitylation using a panel of E2-conjugating enzymes (E2-25K, E2-14K, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH7, and UbcH10). Fig. 2*C* showed that auto-ubiquitylation of GST-NARF-WT occurred most robustly in the presence of E2-25K among the examined E2 enzymes, although other E2 enzymes also mediated weak ubiquitylation. These data show that the auto-ubiquitylating activity of NARF is coordinated with E2-25K, and that the RING finger domain of NARF is indispensable for this reaction.

NARF Ubiquitylates the Transcription Factor TCF/LEF—To clarify the biological role of NARF, we initially searched for target proteins that are ubiquitylated by NARF. Although NARF was isolated as an NLK-associating protein, NARF did not ubiquitylate NLK in an *in vitro* ubiquitylation assay (data not shown). Therefore, we examined further the possibility that NARF may ubiquitylate NLK-associated transcription factors or other Wnt signaling components. We used rabbit reticulocytes to prepare ^{35}S-labeled recombinant proteins for each candidate substrate of NARF-directed ubiquitylation, and incubate each, together with GST-NARF-WT or GST-NARF-CA, in an *in vitro* ubiquitylation assay (data not shown). From this series of assays, we found that NARF-WT could ubiquitylate the transcription factor TCF/LEF, as indicated by the appearance of a ladder of bands (Fig. 3*A*, *lanes 3* and *7*). TCF/LEF ubiquitylation was not observed with NARF-CA, which does not have ubiquitin ligase activity as shown in Fig. 3*B* (Fig. 3*A*, *lanes 4* and *8*). These results indicate that NARF ubiquitylates TCF/LEF and this activity requires its RING finger domain structure.

To ascertain whether TCF/LEF is actually ubiquitylated in cells, T7-tagged TCF4 (T7-TCF4) or LEF1 (T7-LEF1) were co-expressed together with HA-tagged ubiquitin (HA-Ub) and FLAG-tagged NARF (FLAG-NARF-WT) in 293 cells, and cell lysates were immunoprecipitated with anti-T7 antibody. Poly-ubiquitylation of TCF4 or LEF1 was indicated by the appearance of a ladder in Western blots with anti-HA antibody (Fig. 3*B*, *lanes 3* and *7*). When the critical cysteine residues at 17 and 53 within the RING finger domain of NARF were mutated (FLAG-NARF-CA), ubiquitylation of T7-TCF/LEF by FLAG-NARF was abolished to basal levels (Fig. 3*B*, *lanes 4* and *8*). We also examined the association between endogenous TCF4, NLK, and

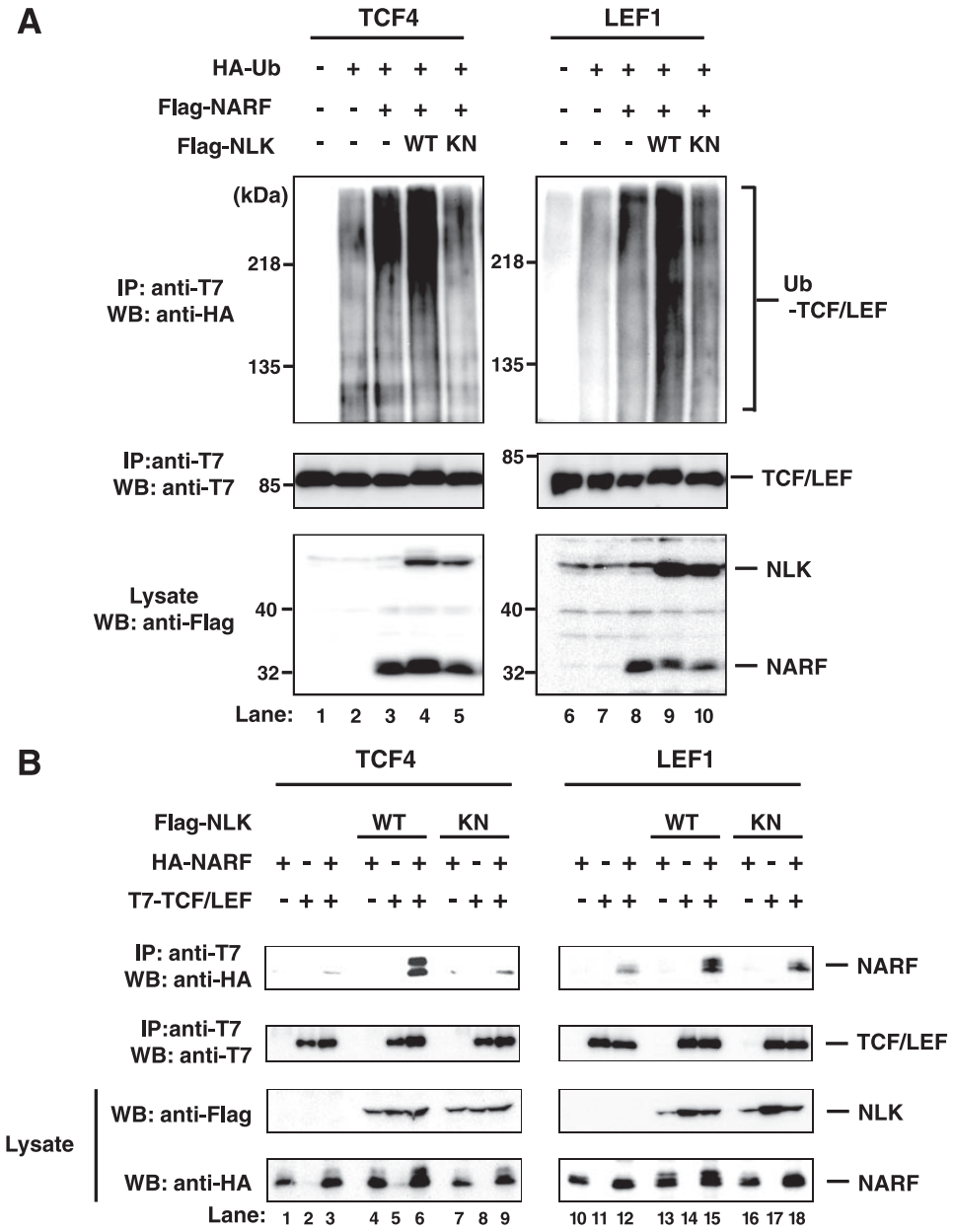

FIGURE 4. **NLK augments the ubiquitylation activity of NARF against TCF/LEF.** *A*, T7- TCF4/LEF1, FLAG-NARF, HA-ubiquitin (*Ub*), and FLAG-NLK wild-type (*WT*) or kinase-negative mutant (*KN*) were co-expressed in 293 cells. T7-TCF4 or T7-LEF1 were immunoprecipitated (*IP*) by an anti-T7 antibody from whole cell lysates and poly-ubiquitylated T7-TCF4 or T7-LEF1 were detected by Western blotting analysis (*WB*) with anti-HA antibody (*top panels*). Western blotting analysis with anti-T7 antibody (*middle panels*) or anti-FLAG antibody (*bottom panels*) using whole cell lysates confirmed that there were equivalent levels of expressed recombinant proteins in each experiment. *B*, HA-NARF and T7-TCF4 or T7-LEF1 were co-expressed with FLAG-NLK wild type (*WT*) or kinase-negative mutant (*KN*) in 293 cells. T7-TCF4 or T7-LEF1 were immunoprecipitated with anti-T7 antibody from whole cell lysates, and the association of HA-NARF with T7-TCF4 or T7-LEF1 was detected by anti-HA antibody (*top panels*). Western blotting analysis with anti-FLAG antibody (*second panels from top*), anti-HA antibody (*second panels from bottom*), or anti-T7 antibody (*bottom panels*) using whole cell lysates confirmed that there were equivalent levels of expressed recombinant proteins in each experiment.

NARF in 293 cells, and detected an interaction between endogenous NLK and TCF4, but not between NARF and TCF4 (data not shown). This result may raise the possibility that TCF4 is rapidly degraded after ubiquitylation by NARF. These data established that TCF/LEF is a candidate target for the ubiquitylation activity of NARF *in vivo*.

To verify the ubiquitylation of TCF/LEF *in vivo*, we attempted to detect ubiquitylation of the endogenous TCF4. When 293 cells were treated with the proteasome inhibitor MG132, endogenous TCF4 levels were markedly increased (Fig. 3*C*, *lane 2*) and an accumulation of poly-ubiquitylated TCF4 was detected with both anti-Ub antibody (Fig. 3*C*, *lane 6*) and anti-TCF4 antibody (Fig. 3*C*, *lane 4*). These results indicate that the ubiquitylation of TCF/LEF can occur under physiological conditions and also suggests that TCF/LEF protein stability is regulated via the ubiquitin-proteasome system.

NLK Augments the Ubiquitylation Activity of NARF against TCF/LEF—To examine the possibility that NLK regulates NARF function in the ubiquitylation of TCF/LEF *in vivo*, either T7-TCF4 or T7-LEF1 were co-expressed with HA-Ub and FLAG-NARF with or without FLAG-NLK in 293 cells. Poly-ubiquitylated T7-TCF4 or T7-LEF1 was immunoprecipitated by anti-T7 antibody and detected as a ladder of bands reacting with anti-HA antibody (Fig. 4*A*, *lanes 3* and *8*). When FLAG-tagged wild-type NLK (FLAG-NLK-WT) was co-expressed, ubiquitylation of T7-TCF4 or T7-LEF1 was significantly augmented (Fig. 4*A*, *lane 3 versus 4* and *lane 8 versus 9*), whereas no enhancement of ubiquitylation was observed when the kinase-inactive mutant FLAG-NLK (FLAG-NLK-KN) was co-expressed (Fig. 4*A*, *lane 3 versus 5* and *lane 8 versus 10*). To assess the cooperation of NLK in the interaction between NARF and TCF/LEF, HA-tagged NARF (HA-NARF) and T7-TCF4 or T7-LEF1 were co-expressed in 293 cells with or without FLAG-NLK. When T7-TCF4 or T7-LEF1 were immunoprecipitated from whole cell lysates with anti-T7 antibody, a weak association of HA-NARF was detected by anti-HA antibody in the absence of NLK co-expression (Fig. 4*B*, *lanes 3* and *12*). When FLAG-NLK wild-type (FLAG-NLK-WT) was co-expressed with HA-NARF and T7-TCF/LEF, the association between HA-NARF and T7-TCF4/LEF1 was apparently enhanced (Fig. 4*B*, *lanes 6* and *15*). However, no such enhancement was seen when kinase-inactive mutant NLK (FLAG-NLK-KN) was co-expressed (Fig. 4*B*, *lanes 9* and *18*). These data clearly indicate that NLK facilitates

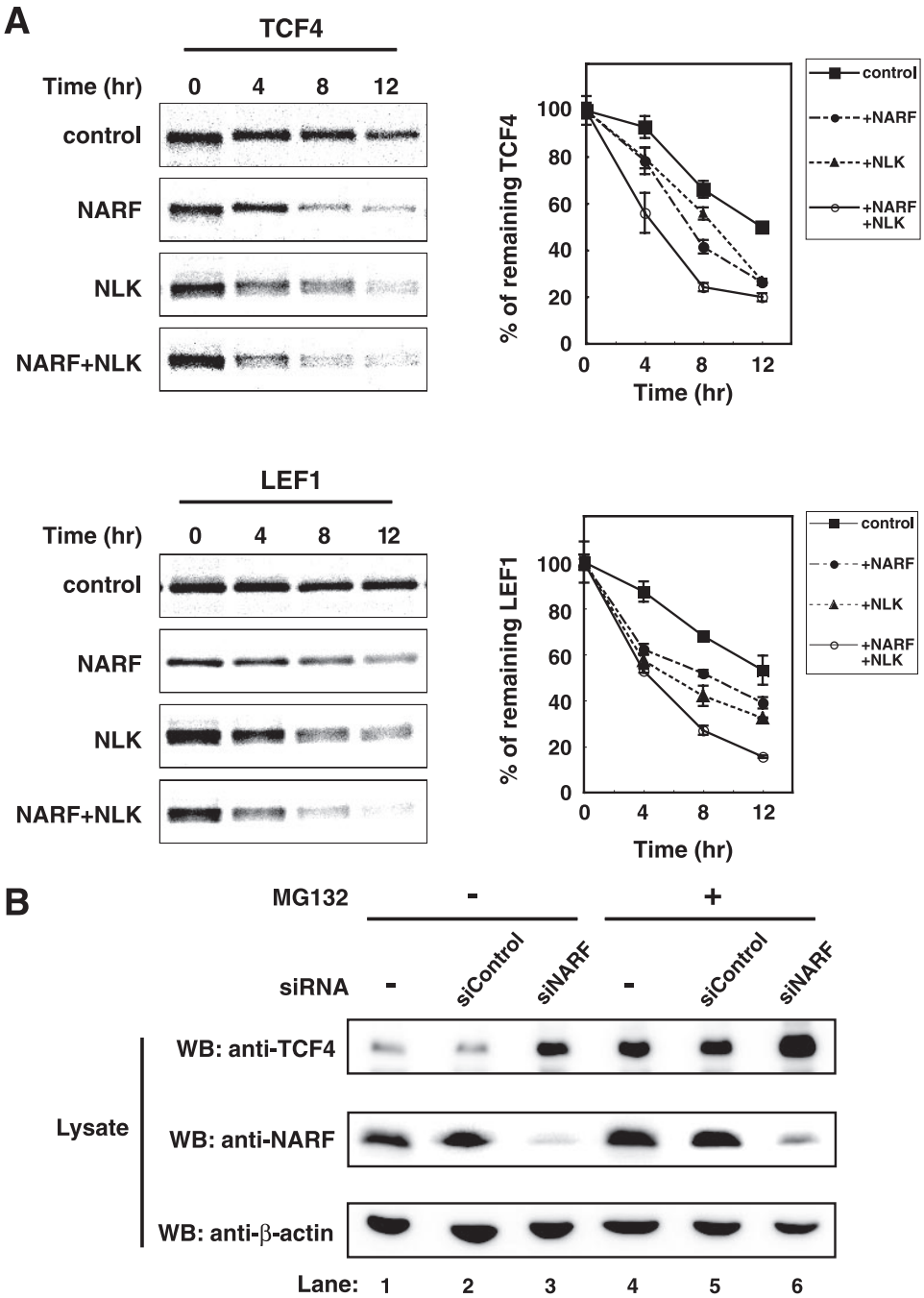

FIGURE 5. **NLK and NARF coordinate the degradation of TCF/LEF.** *A*, T7-TCF4 or T7-LEF1 was expressed with NARF and/or NLK in 293 cells. T7-TCF4 or T7-LEF1 were metabolically labeled with [^{35}S]methionine and cysteine, and chased for the indicated time periods. Cells were lysed in RIPA buffer, and [^{35}S]T7-TCF4 or [^{35}S]T7-LEF1 were immunoprecipitated with anti-T7 antibody. [^{35}S]T7-TCF4 or [^{35}S]T7-LEF1 were detected by autoradiography (*left panels*) and quantified from the intensity of the visualized bands (*right panels*). The half-life of TCF4 was calculated from a linear plot of the rate of [^{35}S]TCF4 decay in cells. *B*, the expression of endogenous NARF was suppressed by siRNA. 293 cells were transfected with siRNAs and treated with 10 μM MG132 or Me_2SO for 4 h. Control siRNA is targeted for luciferase. Expression of endogenous TCF4 and NARF were examined by Western blotting (*WB*) analysis with anti-TCF4 antibody (*top panel*) and anti-NARF peptide antibody (*middle panel*), respectively. β-Actin was used as a loading control (*bottom panel*).

NARF-directed ubiquitylation of TCF/LEF by enhancing the association between NARF and TCF/LEF, and that this enhancement depends on NLK kinase activity.

NLK and NARF Coordinate the Degradation of TCF/LEF—The preceding data demonstrated that TCF/LEF protein levels are regulated via the ubiquitin-proteasome pathway (Fig. 3*C*). To evaluate the effect of NARF and NLK on the protein stability of TCF/LEF, we performed pulse-chase experiments using TCF/LEF metabolically labeled with [^{35}S]methionine/cysteine in 293 cells. During the chase period, the level of ^{35}S-labeled TCF4 and LEF1 declined gradually and their half-lives were estimated to be 12.5 and 12.9 h, respectively (Fig. 5*A*, *control panels*). Interestingly, co-expression of NARF shortened the half-lives of TCF4 and LEF1 to 7.8 and 7.2 h (Fig. 5*A*, *NARF panels*), and co-expression of NLK shortened them to 8.6 and 5.7 h, respectively (Fig. 5*A*, *NLK panels*). Furthermore, it is intriguing that co-expression of both NARF and NLK dramatically reduced the protein stability of TCF4 and LEF1 to half-lives of 4.5 and 4.4 h, respectively. The doublet bands observed for TCF/LEF proteins when NLK was expressed might be due to NLK-induced phosphorylation, as described previously (4). These results are consistent with the data from ubiquitylation analysis, suggesting that NARF and NLK contribute to the regulation of TCF/LEF protein levels via the ubiquitin-mediated degradation pathway.

The physiological involvement of NARF in modulating TCF/LEF protein stability via ubiquitylation and the proteasome degradation was confirmed using siRNA. Introduction of siRNA targeting the human NARF mRNA into 293 cells effectively diminished the amount of endogenous NARF detected by anti-NARF antibody, and caused a significant accumulation of endogenous TCF4 in 293 cells (Fig. 5*B*, *lanes 1* and *2 versus 3*; and *lanes 4* and *5 versus 6*). Proteasome inhibitor treatment further enhanced the stability of endogenous TCF4 (Fig. 5*B*, *lane 1 versus 4*; *lane 2 versus 5*; and *lane 3 versus 6*), indicating that NARF physiologically regulates the fate of TCF4 via proteasome-mediated pathways in 293 cells. Interestingly, the levels of NARF protein also increased when cells were treated with MG132. As we had found that NARF itself was ubiquitylated in an *in vitro* ubiquitylation assay (see Fig. 2, *B* and *C*), we speculate that NARF might also be degraded through the proteasome system.

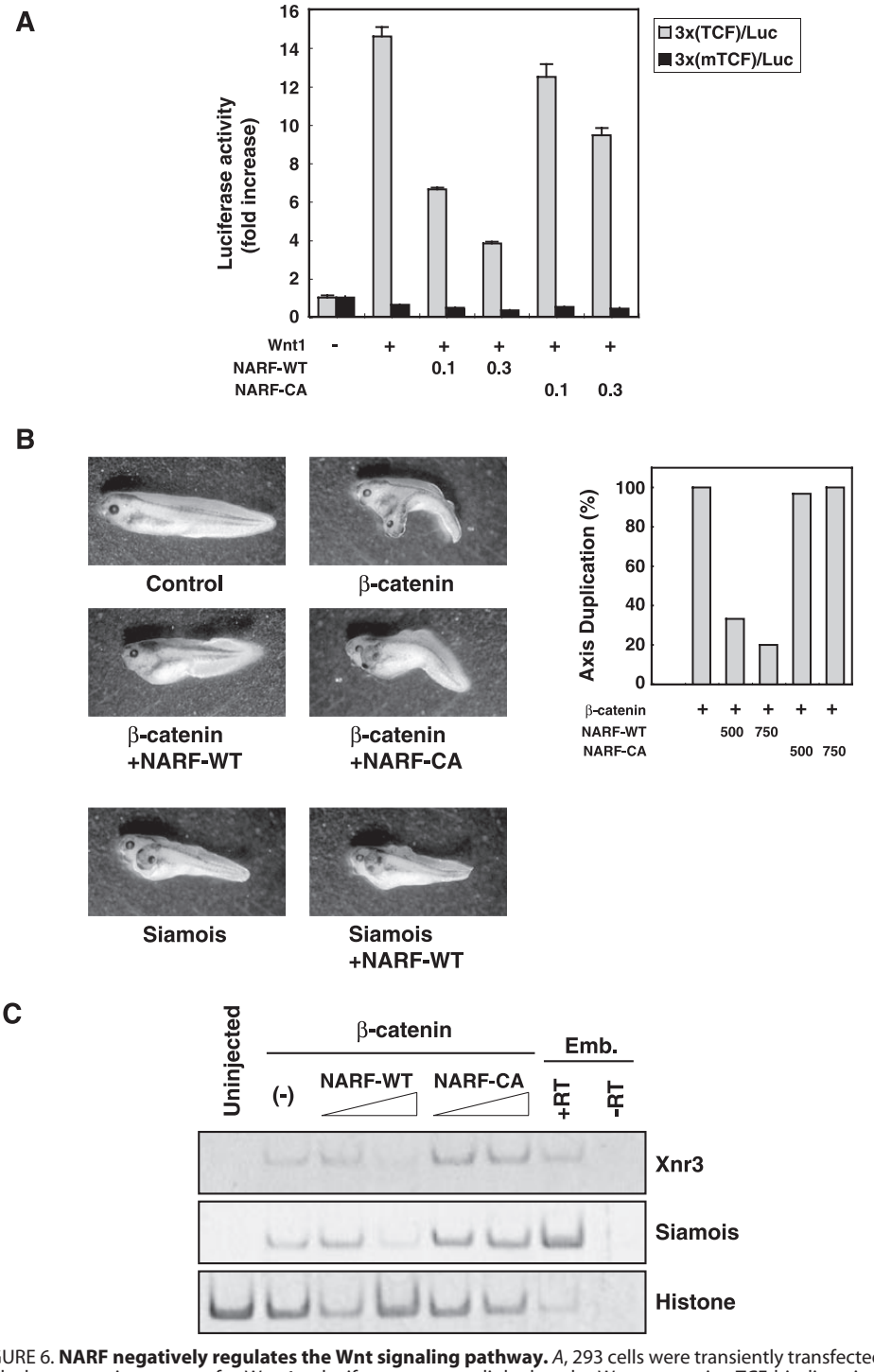

FIGURE 6. **NARF negatively regulates the Wnt signaling pathway.** *A*, 293 cells were transiently transfected with the expression vectors for Wnt-1, a luciferase reporter linked to the Wnt-responsive TCF-binding sites (*3x(TCF)/Luc*) or the mutated sites (*3x(mTCF)/Luc*), and either NARF wild-type (*NARF-WT*) or RING finger domain mutant (*NARF-CA*). Cells were harvested after 24 h post-transfection and assayed for luciferase activity. The transfection efficiency was normalized with the activity of co-transfected *Renilla* luciferase vector controlled by the EF-1α promoter. Values are expressed as the –fold increase in luciferase activity relative to the level of activity with reporter plasmid alone. *B*, synthetic mRNAs encoding *β-catenin* (100 pg) or *Siamois* (0.5 pg) were microinjected with *NARF-WT* mRNA (500 and 700 pg) or RING finger domain mutant mRNA (500 and 750 pg) into the ventral equatorial region of the 4-cell stage *Xenopus* embryos to induce secondary axis formation as indicated. The representative embryos are shown (*left panels*). The ectopic axis formations were counted at the tadpole stage and expressed as the ratio of axis-formed embryos to total examined numbers ($n = 30$, axis duplication %, *right panel*). *C*, RT-PCR analysis in animal caps. Synthetic mRNA encoding *β-catenin* (100 pg) was microinjected with *NARF-WT* mRNA (50 and 300 pg) or RING finger domain mutant mRNA (50 and 300 pg) into the animal pole of two blastomeres at the 2-cell stage *Xenopus* embryos. Animal cap explants were removed at the blastula stage. Total RNAs were prepared and analyzed by RT-PCR for the expression of Wnt target genes, *Xnr3* and *Siamois*. Histone was used as a loading control. *Emb* indicates whole embryo control with (+*RT*) or without (−*RT*) at the RT step.

NARF Negatively Regulates the Wnt Signaling Pathway—To investigate the effect of NARF on Wnt-induced, TCF/LEF-dependent transcriptional activity, a luciferase reporter plasmid containing Wnt-responsive TCF-binding sites, 3x(TCF)/Luc (38), was transfected into 293 cells, and the transcriptional activity of TCF/LEF was measured as shown in Fig. 6*A*. When an expression vector encoding wild-type NARF (*NARF-WT*) was co-transfected together with the reporter plasmid, Wnt-induced TCF/LEF-dependent transcriptional activity was suppressed in a dose-dependent manner down to 75% of the control. In contrast, expression of RING finger domain mutant NARF (*NARF-CA*) exhibited little effect on the TCF/LEF transcriptional activity. To further address the possibility that NARF suppresses the Wnt-β-catenin-mediated signaling pathway, NARF mRNA was co-injected into *X. laevis* embryos. Expression of the injected mRNAs in embryos was confirmed by Western blotting (data not shown). Normally, the secondary axis in *X. laevis* embryos is induced by ectopic expression of β-catenin. However, injection of wild-type NARF (*NARF-WT*) mRNA into the embryos significantly inhibited secondary axis formation and the target gene expression (*Xnr3* and *Siamois*) induced by β-catenin in a dose-dependent manner. In contrast, injection of the RING finger domain mutant NARF (*NARF-CA*) mRNA had no inhibitory effect on axis formation and gene expression (Fig. 6, *B* and *C*). Furthermore, injection of *NARF* or *NLK* mRNA into the 4-cell stage embryos did not produce any discernible phenotypic change in the embryos (data not shown). Moreover, injection of *NARF-WT* mRNA did not inhibit axis formation induced by Siamois (Fig. 6*B*). These results indicate that NARF specifically inhibits secondary axis formation and gene expression induced by β-catenin, and does

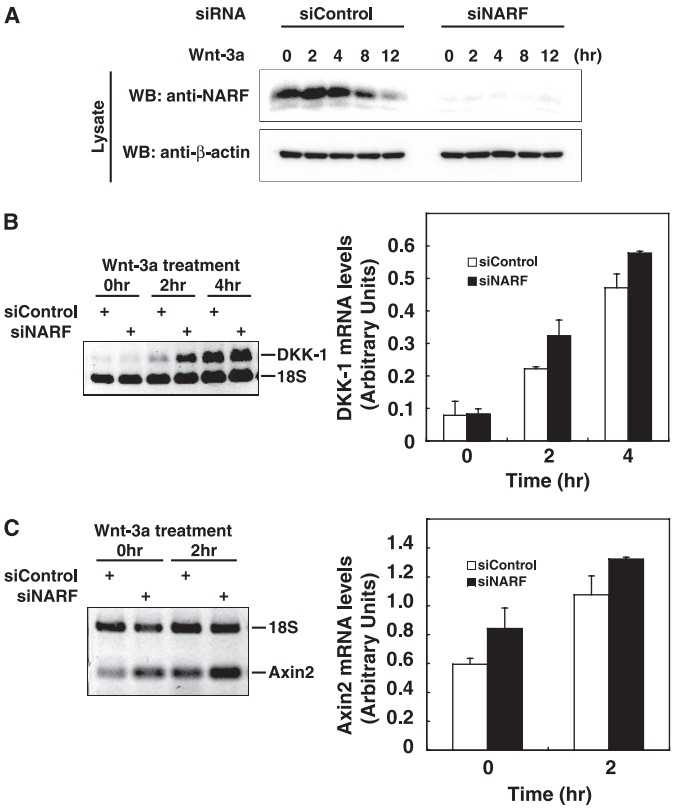

FIGURE 7. **Knockdown of the endogenous NARF enhances Wnt-3a-dependent *DKK-1* and *Axin2* gene expression.** *A*, HeLaS3, transiently transfected with control (*siControl*) or specific human NARF siRNA (*siNARF*), were treated with Wnt-3A conditioned medium for the indicated periods, and total cell lysates were subjected to SDS-PAGE and Western blotting (*WB*) analysis with anti-NARF antibody (*upper panel*). The loading control was carried out with an anti-β-actin antibody (*lower panel*). *B* and *C*, the effect of *siNARF* on Wnt-3A-induced *DKK-1* and *Axin2* mRNA expression. siRNA-treated HeLaS3 cells were incubated with Wnt-3A CM for the indicated time periods. Semi-quantitative multiplex RT-PCR was performed for 30 cycle amplifications with *DKK-1* primers or 35 cycle amplifications with *Axin2* primers, and Quantum RNA 18 S internal standards II. Representative SYBR Green I-stained PCR products are shown (*left panel*). *DKK-1* and *Axin2* expression were normalized to expression of the 18 S ribosomal RNA and presented as the ratio of fluorescence intensity of the *DKK-1* or *Axin2 versus* 18 S bands (*right panel*). Data are shown as the mean ± S.D. of the three separate experiments.

not act at some downstream signaling step, suggesting that NARF can negatively regulate Wnt-β-catenin signaling *in vivo*.

Knockdown of the Endogenous NARF Enhances Wnt-3a-dependent Genes Expression—To evaluate the physiological relevance of NARF to the canonical Wnt/β-catenin signaling pathway, we used siRNA to suppress expression of the endogenous NARF protein in HeLaS3 cells. We examined the effect of NARF siRNA on the expression of *Dickkopf-1* (*DKK-1*) and *Axin2*, target genes of Wnt/β-catenin signaling (39, 40). As shown in Fig. 7, *B* and *C*, Wnt-3a treatment induced the expression of the *DKK-1* and *Axin2* genes. When endogenous NARF expression was suppressed with NARF siRNA (siNARF) (Fig. 7*A*), Wnt-dependent expression of *DKK-1* and *Axin2* mRNAs were further enhanced as compared with the control (siControl) at all time points examined (Fig. 7, *B* and *C*). The data presented here are consistent with our notion that NARF is physiologically required for suppression of the Wnt/β-catenin-mediated signaling pathway.

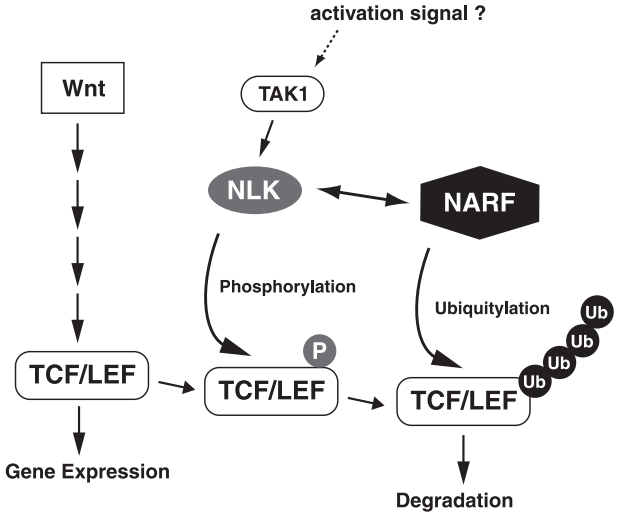

FIGURE 8. **A proposal model for the roles of NARF in the suppression of the Wnt-β-catenin signaling pathways.**

DISCUSSION

In the present study, we identified NARF as a NLK-binding protein and characterized its functions. Our results suggest that NARF is a RING finger-type E3 ubiquitin-ligase and is involved in the ubiquitylation of TCF/LEF. Furthermore, we demonstrate that NARF and E2-25K are indispensable for ubiquitylation and degradation of TCF/LEF. These activities require NLK-induced phosphorylation of TCF/LEF, as *in vitro* kinase assays demonstrated that NLK could phosphorylate TCF/LEF but not NARF (data not shown). These findings provide the first evidence that NARF functions in the specific ubiquitylation and degradation of TCF/LEF *in vivo*, and demonstrate the significance of NARF in Wnt signaling. Based on these data, we propose a model for the function of NARF in the ubiquitylation and degradation of TCF/LEF (Fig. 8). In cells at a steady state, NARF complexes with NLK. Activation of NLK induced by unknown ligands leads to the phosphorylation of TCF/LEF. NARF then acts on TCF/LEF as an E3 ubiquitin-ligase and, together with E1 and E2 ubiquitylation enzymes, catalyze the ubiquitylation of TCF/LEF. Finally, ubiquitylated TCF/LEF is degraded by the 26 S proteasome. Thus, we suggest that NARF is a key component that regulates the degradation of TCF/LEF and acts as a negative regulator of Wnt signaling.

We analyzed NARF-binding proteins using an LC-MS/MS system and identified E2-25K, an E2 ubiquitin-conjugating enzyme for ubiquitylation. This suggests that NARF is an E3 ubiquitin-ligase. We have previously shown that NLK, and TCF/LEF phosphorylation, negatively regulate Wnt signaling. These facts raise the possibility that TCF/LEF or other molecules involved in Wnt signaling may be targets of NARF E3 ubiquitin-ligase activity. We observed that TCF/LEF was ubiquitylated by NARF *in vitro* and *in vivo*. We also demonstrated that suppression of NARF expression by siRNA results in increased levels of expression of the Wnt-induced genes, DKK-1 and Axin2 (Fig. 7). This indicates that NARF plays a critical role in the endogenous regulation of Wnt signaling. Several reports have indicated that the sumoylation and acetylation of TCF/LEF regulates its subcellular localization and transcrip-

tional activity (41–43). Our results provide new evidence that ubiquitylation of TCF/LEF also plays a role in the regulation of cellular signaling. Although the biological significance of ubiquitylation of TCF/LEF is unclear at present, it is certain that NARF is involved in the degradation of TCF/LEF through the action of a yet to be identified ligand(s) that negatively regulates the Wnt signaling pathway. Further studies will be needed to identify the precise ligand that induces the activity of the NARF E3 ubiquitin-ligase involved in TCF/LEF ubiquitylation.

Our studies demonstrate that among the members of the Ubc family of E2 ubiquitin-conjugating enzymes, E2-25K can specifically support the ubiquitylation of TCF/LEF. Previous biochemical analyses have demonstrated that E2-25K is a unique E2 protein in the ubiquitin/proteasome system that is involved in the synthesis of poly-ubiquitin chains *in vitro* from mono- or poly-ubiquitin, E1, and ATP (44–46). Previous studies also indicated biological roles for E2-25K in neurodegenerative diseases, as a regulator of huntingtin in Huntingtin disease (47) and amyloid-β peptide neurotoxicity in Alzheimer disease (48). TCF/LEF is the first protein to be directly and specifically shown to be a substrate for E2-25K-dependent ubiquitylation by NARF, although it has not been definitively proven whether NARF, an E3 ubiquitin-ligase, is involved in any neurodegenerative disease at present.

The function of TCF/LEF in embryonic development has been intensively studied. We have shown previously that NLK plays an essential role in axis formation, mesoderm induction, and neural development in *Xenopus* embryos (4, 6, 11). Moreover, *Xenopus* NARF mRNA was expressed maternally and throughout early development (data not shown). Expression of NARF was observed to inhibit the secondary axis formation induced by ectopic expression of β-catenin in *Xenopus* embryos. These observations led us to hypothesize that NARF, a post-translational regulator of TCF/LEF stability, may also participate in the control of embryonic development. Further elucidation of the function of NARF may lead to the identification of novel mechanisms by which ubiquitylation induced by unknown ligands negatively regulates Wnt signaling. However, it remains to be shown how the specific ubiquitylation of TCF/LEF contributes to the diverse early developmental processes regulated by NARF in *Xenopus* embryos. Additional experiments will be required to assess the role of NARF in *Xenopus* development.

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