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# NEDD4L regulates convergent extension movements in *Xenopus* embryos via Disheveled-mediated non-canonical Wnt signaling

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ABSTRACT

During the early vertebrate body plan formation, convergent extension (CE) of dorsal mesoderm and neurectoderm is coordinated by the evolutionarily conserved non-canonical Wnt/PCP signaling. Disheveled (Dvl), a key mediator of Wnt/PCP signaling, is essential for the medial–lateral polarity formation in the cells undergoing convergent extension movements. NEDD4L, a highly conserved HECT type E3 ligase, has been reported to regulate the stability of multiple substrates including Dvl2. Here we demonstrate that NEDD4L is required for the cellular polarity formation and convergent extension in the early *Xenopus* embryos. Depletion of NEDD4L in early *Xenopus* embryos results in the loss of mediolateral polarity of the convergent-extending mesoderm cells and the shortened body axis, resembling those defects caused by the disruption of non-canonical Wnt signaling. Depletion of xNEDD4L also blocks the elongation of the animal explants in response to endogenous mesoderm inducing signals and partially compromises the expression of *Brachyury*. Importantly, reducing Dvl2 expression can largely rescue the cellular polarity and convergent extension defects in NEDD4L-depleted embryos and explants. Together with the data that NEDD4L reduces Dvl2 protein expression in the frog embryos, our findings suggest that regulation of Dvl protein levels by NEDD4L is essential for convergent extension during early *Xenopus* embryogenesis.



## Introduction

The body plan formation of early *Xenopus* embryos involves both cell fate specification and tissue rearrangement in a time- and space-coordinated manner (Heasman, 2006). During mid- to late-blastula, mesoderm is induced to form around the equatorial zone by the signals including Nodal family ligands emitted from the vegetal pole. During gastrulation, mesoderm cells involute through blastopore lip and undergo extensive tissue rearrangement. In particular, chordamesoderm precursors converge in the mediolateral direction through cell–cell intercalation while collectively migrating towards the dorsal side of the embryo, resulting in the extension of the forming head to tail axis. The mediolateral polarity formation of intercalating cells in the direction normal to the antero-posterior axis is essential for ensuring the proper convergent-extension movements and body plan formation (Tada and Heisenberg, 2012; Keller et al., 2000). All these aspects of cellular function during convergent extension seem to depend on the balanced activity of small Rho GTPases (RhoA, Rac1 and Cdc42), central regulators of actin dynamics (Tanegashima et al., 2008; Tahinci and Symes, 2003; Keller, 2002).

Numerous studies have implicated essential roles for β-catenin-independent non-canonical Wnt/PCP signaling in CE movements in multiple model organisms (Goodrich and Strutt, 2011; Angers and Moon, 2009). The multi-domain cytoplasmic protein, Disheveled (Dvl), plays pivotal roles in both canonical and non-canonical Wnt pathways (Gao et al., 2010; Gao and Chen, 2010; van Amerongen and Nusse, 2009; Wallingford and Habas, 2005; Wharton, 2003). Evidence has been reported that Dvl may lie upstream of Rho A in the dynamic control of cellular functions during mesodermal convergent extension (Tanegashima, et al., 2008; Habas et al., 2003, 2001). Perturbation of Dvl protein levels or its membrane localization leads to the loss of mediolateral polarity in chordamesoderm cells and impedes convergent extension of axial tissues, often resulting in a failure of blastopore/neural tube closure and a drastically shortened body axis in *Xenopus* embryos (Kinoshita et al., 2003; Park et al., 2005; Wallingford et al., 2000; Wang et al., 2006). Therefore,

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dissecting the mechanism underlying the control of Dvl production is of central importance in understanding CE movements.

NEDD4L (neural precursor cell expressed, developmentally down-regulated 4-like), a HECT-type E3 ubiquitin ligase, has been shown to be essential for the stability control of multiple cellular proteins including ENαC, activated Smad2/3 and Dvl (Yang and Kumar, 2009; Boase et al., 2011; Kimura et al., 2011; Lee et al., 2009; Rotin and Kumar, 2009; Gao et al., 2009; Ding et al., 2013). In this report we tested the function of NEDD4L in the context of mesodermal convergent extension using *Xenopus* model organism. Serial in vivo and ex vivo analyses revealed that NEDD4L is essential for the mediolateral polarity formation in the chordamesoderm and normal convergent extension. Simultaneous knockdown of Dvl2 or over-expression of constitutively active RhoA could largely rescue the mediolateral polarity formation when NEDD4L was depleted. In addition, NEDD4L is required for the expression of pan-mesoderm gene *Brachyury* in the dorsal region.

## Materials and methods

### *Xenopus laevis embryos and explants*

*Xenopus laevis* embryos were obtained through standard procedures (Sive et al., 2000) and staged according to Nieuwkoop's standard (Nieuwkoop and Faber, 1994).

For injection of MO or mRNA, fertilized eggs were dejellyed using 2% cysteine (pH8.0) at one cell stage and thoroughly washed with 0.1 × MMR (Marc's modified ringer solution, Sive et al., 2000) and then transferred into 2% Ficoll made in 0.5 × MMR. xNEDD4L MO was always injected with 5 ng of rhodamine-lysinated-dextran (RLDx) or fluorescent-lysinated-dextran (FLDx) as a lineage tracer. Embryos injected with xNEDD4L MO and RLDx or FLDx were always scored under a fluorescent stereoscope at st10 to ensure the accurate targeting of dorsal mesoderm.

For Activin induction assay, animal caps were excised by a pair of sharp forceps from injected and uninjected embryos at st9, and then transferred into 0.5 × oocyte culture medium (OCM) supplemented with or without Activin. The animal caps were cultured to sibling stage of st11 for in situ hybridization or quantitative RT-PCR analysis and st18 for phenotyping respectively.

For Keller's sandwich assay, the dorsal marginal zone of injected and uninjected embryo were cut at st10.25, the sandwiches were then made by facing two pieces of dorsal marginal zone against each other and cultured under pressure from a tiny piece of cover glass using Vaseline as spacers to the sibling stage of st16–18. To prepare open-faced Keller's explants, the dorsal marginal zones were dissected at st10.25 and then cultured on the coverslip precoated with fibronectin (Sigma F1056).

For Nieuwkoop assay, all vegetal bases were prepared at st8.5–9, and animal caps were dissected from uninjected or MO-injected embryos at st8.5–9. The Nieuwkoop conjugates were then cultured in 1 × MMR to the equivalent stage 11 for *Xbra* in situ hybridization or the equivalent stage 16 for monitoring the elongation of animal caps in response to the mesoderm inducing activity of the vegetal base. For the quantitative analysis of gene expression, the animal caps from the Nieuwkoop conjugates were stripped off at the equivalent stage 11 and subjected to RT-qPCR analysis.

### *Oligos, mRNAs, whole mount in situ hybridization and quantitative RT-PCR*

All antisense MO oligos were purchased from Gene Tools (Oregon, USA). The sequences of MOs were as follows: xNEDD4L: 5'-gccgccatggggagagttggggtgc-3'; xDvl2 MO: 5'-ggtaaatcacttt-agtctccgccat-3' (Gray et al., 2009). RT-PCR primers used in this study are listed in Table S1.

*Xenopus* and human NEDD4L (isoform 2) cDNAs were cloned into pCS2 vector. A MO-resistant form of xNEDD4L was generated a homolog of human NEDD4L isoform 2 through removing the 5' part encoding the C2 domain. For in vitro mRNA synthesis, pCS2-xNEDD4L or hNEDD4L, pCS2-V14-RhoA was linearized with *Not*I and transcribed using SP6 message machine kit (Ambion). mRNAs were purified through proteinase K treatment, phenol/chloroform extraction and alcohol precipitation and resuspended in nuclease free water. For xNEDD4L in situ probe labeling, pCS2-xNEDD4L was linearized with *Hind*III and then transcribed with Promega T7-Dig-UTP labeling kit (Promega). Whole mount in situ hybridizations were carried out through a standard protocol as described in Birsoy et al. (2005) and Sive et al. (2000). For the quantitative RT-PCR, embryos or explants at indicated stages were lysed with proteinase K containing buffer. Total RNAs were then isolated and purified as described previously (Kofron et al., 1999). The quantitative RT-PCR was carried out using a Roche LightCycler following the procedures as described (Kofron et al., 1999; Tao et al., 2005).

### *F-actin staining, Dvl2-GFP reporter assay and confocal imaging*

For F-actin staining, embryos at indicated stages were fixed with 3.7% formaldehyde, 0.25% glutaraldehyde in PBS/Tween 20 (0.1%) at room temperature for 45 min followed by washing in PBSTw for 3 times, 20 min each. Then the fixed embryos were transverse sectioned using a sharp razor blade. Samples were then stained with Alexa-488-conjugated phalloidin (5 U/ml in PBSTw, Molecular Probe) at 4 °C overnight and washed with PBSTw for 1 h. Embryos were dehydrated in an isopropanol series and cleared in Murray's Clear (1:2 benzyl alcohol and benzyl benzoate). For Dvl2-GFP/mRFP reporter assay, injected and uninjected embryos were cultured to st13, and the neural plates were dissected and fixed with 4% PFA in PBS for 45 min at room temperature and then thoroughly washed with 1 × PBS. The confocal images were acquired on a Zeiss LSM710 using a 100 × NA1.4 oil objective for images in Figs. 6 and 8 and a 40 × NA0.8 water objective for images in Fig. 2D, Figs. S5 and S6 with appropriate settings.

### *Co-immunoprecipitation*

HEK293T cells were transiently transfected with HA-Dvl2, hNEDD4L C821A-FLAG plasmids at 70% confluence and harvested after 40 h. The cells were then lysed with lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 25 mM NaF, 1% Triton X-100) plus protease inhibitors (Sigma) for 30 min at 4 °C. After 12,000*g* centrifugation for 15 min, the lysates were then incubated with anti-HA antibodies and protein G beads. The immunocomplexes were then washed with washing buffer (20 mM Tris–HCl, 150 mM NaCl, pH 7.4, 2 mM EDTA, 25 mM NaF, 1% Triton X-100) for 3 times and then separated with 8% SDS-PAGE.

### *Ubiquitination assay*

At 40 h post-transfection, HEK293T cells were treated with ALLN for 4 h. Then the cell were lysed by boiling for 5 min in 1% SDS. After 10-fold dilution with the buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 25 mM NaF, 1% Triton X-100) plus protease inhibitors (Roche), Dvl2 was then precipitated followed by immunoblotting.

## Results

### *Spatial–temporal expression of NEDD4L during Xenopus embryonic development*

Peptide sequence comparison indicated that *Xenopus* NEDD4L (xNEDD4L) encodes a HECT class E3 ligase, sharing 89% and 62%