THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 12, pp. 8289–8298, March 22, 2013

HECT Domain-containing E3 Ubiquitin Ligase NEDD4L Negatively Regulates Wnt Signaling by Targeting Dishevelled for Proteasomal Degradation*

Received for publication, November 1, 2012, and in revised form, February 8, 2013 Published, JBC Papers in Press, February 9, 2013, DOI 10.1074/jbc.M112.433185

Yi Ding[‡1], **Yan Zhang**[§1], **Chao Xu**[‡], **Qing-Hua Tao**[§2], **and Ye-Guang Chen**[‡3]

From [‡]*The State Key Laboratory of Biomembrane and Membrane Biotechnology, Tsinghua-Peking Center for Life Sciences, and* [§]*School of Life Sciences, Tsinghua University, Beijing 100084, China*

Background: Dishevelled is a critical component of Wnt signaling; however, its stability control is not fully understood.
Results: NEDD4L regulates Wnt signaling through Dishevelled2 degradation, and Wnt5a-induced NEDD4L phosphorylation by JNK1 is required for this process.
Conclusion: NEDD4L modulates Wnt signaling through a negative feedback mechanism.
Significance: Our findings shed light on the understanding of Dishevelled2 stability control and NEDD4L-associated diseases.

Wnt signaling plays a pivotal role in embryogenesis and tissue homeostasis. Dishevelled (Dvl) is a central mediator for both Wnt/β-catenin and Wnt/planar cell polarity pathways. NEDD4L, an E3 ubiquitin ligase, has been shown to regulate ion channel activity, cell signaling, and cell polarity. Here, we report a novel role of NEDD4L in the regulation of Wnt signaling. NEDD4L induces Dvl2 polyubiquitination and targets Dvl2 for proteasomal degradation. Interestingly, the NEDD4L-mediated ubiquitination of Dvl2 is Lys-6, Lys-27, and Lys-29 linked but not typical Lys-48-linked ubiquitination. Consistent with the role of Dvl in both Wnt/β-catenin and Wnt/planar cell polarity signaling, NEDD4L regulates the cellular β-catenin level and Rac1, RhoA, and JNK activities. We have further identified a hierarchical regulation that Wnt5a induces JNK-mediated phosphorylation of NEDD4L, which in turn promotes its ability to degrade Dvl2. Finally, we show that NEDD4L inhibits Dvl2-induced axis duplication in *Xenopus* embryos. Our work thus demonstrates that NEDD4L is a negative feedback regulator of Wnt signaling.

Wnt Signaling directs cell proliferation, polarity, and fate determination during embryonic development and tissue homeostasis. Mutations in the Wnt pathway components are often linked to various human diseases (1). Wnt signaling is initiated by binding of extracellular Wnt ligands to Frizzled transmembrane receptors, leading to membrane recruitment and activation of Dishevelled (Dvl).[4] Downstream of Dvl, Wnt signals diverge into the canonical Wnt/β-catenin and noncanonical Wnt pathways including the Wnt/planar cell polarity (PCP) pathway. Wnt/β-catenin signaling prevents β-catenin from ubiquitination and degradation by destruction of a complex composed of Axin, adenomatous polyposis coli, glycogen synthase kinase 3β, and casein kinase 1, leading to the nuclear accumulation of β-catenin, and eventually β-catenin/T cell factor-mediated transcription. In contrast, Wnt/PCP signaling is involved in cytoskeleton remodeling and coordinates dynamic cell-cell adhesion and cell migration via the small GTPases RhoA and Rac1 and c-Jun N-terminal kinases (JNK) (2–6).

As Dvl is a pivotal component of Wnt signaling, regulation of its stability is of great significance for proper signal transduction. Dvl undergoes degradation through two distinct pathways: the ubiquitin-proteasomal pathway and the autophagy-lysosomal pathway (7–9). Several ubiquitin ligases have been suggested to regulate Dvl stability, including KLHL12-Cullin-3 ubiquitin ligase (10), NEDL1 (11), adenomatous polyposis coli/C (12), pVHL (8), Malin (13), and ITCH (14). These E3 ubiquitin ligases were reported to promote ubiquitination and degradation of Dvl under various physiological conditions. However, the mechanisms underlying Dvl stability control is still not fully understood.

NEDD4 family ubiquitin ligases contain nine members with distinct physiological functions (15). NEDD4L (neural precursor cell expressed, developmentally down-regulated 4-like, also called NEDD4-2) is a member of the NEDD4 family ubiquitin ligases (16, 17). It consists of an N-terminal calcium/lipid-binding C2 domain, four WW domains, and a C-terminal HECT (homologous to E6-AP C terminus of the human papilloma virus) ubiquitin-ligase domain. The WW domains of NEDD4L have been shown to mediate its binding to substrates containing PP*X*Y (PY) motifs. By far, several proteins have been described as the substrates of NEDD4L, including ion and neurotransmitter channels, growth factor receptors, signaling intermediates, and tight junction molecules (17–20). NEDD4L knock-out mice die perinatally due to impaired lung function (21, 22).

In this study, we reported that NEDD4L could directly bind Dvl2 and target Dvl2 for proteasomal degradation through Lys-

* This work was supported by National Key Basic Research Program of China (973 Program) Grants 2011CB943800 (to Y. G. C. and Q. H. T.) and National Natural Science Foundation of China Grants 30930050 and 30921004 (to Y. G. C.) and 30930012 (to Q. H. T.).
[1] Both authors contributed equally to this work.
[2] To whom correspondence may be addressed. Tel.: 86-10-62788745; Fax: 86-10-62792749; E-mail: qhtaolab@tsinghua.edu.cn.
[3] To whom correspondence may be addressed. Tel.: 86-10-62795184; Fax: 86-10-62794376; E-mail: ygchen@tsinghua.edu.cn.
[4] The abbreviations used are: Dvl, Dishevelled; PCP, planar cell polarity; JNK, c-Jun N-terminal kinases; GST, glutathione *S*-transferase; TRITC, tetramethylrhodamine isothiocyanate.

6-, Lys-27-, and Lys-29-linked atypical ubiquitination. The regulation of Dvl2 by NEDD4L was required for both Wnt/β-catenin and Wnt/PCP signaling. We further demonstrated that Wnt5a-induced phosphorylation of NEDD4L by JNK1 was required for it to regulate Dvl stability. NEDD4L also negatively regulated Dvl2-induced axis duplication in *Xenopus* embryos. Thus NEDD4L plays an essential role in Wnt signaling through a negative feedback mechanism.

EXPERIMENTAL PROCEDURES

Plasmids and RNA Interference—Human NEDD4L isoform 2 (NM_001144964.1) was cloned into ClaI and XbaI sites of pCS2$^+$-HA or pCS2$^+$-FLAG vectors or into BglII and SalI sites of pEGFP-C3 vector or into XbaI and SalI sites of the pGEX-4T1 vector. pCS2$^+$-HA-Dvl2, pCS2$^+$-Flag-Dvl2, and pDsRed-Dvl2 plasmids were described previously (8). Various point mutants were generated using the QuikChange Site-directed Mutagenesis Kit (Stratagene). Nonspecific and NEDD4L shRNAs were described previously (8, 18). To generate a NEDD4L shRNA1-resistant construct, the target sequence of NEDD4L shRNA1 was mutated to 5′-gctaggctatggatcgagt-3′ (sense); for generation of the NEDD4L shRNA2-resistant construct, the target sequence of NEDD4L shRNA2 was mutated to 5′-tgaggaccacttatcatac-3′ (sense).

Cell Culture and Transfection—HEK293T and HeLa cells were maintained in DMEM supplemented with 10% FBS (Hyclone) in a 37 °C humidified incubator containing 5% CO_2. Transient transfection was performed with VigoFect (Vigorous).

Reagents and Antibodies—Chloroquine, bafilomycin A1, ALLN, lactacystin, JNK inhibitor SP600125, and Hanks' balanced salt solution were purchased from Sigma. Wnt5a protein was from Millipore. Antibodies were from various sources: Cell Signaling (anti-NEDD4L, anti-Dvl2, and anti-phospho-JNK), BD Biosciences (anti-Rac1), Sigma (anti-FLAG M2, anti-Ser(P), and anti-Thr(P)), Santa Cruz (anti-RhoA, anti-total JNK, anti-tubulin, anti-HA, and anti-Myc), and Jackson ImmunoResearch (Aminomethylcoumarin Acetate-conjugated anti-rabbit, Aminomethylcoumarin Acetate-conjugated anti-mouse, fluorescein isothiocyanate-conjugated anti-mouse, TRITC-conjugated anti-goat, TRITC-conjugated anti-rabbit).

Real-time RT-PCR—Quantitative RT-PCR analysis was carried out as described previously (8). The primers used for quantitative RT-PCR in cultured cells were as follows: human *β-ACTIN* (5′-gtaccactggcatcgtgatggact-3′ and 5′-ccgctcattgccaatggtgat-3′), human *DVL2* (5′-gcttccacatggccatgggc-3′ and 5′-tggcactgctggtgagagtcacag-3′), human *NEDD4L* (5′-tccaatggtcctcagctgttta-3′ and 5′-attttccacggccatgaga-3′), human *AXIN2* (5′-agtgtgaggtccacggaaac-3′ and 5′-cttcacactgcgatgcattt-3′), human *c-MYC* (5′-tctccttgcagctgcttag-3′ and 5′-gtcgtagtcgaggtcatag-3′), and human *DKK1* (5′-tcccctgtgattgcagtaaa-3′ and 5′-tccaagagatccttgcgttc-3′).

Reporter Assay, Immunoblotting, Immunofluorescence, and Immunoprecipitation—They were performed as described previously (8).

In Vitro Pulldown Assay—GST-NEDD4L was purified from *Escherichia coli* and immobilized for 1 h on glutathione-Sepharose beads (Amersham Biosciences) at 4 °C. The beads were washed extensively with binding buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl) and subsequently incubated for 90 min with FLAG-tagged Dvl2, which was *in vitro* translated by a reticulocyte lysate system (Promega). Bound proteins were extracted with loading buffer and analyzed by immunoblotting.

Ubiquitination Assay—In vivo ubiquitination was performed as follows. HEK293T cells were transfected with pCMV5-His-Myc-ubiquitin along with other expression vectors as indicated. At 40 h post-transfection, HEK293T cells were treated with ALLN for 4 h. The cells were lysed and cell lysates were boiled for 5 min in 1% SDS. After 10-fold dilution of the lysate with lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 25 mM NaF, 1% Triton X-100) plus protease inhibitors (Roche Applied Science) for 30 min at 4 °C, Dvl2 was immunoprecipitated and followed by immunoblotting. The *in vitro* ubiquitination assay was performed as described previously with modifications that 500 ng of recombinant human UbcH5b (E2; Enzo Life Science) and 2 μg of purified GST-NEDD4L were used in the reaction (8).

Embryo Microinjections—Xenopus laevis embryos were obtained and maintained as described (23). Embryos were microinjected in 2% Ficoll solution with mRNA at the required stages and cultured in 0.1 × MMR (Marc's Modified Ringers). Capped synthetic RNAs were generated by *in vitro* transcription using the mMessage mMachine kit (Ambion).

Statistical Analysis—Statistical analyses were performed with a two-tailed unpaired *t* test. $p < 0.05$ was considered statistically significant.

RESULTS

NEDD4L Interacts and Colocalizes with Dvl2—As the C-terminal of Dvl2 harbors a PY motif (PP*X*Y), which is highly conserved among three human Dvl proteins and *Drosophila* Dsh (Fig. 1*A*); we asked whether this PY motif (PP*X*Y) could be recognized by WW domain-containing NEDD4L through a physical interaction. Coimmunoprecipitation revealed the interaction between endogenous NEDD4L and endogenous Dvl2 in HEK293T cells (Fig. 1*B*). Purified GST-NEDD4L could interact with *in vitro* translated Dvl2, indicating the interaction is direct (Fig. 1*C*). The interaction was also confirmed when Dvl2 was overexpressed together with the NEDD4L C821A mutant (CA mutant) (Fig. 1*E*), which lacked ubiquitin ligase activity (17). Domain mapping revealed that the WW3 domain of NEDD4L alone had a strong interaction with Dvl2, and the WW2 domain had a weaker binding affinity, whereas both the WW1 and WW4 domains failed to bind to Dvl2 (Fig. 1*D*). Furthermore, the interaction between Dvl2 and NEDD4L was abrogated when the conserved residues (WW . . . P) in the WW3 domain were substituted (AA . . . F) (Fig. 1*E*). Consistent with the reported interaction between WW domains of NEDD4L and PY motifs of its substrates (15, 17), mutation of the PY motif (PPGY to AAGY, AY mutant) of Dvl2 abolished its interaction with full-length NEDD4L (Fig. 1*F*) or the WW3 domain (Fig. 1*G*). These data indicate that NEDD4L directly interacts with Dvl2 through the WW3 domain of NEDD4L and the PY motif of Dvl2.

We then analyzed the subcellular localization of NEDD4L and Dvl2. When expressed alone in HeLa cells, both wild-type

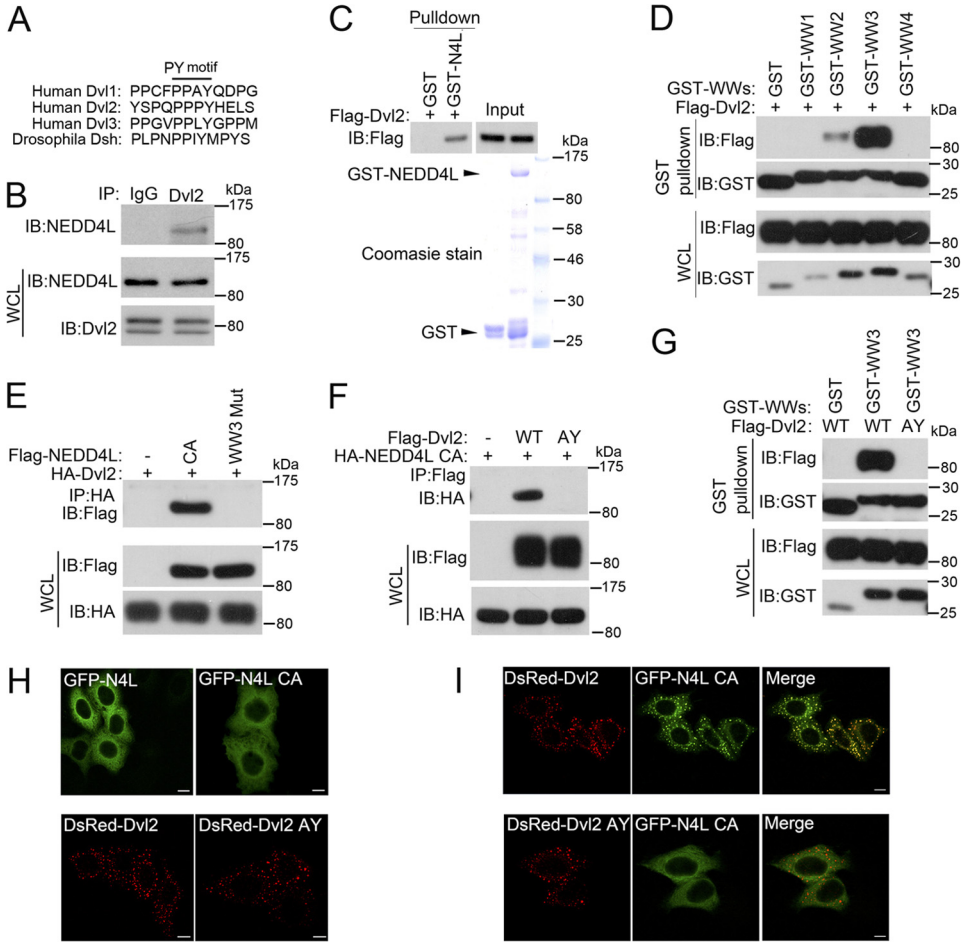

FIGURE 1. **NEDD4L interacts with Dvl2.** *A*, the PY motif (PP*XY*) is highly conserved among three human Dvl proteins and the *Drosophila* Dishevelled protein. *B*, endogenous NEDD4L interacts with Dvl2. HEK293T cell lysates were subjected to immunoprecipitation (*IP*) with control IgG or anti-Dvl2 antibodies followed by anti-NEDD4L immunoblotting (*IB*). *C*, NEDD4L interacts with Dvl2 *in vitro*. GST pulldown assay using *in vitro* translated FLAG-Dvl2 and immobilized GST or GST-NEDD4L fusion protein is shown. Precipitated proteins were detected with anti-FLAG antibody. Loading of GST and GST-NEDD4L protein was shown by Coomassie staining. *D*, WW3 and WW2 domains of NEDD4L bind to Dvl2. After transfection with different GST-NEDD4L WW domains together with FLAG-Dvl2 for 48 h, HEK293T cells were harvested for GST pulldown followed by anti-FLAG immunoblotting. Total protein expression was confirmed by immunoblotting with whole cell lysates (WCL). *E*, the WW3 domain of NEDD4L is required for its interaction with Dvl2. After transfection with FLAG-NEDD4L CA or WW3 mutant together with HA-Dvl2 for 48 h, HEK293T cells were harvested for anti-HA immunoprecipitation followed by anti-FLAG immunoblotting. Total protein expression was confirmed by immunoblotting with whole cell lysates (*WCL*) (*lower panels*). *F*, the PY motif of Dvl2 is essential for its interaction with NEDD4L. The experiment was performed similarly as in *D*. *G*, the WW3 domain binds to wild-type, but not the AY mutant Dvl2. After transfection with GST-NEDD4L (WW3) together with wild-type or AY mutant FLAG-Dvl2 for 48 h, HEK293T cells were harvested for GST pulldown followed by anti-FLAG immunoblotting. Total protein expression was confirmed by immunoblotting with whole cell lysates. *H*, Cellular distribution of wild-type, CA mutant NEDD4L, wild-type and AY mutant Dvl2. HeLa cells transfected with wild-type or mutant GFP-NEDD4L or DsRed-Dvl2 individually were processed for immunofluorescence. *Scale bar*: 10 μm. *I*, wild-type, but not AY mutant Dvl2, recruits NEDD4L to puncta. HeLa cells transfected with wild-type or AY mutant DsRed-Dvl2 together with EGFP-NEDD4L CA were processed for immunofluorescence. *Scale bar*, 10 μm.

and AY mutant Dvl2 appeared as puncta in the cytoplasm, whereas wild-type and the NEDD4L CA mutant were diffused throughout the cytoplasm (Fig. 1*H*). Strikingly, when co-expressed with wild-type Dvl2, NEDD4L (CA) formed puncta and colocalized well with Dvl2 (Fig. 1*I*). However, coexpression with the Dvl2 (AY) mutant did not induce punctate distribution of NEDD4L (Fig. 1*I*), consistent with that Dvl2 (AY) did not interact with NEDD4L. Together, these results demonstrate that NEDD4L and Dvl2 interact with each other.

NEDD4L Ubiquitinates Dvl2 and Induces Its Degradation—As NEDD4L is a HECT-domain E3 ubiquitin ligase, we then tested whether NEDD4L could ubiquitinate Dvl2. Indeed, wild-type NEDD4L, but not the CA mutant, efficiently promoted Dvl2 ubiquitination *in vivo* (Fig. 2*A*). *In vitro* ubiquitination reconstitution with recombinant proteins also confirmed that Dvl2 is a direct target of NEDD4L (Fig. 2*B*). Consistently, Dvl2 (AY) could not be ubiquitinated by NEDD4L (Fig. 2*C*), and knockdown of NEDD4L decreased Dvl2 ubiquitination (Fig. 2*D*).

Lys-48-linked ubiquitination is mainly to target the substrates for proteasomal degradation, whereas Lys-63-linked ubiquitination is involved in regulation of protein activity (24). It has been reported that NEDD4L promotes Lys-11-, Lys-48-, and Lys-63-linked ubiquitination (25). However, overexpression of K11R, K48R, and K63R ubiquitin mutants did not abolish the NEDD4L-mediated ubiquitination of Dvl2 (Fig. 2*E*). This raised the intriguing question, what type of ubiquitination NEDD4L induces on Dvl2? To address this, we created a series of ubiquitin mutants possessing a single lysine and found that NEDD4L promoted Lys-6-, Lys-27-, and Lys-29-linked polyubiquitination of Dvl2 (Fig. 2*F*). These results suggest that NEDD4L is an ubiquitin ligase for

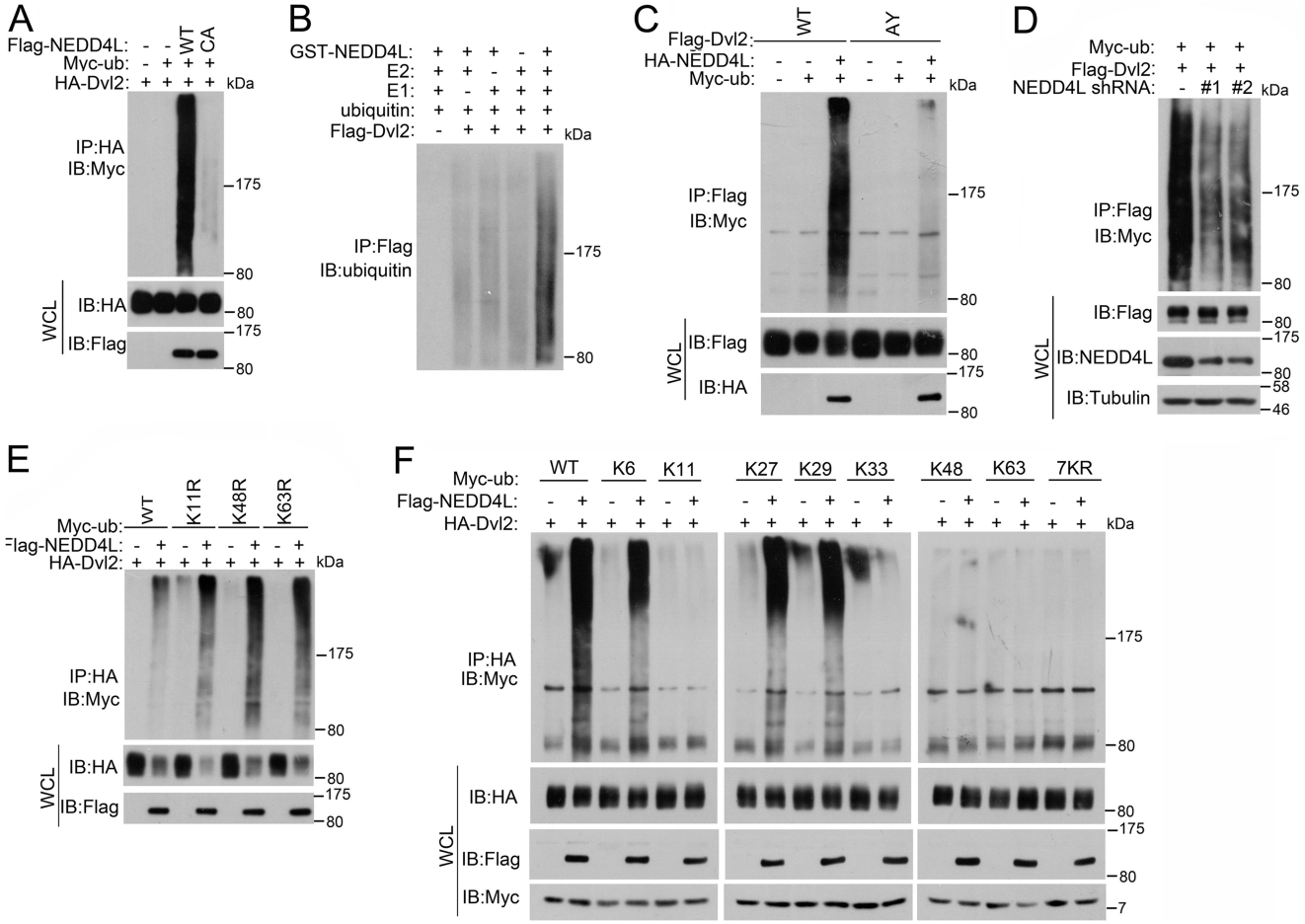

FIGURE 2. **NEDD4L ubiquitinates Dvl2.** *A,* NEDD4L promotes Dvl2 ubiquitination *in vivo*. After HEK293T cells were transfected with the plasmids as indicated for 40 h, cells were treated with ALLN (30 μM) for 4 h. Cells were harvested for anti-HA immunoprecipitation (*IP*) followed by anti-Myc immunoblotting (*IB*). Total protein expression was confirmed by immunoblotting with whole cell lysates (*WCL*). *B, in vitro* ubiquitination of Dvl2 by NEDD4L. *In vitro* translated FLAG-Dvl2 was incubated with or without purified E1, E2, and E3 ligase NEDD4L as indicated. The reaction was subjected to anti-FLAG immunoprecipitation, followed by anti-ubiquitin immunoblotting. *C*, NEDD4L enhances ubiquitination of wild-type but not AY mutant Dvl2. The experiment was performed similarly as in *A*. *D,* NEDD4L knockdown decreases Dvl2 ubiquitination. After HEK293T cells were transfected with FLAG-Dvl2, Myc-ubiquitin, and NEDD4L shRNAs for 60 h, cells were treated with ALLN (30 μM) for 4 h. Cell extracts were then subjected to anti-FLAG immunoprecipitation followed by anti-Myc immunoblotting. Total protein expression was confirmed by immunoblotting with whole cell lysates. *E,* ubiquitin K11R, K48R, and K63R mutants have no effect on Dvl2 ubiquitination induced by NEDD4L. The experiment was performed similarly as in *A*. *F,* NEDD4L promotes Lys-6, Lys-27, and Lys-29-linked ubiquitination of Dvl2. The experiment was performed similarly as in *A*.

Dvl2 and catalyzes Lys-6-, Lys-27-, and Lys-29-linked ubiquitination.

We then examined whether NEDD4L regulated Dvl stability via its ubiquitin ligase activity. Overexpression of NEDD4L down-regulated the protein levels of all three Dvl members albeit less efficiently on Dvl3 in HEK293T cells (Fig. 3*A*). Consistently, the half-life of Dvl2 was greatly reduced by NEDD4L (Fig. 3*B*). Moreover, down-regulation of the Dvl2 protein by NEDD4L was blocked by proteasome inhibitors lactacystin and ALLN, but not by lysosome inhibitors bafilomycin A1 and chloroquine (Fig. 3*C*), indicating that NEDD4L promoted Dvl2 degradation through the proteasomal pathway. Conversely, knockdown of NEDD4L significantly increased the Dvl2 protein level without affecting the Dvl2 mRNA levels (Fig. 3, *D–F*). The intact PY motif, which was required for the association of Dvl2 with NEDD4L, was necessary for NEDD4L-promoted Dvl2 degradation (Fig. 3, *G* and *H*). In line with this, both the WW3 mutant NEDD4L that was incapable of binding Dvl2 and CA NEDD4L that was unable to ubiquitinate Dvl2 failed to degrade Dvl2 (Fig. 3*I*).

Several point mutations of NEDD4L have been indicated to be associated with epilepsy (26). We then assessed if these mutations would affect its ability to regulate Dvl2 turnover and found that these mutations indeed attenuated the ability of NEDD4L to promote Dvl2 degradation (Fig. 3*J*). Interestingly, these mutations did not affect the interaction between NEDD4L and Dvl2 (Fig. 3*K*). Collectively, our results indicate that NEDD4L promotes Dvl2 degradation through the proteasomal pathway and the interaction between these two proteins is needed for this process.

NEDD4L Negatively Regulates Wnt/β-Catenin Signaling—Given that NEDD4L targets Dvl2 for proteasomal degradation and Dvl2 is a critical component of both Wnt/β-catenin and Wnt/PCP pathways, we assessed whether NEDD4L modulated the Wnt/β-catenin signaling. Indeed, overexpression of wild-type NEDD4L, but not the CA or WW3 mutant NEDD4L, decreased Wnt3a-induced Topflash reporter expression (Fig. 4*A*). Moreover, NEDD4L inhibited wild-type, but not the AY mutant, Dvl2 induced Topflash reporter expression (Fig. 4*B*). However, NEDD4L had no effect on reporter expression

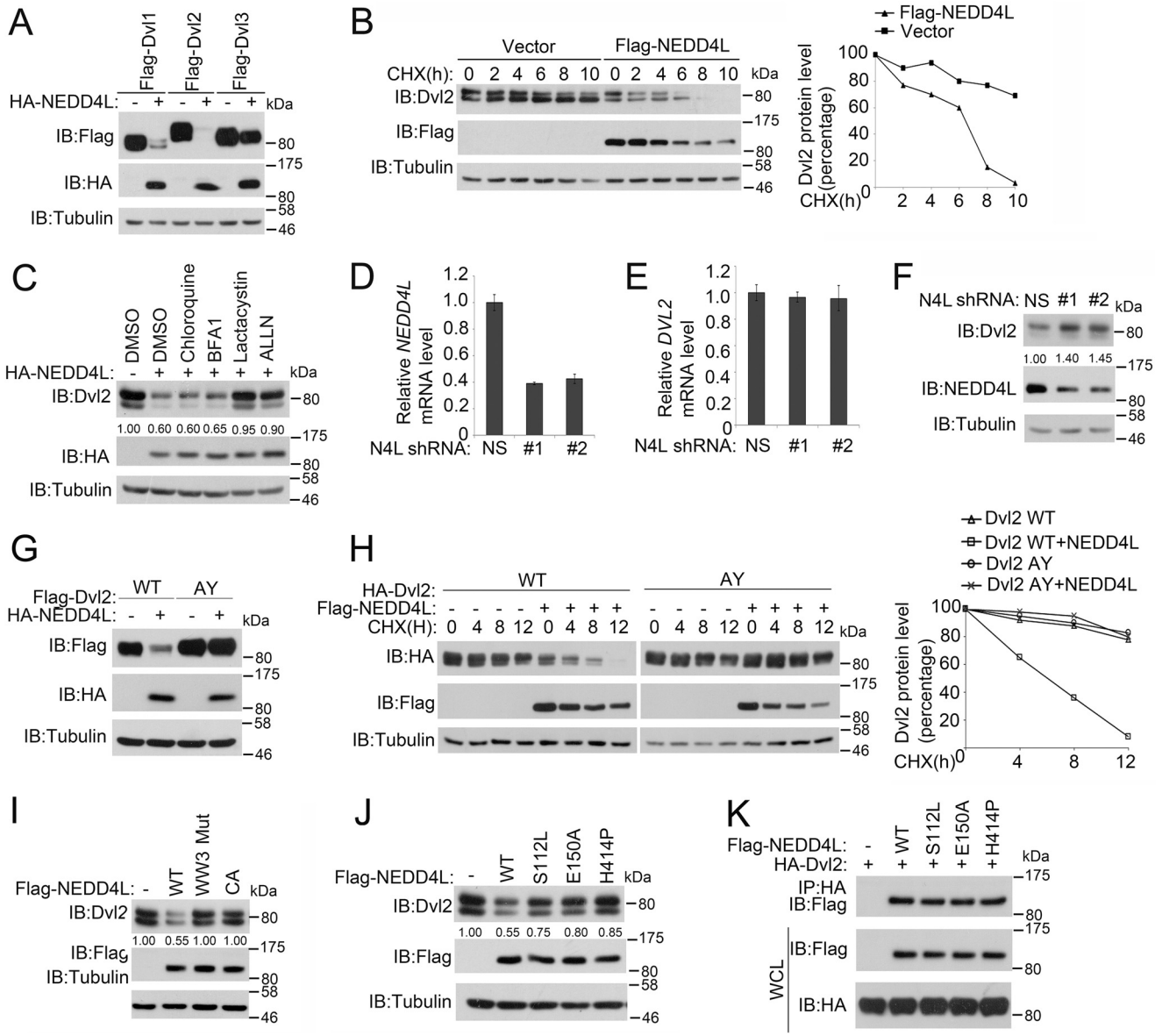

FIGURE 3. **NEDD4L promotes Dvl2 degradation.** *A*, NEDD4L induces degradation of three Dvl members. After transfection with human FLAG-Dvl1, -2, and -3 together with or without HA-NEDD4L for 48 h, HEK293T cells were harvested for immunoblotting (*IB*). Tubulin served as a loading control. *B*, NEDD4L decreases Dvl2 half-life. After HEK293T cells were transfected with FLAG-NEDD4L for 36 h, cells were treated with cycloheximide (*CHX*) (20 μg/ml) for the indicated times and harvested for immunoblotting. Tubulin served as a loading control. Dvl2 levels were quantified. *C*, NEDD4L degrades Dvl2 through the ubiquitin-proteasome pathway. HEK293T cells transfected with HA-NEDD4L were treated with lysosome inhibitors bafilomycin A1 (0.2 μM) and chloroquine (50 μM) or proteasome inhibitors lactacystin (10 μM) and ALLN (30 μM) for 4 h before harvesting for immunoblotting. Tubulin served as a loading control. The *lower panel* is the quantitation of Dvl2 band density. *D* and *E*, NEDD4L knockdown has no effect on the Dvl2 mRNA level. After HEK293T cells were transfected with nonspecific (*NS*) or two independent NEDD4L shRNAs for 72 h, total RNA was extracted and subjected to quantitative RT-PCR for examine NEDD4L (*D*) or Dvl2 (*E*) mRNA expression. The results show mean ± S.D. ($n = 3$). *F*, NEDD4L knockdown increases the Dvl2 level. After HEK293T cells were transfected with NS or two independent NEDD4L shRNAs for 72 h, cells were harvested for immunoblotting. Tubulin served as a loading control. *G*, the PY motif of Dvl2 is necessary for NEDD4L-induced degradation. After wild-type or AY mutant FLAG-Dvl2 was transfected with or without HA-NEDD4L into HEK293T cells for 48 h, cells were harvested for immunoblotting. Tubulin served as a loading control. *H*, NEDD4L decreases the half-life of wild-type Dvl2, but not Dvl2 (AY). The experiment was performed similarly as in *B*. *I*, the WW3 domain and ubiquitin ligase activity of NEDD4L are required for its induction of Dvl2 degradation. After wild-type or WW3 mutant or CA FLAG-NEDD4L was transfected into HEK293T cells for 48 h, cells were harvested for immunoblotting. Tubulin served as a loading control. *J*, point mutations of NEDD4L associated with epilepsy attenuate its ability to degrade Dvl2. The experiment was preformed similarly as in *I*. *K*, mutations of NEDD4L associated with epilepsy do not affect the interaction with Dvl2. HEK293T cells were transfected with the indicated constructs. After 36 h, the cells were treated with 30 μM ALLN for 4 h before harvesting for anti-HA immunoprecipitation (*IP*) followed by anti-FLAG immunoblotting. Total protein expression was confirmed by immunoblotting with whole cell lysates (*WCL*) (*lower panels*).

induced by the glycogen synthase kinase 3β inhibitor LiCl and a constitutively active (S37A) β-catenin (Fig. 4, *C* and *D*). Conversely, knockdown of NEDD4L enhanced Wnt3a-induced Topflash reporter expression, which was blocked by expression of shRNA-resistant NEDD4L (Fig. 4, *E* and *F*). Consistently, knockdown of NEDD4L increased the expression of Wnt/β-catenin signaling target genes *AXIN2*, *DKK1*, and *C-MYC* (Fig. 4, *G–I*). Accordingly, overexpression and knockdown of NEDD4L decreased and increased cellular β-catenin levels, respectively (Fig. 4, *J* and *K*). Taken together, these results indicate that NEDD4L is a negative regulator of Wnt/β-catenin signaling, functioning at the level of Dvl.

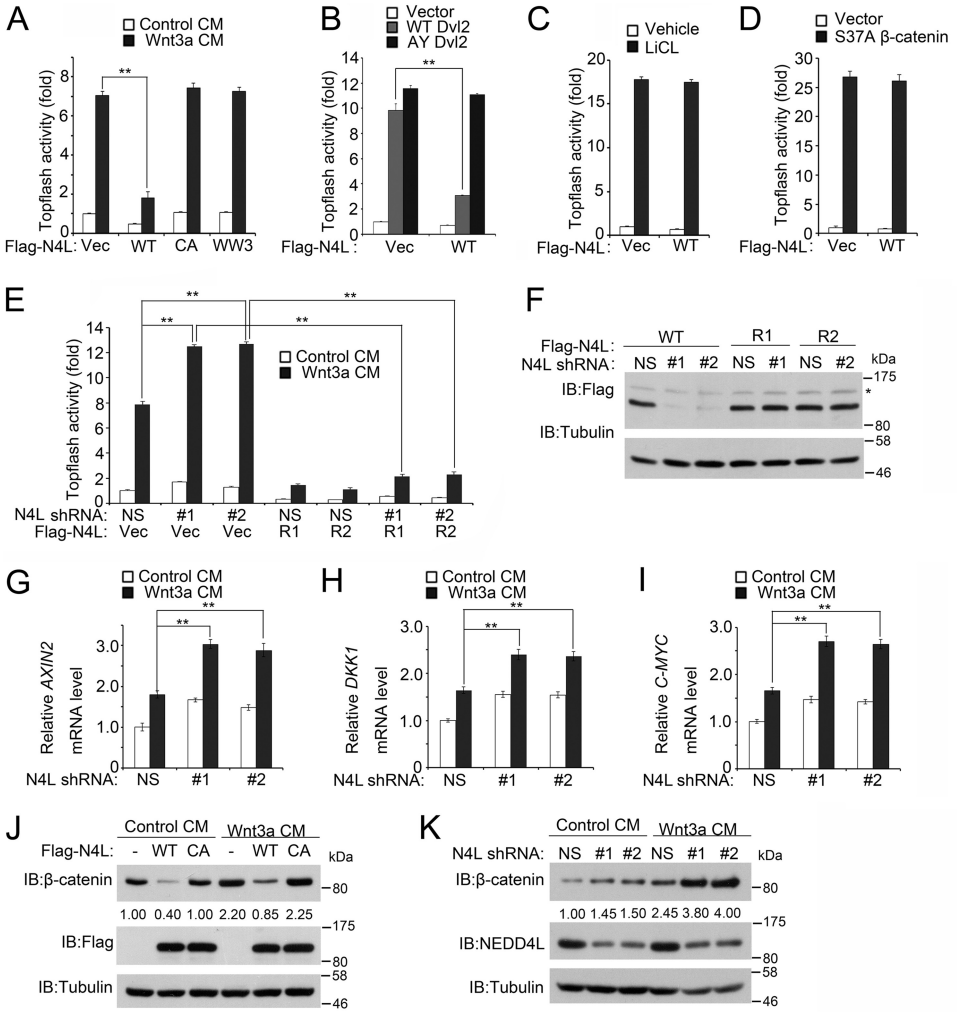

FIGURE 4. **NEDD4L attenuates Wnt/β-catenin signaling.** *A*, wild-type NEDD4L, but not CA or WW3 NEDD4L mutants, attenuates Wnt3a-induced Topflash reporter expression. After transfection with the Topflash luciferase reporter with or without FLAG-NEDD4L variants for 36 h, HEK293T cells were treated with control medium or Wnt3a conditioned medium (*CM*) overnight and then luciferase activity was measured. The pRL-TK *Renilla* reporter was co-transfected to normalize transfection efficiency. The experiment was performed in triplicate, and data were represented as the mean ± S.D. after normalized to *Renilla* activity (**, $p < 0.01$). *B*, NEDD4L decreases wild-type Dvl2, but not AY mutant Dvl2, induced Topflash reporter activity. The experiment was performed similarly as in *A* except that Dvl2 was transfected to activate the reporter activity. *C*, NEDD4L has no effect on LiCl-induced Topflash reporter activity. The experiment was performed similarly as in *A*, except that 20 mM LiCl was used to activate the reporter activity. *D*, NEDD4L has no effect on β-catenin (S37A)-induced Topflash reporter activity. The experiment was performed similarly as in *A*, except that β-catenin (S37A) was transfected to activate reporter activity. *E*, NEDD4L knockdown increases Topflash reporter activity. The experiment was performed as in *A*, except that various plasmids and nonspecific (*NS*) or NEDD4L shRNAs were transfected for 60 h. FLAG-NEDD4L R1 and R2 were resistant to NEDD4L shRNA1 and shRNA2, respectively. *F*, NEDD4L shRNAs have no effect on expression of shRNA-resistant NEDD4L. HEK293T cells transfected in *E* were harvested for immunoblotting (*IB*) with anti-FLAG antibody. Tubulin served as a loading control. The *asterisk* indicates a nonspecific band. *G–I*, NEDD4L knockdown increases the expression of *AXIN2*, *DKK1*, and *C-MYC*. The experiments were performed similarly as in Fig. 3*D*. *J*, NEDD4L overexpression decreases the β-catenin level. After transfection with wild-type or CA FLAG-NEDD4L for 40 h, HEK293T cells were treated with control medium or Wnt3a condition medium (*CM*) for 6 h and then harvested for immunoblotting. Tubulin served as a loading control. *K*, NEDD4L knockdown increases the β-catenin level. The experiment was performed as in *J*, except that nonspecific or NEDD4L shRNAs were transfected for 60 h.

NEDD4L Regulates Activities of the Wnt/PCP Pathway Components RhoA, Rac1, and JNK—To explore the function of NEDD4L in Wnt/PCP signaling, we tested whether the activities of Rac1 and RhoA, which can be activated by Wnt5a and act downstream of Dvl2, were altered in response to NEDD4L depletion. Using GST-PBD (p21 binding domain) and GST-RBD (Rho binding domain) fusion proteins to pull down the active form of Rac1 and RhoA, respectively, we found that NEDD4L depletion dramatically increased the Rac1-GTP level and decreased the RhoA-GTP level both in the basal level and upon Wnt5a stimulation (Fig. 5, *A* and *B*). Rac1 activation resulted from NEDD4L depletion was further corroborated by enhanced phosphorylation of JNK, an effector of Rac1-GTP (Fig. 5*C*) and elevated expression of the JNK-responsive AP1 reporter (Fig. 5*D*). These results are in agreement with the observation that the increased Dvl protein level stimulated JNK activity, whereas impairing Rho activity (27). These data together indicate that NEDD4L could regulate PCP signaling via Dvl.

JNK-mediated Phosphorylation Is Required for NEDD4L to Down-regulate Dvl2—Several kinases have been reported to regulate NEDD4L activity. For instance, JNK1 can phosphorylate NEDD4L at three conserved serine and threonine residues (Ser-176, Thr-291, and Thr-882), leading to stimulation of its

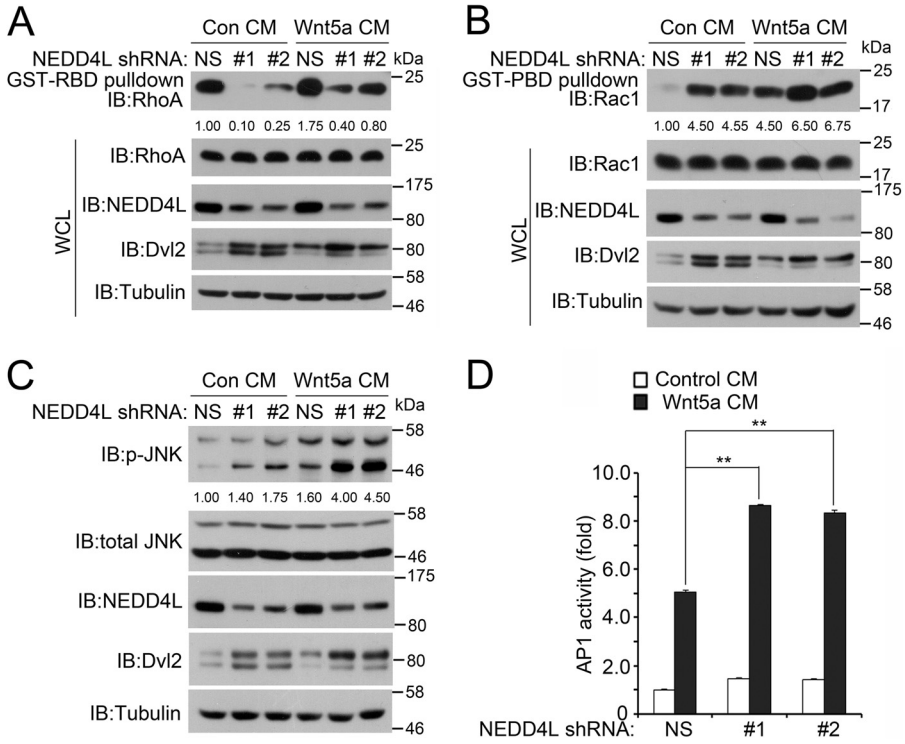

FIGURE 5. **NEDD4L regulates the activities of RhoA, Rac1, and JNK.** *A,* NEDD4L knockdown decreases RhoA activity. HEK293T cells were transfected with nonspecific (*NS*) shRNA or NEDD4L shRNAs. At 40 h post-transfection, the cells were serum-starved for 20 h and then treated with control medium or Wnt5a medium for 4 h. The cells were then lysed, and active GTP-bound RhoA was immunoprecipitated using purified GST-RBD protein and detected by anti-RhoA antibody. Total protein expression was confirmed by immunoblotting with whole cell lysates (*WCL*). *B,* NEDD4L knockdown increases Rac1 activity. As in *A*, except that active GTP-bound Rac1 was immunoprecipitated using purified GST-PBD protein and detected by anti-Rac1 antibody. *C*, NEDD4L knockdown increases JNK activity. HEK293T cells were transfected, serum starved, and treated with control medium or Wnt5a medium as in *A*. Then cells were harvested for immunoblotting (*IB*). Tubulin serves as a loading control. *D,* NEDD4L knockdown increases AP1 luciferase-reporter activity. HEK293T cells were transfected with NS or NEDD4L shRNA together with AP-1 reporter and *Renilla*. The cells were then serum-starved and treated with control medium or Wnt5a medium as in *A* and harvested for luciferase assay. The experiment was performed in triplicate, and data represented as the mean ± S.D. after normalized to *Renilla* activity (**, $p < 0.01$).

ubiquitin ligase activity (28, 29). As JNK1 activity is regulated by Wnt5a, we then asked whether Wnt5a could affect NEDD4L phosphorylation. Indeed, Wnt5a treatment induced NEDD4L phosphorylation, which was abolished by the JNK inhibitor SP600125, indicative of JNK involvement. Consistently, mutation of the three JNK1-phosphorylated serine and threonine residues to alanines (AAA mutant) abolished Wnt5a-induced phosphorylation (Fig. 6*A*). In agreement with the previous report (28), NEDD4L(AAA) showed impaired autoubiquitination, suggesting that JNK1-mediated phosphorylation is required for its ubiquitin ligase activity (Fig. 6*B*). We then assessed whether NEDD4L phosphorylation affected its ability to induce Dvl2 degradation. Compared with the wild-type, NEDD4L(AAA) was less effective in promoting Dvl2 turnover (Fig. 6*C*). This was not due to impaired interaction between NEDD4L and Dvl2, as Dvl2 showed similar binding affinity with wild-type and the AAA mutant NEDD4L (Fig. 6*D*). Moreover, NEDD4L(AAA) did not promote Dvl2 ubiquitination as efficiently as wild-type NEDD4L (Fig. 6*E*). Consistently, co-expression of constitutively active JNK1 (30, 31) facilitated Dvl2 degradation by wild-type NEDD4L, but not the AAA mutant NEDD4L (Fig. 6*C*). Finally, we assessed the temporal order of JNK activation and NEDD4L phosphorylation. Upon Wnt5a treatment, JNK was fast activated and then gradually inactivated. The phosphorylation of NEDD4L correlated well with JNK activity, suggesting that JNK may act as an upstream kinase for NEDD4L. Activated NEDD4L then facilitated Dvl2 degradation (Fig. 6*F*). Collectively, these results indicate that Wnt5a-induced JNK activation and subsequent NEDD4L phosphorylation were critical for NEDD4L to promote Dvl2 ubiquitination and degradation.

NEDD4L Inhibits Dvl2-induced Axis Duplication in Xenopus—Finally, we assessed the physiological significance of NEDD4L-regulated Wnt/β-catenin signaling *in vivo*. Wnt/β-catenin signaling activation induces dorsal axis duplication in *Xenopus* embryos (5, 9, 12). Consistent with previous reports (12), injection of wild-type or AY mutant Dvl2 mRNA could induce a second axis. However, co-injection of NEDD4L mRNA repressed axis duplication induced by wild-type Dvl2, but not by the AY mutant Dvl2 (Fig. 7). Interestingly, injection of NEDD4L mRNA alone also resulted in bent axis, indicating a role of NEDD4L in axis formation.

DISCUSSION

NEDD4L is an ubiquitin ligase implicated in several cellular or physiological processes (16, 17). Here we provide multiple lines of evidence to show a novel function of NEDD4L to regulate Wnt signaling. NEDD4L ubiquitinates Dvl and promotes its degradation and then controls the cellular β-catenin level and the activities of Rac1, RhoA, and JNK. We further demonstrated that JNK1 could potentiate NEDD4L ubiquitin ligase activity through phosphorylation, implicating a possible nega-

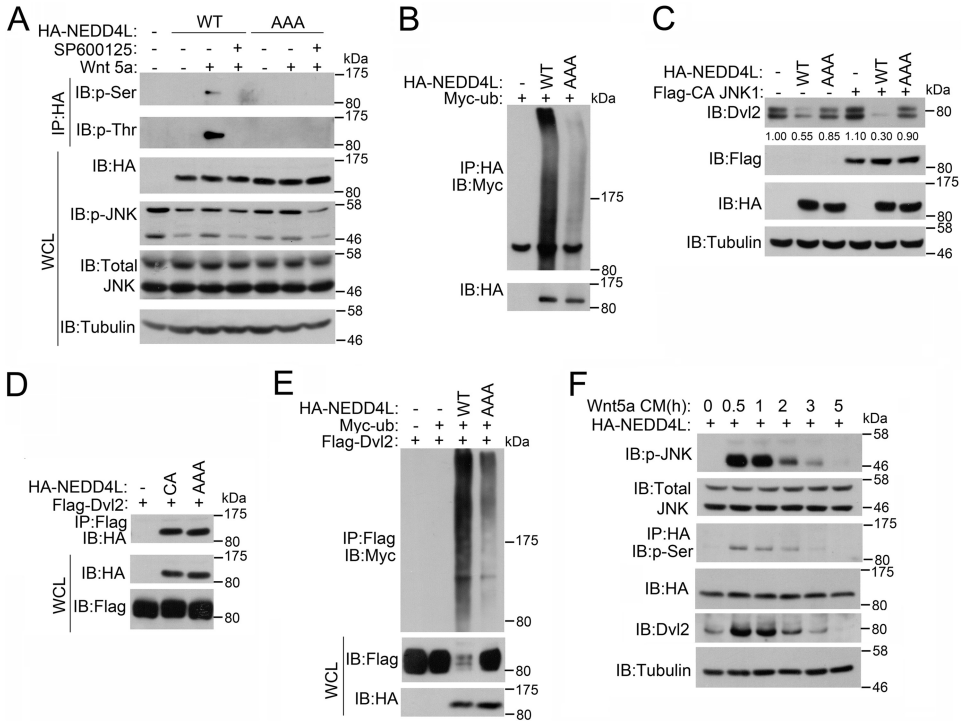

FIGURE 6. **JNK1-mediated phosphorylation is required for NEDD4L to promote Dvl2 degradation.** *A*, Wnt5a induces NEDD4L phosphorylation in a JNK-dependent manner. After HEK293T cells were transfected wild-type or AAA mutant HA-NEDD4L for 40 h, cells were treated with Wnt5a (100 ng/ml) and JNK inhibitor (20 μM) for 4 h. Cells were then harvested for anti-HA immunoprecipitation (*IP*) followed by anti-Ser(P) or anti-Thr(P) immunoblotting (*IB*). Total protein expression was confirmed by immunoblotting with whole cell lysates (*WCL*). *B*, mutation of JNK1 phosphorylation sites disrupts ubiquitin ligase activity of NEDD4L. The experiment was performed similarly as in Fig. 3*A*. *C*, JNK1 phosphorylation of NEDD4L triggers its degradation of Dvl2. HEK293T cells were transfected with the indicated plasmids. After 48 h, cells were harvested for immunoblotting. Tubulin served as a loading control. *D*, mutation of JNK1 phosphorylation sites of NEDD4L has no effect on its interaction with Dvl2. The experiment was performed similarly as in Fig. 1*E*. *E*, JNK1 phosphorylation of NEDD4L is requisite for its ubiquitination of Dvl2. The experiment was performed similarly as in Fig. 2*A*. *F*, NEDD4L phosphorylation is correlated with JNK activation. HEK293T cells were transfected with wild-type NEDD4L. At 40 h post-transfection, the cells were nutrient starved in Hanks' balanced salt solution for 6 h, treated with Wnt5a conditional medium, and then harvested at the indicated time points for immunoblotting. Tubulin served as a loading control.

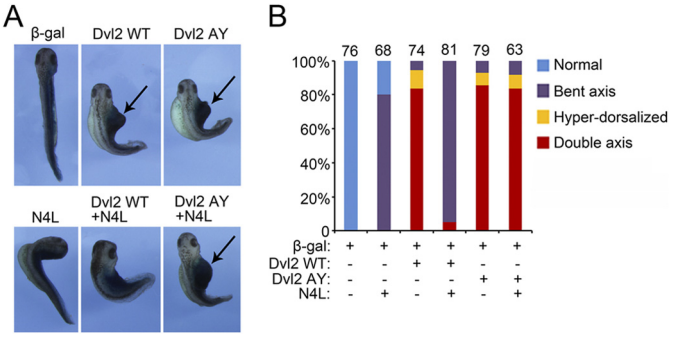

FIGURE 7. **NEDD4L antagonizes Dvl2-induced axis duplication in *Xenopus* embryos.** *A*, NEDD4L blocks axis duplication induced by wild-type Dvl2, but not by AY mutant. mRNA (1 ng) encoding wild-type or AY mutant human Dvl2 was injected alone or together with 500 pg of human NEDD4L mRNA into the ventral marginal zone at the 4-cell stage *Xenopus* embryos, and β-galactosidase mRNA (500 pg) was coinjected as a lineage marker. The phenotype was scored at the tadpole stage. *B*, quantitative analysis of the phenotype shown in *A*. The total number of injected embryos of each group was indicated.

tive feedback regulation of Wnt signaling. Finally we showed that NEDD4L antagonized Dvl2-induced axis duplication in *Xenopus* embryos.

The activity and stability of Dvl proteins are tightly regulated by many cellular proteins. They can undergo either proteasomal degradation or autophagy-lysosomal degradation upon ubiquitination induced by various E3 ligases (7–9). Recent studies have suggested a role of NEDD4 family ubiquitin ligases in regulation of Dvl stability. NEDL1 has been reported to target Dvl1 for degradation (11, 32). NEDD4 was shown to degrade Dvl1, but not Dvl2 and Dvl3 (32). However, we observed that, like NEDD4L, NEDD4 could interact with Dvl2 and promote its degradation through ubiquitination (data not shown). ITCH specifically regulates turnover of phosphorylated Dvl (14). In this study, we provide compelling evidence that NEDD4L is a genuine ubiquitin ligase for Dvl2 and regulates both Wnt/β-catenin and Wnt/PCP pathways. *In vivo* studies using mouse models suggest that these NEDD4 family members have distinct functions (15). *NEDL1* transgenic mice mainly show muscle atrophy and motor neuron degeneration (33). Disruption of NEDD4 in mice causes embryonic lethality at midgestation and severe heart defects and vasculature abnormalities due to up-regulation of the Tsp-1 protein (34). It has also been reported that *NEDD4* knock-out mice show delayed embryonic development and growth retardation resulting from reduced insulin-like growth factor 1 and insulin signaling (35). *ITCH* knock-out mice primarily show defects in the immune system through regulation of diverse substrates, including c-Jun, JunB, and PLCγ1 (36, 37). Recently it was shown that *ITCH* knock-out mice also showed age-dependent alterations in spermatogenesis (38). *NEDD4L* knock-out mice died perinatally due to disrupted lung function (21, 22). Therefore, it remains to clarify whether NEDD4 family ubiquitin ligases play a unique or redundant role in regulating stability of Dvl family proteins and

thus examine their relevant contributions to the related physiological and pathological processes.

E3 ligases can promote the formation of polyubiquitin chains on substrates through any of the seven lysines present on ubiquitin molecules (24, 39). NEDD4L activity has been associated with chain formation mainly via Lys-11, Lys-48, and Lys-63 linkages (25). However, none of the linkages was used in the ubiquitination of Dvl2 by NEDD4L. In contrast, NEDD4L promoted Dvl2 ubiquitination via atypical ubiquitin chain formation involving residues Lys-6, Lys-27, and Lys-29, leading to its proteasomal degradation. Our study uncovered a novel role of NEDD4L to catalyze Lys-6, Lys-27, and Lys-29 ubiquitin chain formation and showed that these atypical ubiquitin chains played an important role in the regulation of Dvl stability.

NEDD4L gene mutations have been associated with neurological disorders. It was reported that NEDD4L has been implicated to be a susceptibility locus for bipolar affective disorder (40). NEDD4L was also identified as a candidate gene for dyslexia susceptibility and a modifier of age at neurological onset in Huntington disease (41). In addition, point mutations of NEDD4L have also been identified in idiopathic generalized epilepsies in patients (26). However, the role of NEDD4L in the pathogenesis and progression of these disorders is unknown. We show here that its point mutations associated with epilepsy, which did not affect its regulation of ion channels (26), impaired its ability to degrade Dvl2. Taken together, given the critical role of Wnt signaling in neural development and diseases, our findings may shed light on the role of NEDD4L in neurological disorders. Furthermore, down-regulation or loss of function of NEDD4L has been proposed to be associated with the malignancy of prostate cancer, non-small cell lung cancer, and glioma (42–44). Interestingly, Dvl protein levels have been reported to be elevated in these cancers (45–47). Therefore, the NEDD4L/Dvl axis may also have an important function in tumorigenesis.

Acknowledgments—We thank Dr. Lingqiang Zhang for providing human NEDD4 plasmid, Dr. Long Yu for providing NEDD4 antibody, Dr. Wei Wu for providing Rac1 antibody, and Dr. He Li for help in purifying NEDD4L protein.

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