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Supplementary Information for

Mechanistic insights into the interactions of dynein regulator Ndel1 with neuronal ankyrins and implications in polarity maintenance

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SI Materials and Methods

Constructs, Protein expression and purification.

The full-length Ndel1 gene was PCR amplified from a mouse cDNA library (NCBI: NP_076157.2, 345 aa). Full-length Nde1 gene was PCR amplified from a human cDNA library (UniProt: Q9NXR1, 335 aa). Full-length rat 480 kDa ankyrin-G and full-length human 440 kDa ankyrin-B are generous gifts from Dr. Vann Bennett (Duke University). Various mutations or shorter fragments of AnkG, AnkB, Nde1, and Ndel1 were generated using standard PCR-based methods and confirmed by DNA sequencing. All of these coding sequences were cloned into a home-modified pET32a vector for protein expression. The N-terminal thioredoxin-His_6-tagged proteins were expressed in *Escherichia coli* BL21 (DE3) cells in LB medium at 16 °C and purified using a nickel-NTA agarose column followed by size exclusion chromatography (Superdex 200 or Superdex 75) with a column buffer containing 50 mM Tris pH 7.8, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT. The thioredoxin-His_6 tag was removed by incubation with HRV 3C protease and separated by size exclusion columns when needed.

Isothermal Titration Calorimetry Assay.

Isothermal titration calorimetry (ITC) measurements were carried out on a VP-ITC Microcal calorimeter (Malvern) at 25 °C. All proteins were in 50 mM Tris buffer containing 100 mM NaCl, 1 mM EDTA, and 1 mM DTT at pH 7.8. Each titration point was performed by injecting a 10 μL aliquot of AnkG or AnkB protein into Nde1 or Ndel1 protein samples in the cell at a time interval of 180 seconds to ensure that the titration peak returned to the baseline. The titration data were analyzed using the program Origin7.0 and fitted by the one-site binding model.

Analytical Gel Filtration Chromatography Coupled with Static Light Scattering

The analysis was performed on an AKTA Pure system (GE Healthcare) coupled with a static light scattering detector (miniDawn, Wyatt) and a differential refractive index detector (Optilab, Wyatt). Protein samples (concentration of 100 μM for AnkG, AnkB and 100 μM or 200 μM or 300 μM for Nde1, Ndel1) were filtered and loaded into a Superose

12 10/300 GL column pre-equilibrated by a column buffer composed of 50 mM Tris pH 7.8, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT. Data were analyzed with ASTRA7 (Wyatt).

Circular Dichroism Measurements

The CD spectra of the proteins were acquired on a Chirascan™-plus CD Spectrometer at the room temperature. AnkG-NIR, AnkB-NIR, Nde1 CT-CC, and Ndel1 CT-CC proteins were purified, tag-cleaved, and diluted into 20 mM in a buffer containing 25 mM Tris pH 7.8, 50 mM NaCl, 0.5 mM EDTA and 0.5 mM DTT. For urea-induced denaturation assay, the ellipticity values of the samples at 222 nm were recorded with the increasing concentrations of urea in the same buffer.

NMR spectroscopy

NMR samples contained 1 mM of the AnkG fused with Ndel1 protein in 100 mM potassium phosphate at pH6.5. NMR spectra were acquired at 30°C on Varian Inova 750- and 800-MHz spectrometers each equipped with an actively z-gradient shielded triple resonance probe. Backbone and sidechain resonance assignments were achieved by the standard heteronuclear correlation experiments. To simplify the NOE assignments, single chain fusion protein was designed. The AnkG-NIR was fused N-terminal to two copies of Ndel1 CT-CC with the thrombin cleavable linker sequences of "GSLVPRGS".

Crystallography

Crystallization of the AnkB/Ndel1 complex was performed using the hanging drop vapor diffusion method at 16 °C. Crystals of the AnkB/Ndel1 complex were obtained from the crystallization buffer containing 2.0 M ammonium sulfate, 0.2 M potassium sodium tartrate tetrahydrate, and 0.1 M sodium citrate tribasic dihydrate at pH 5.6. The diffraction data were collected at Shanghai Synchrotron Radiation Facility and processed and scaled using HKL3000 (1). By using the ab initio phasing program ARCIMBOLDO (2), an initial model containing four α-helices were found. Subsequent model autobuilding was carried

out by Buccaneer (3). Further manual model adjustment and refinement were completed iteratively using COOT (4) and Refmac5 (5). The final model was validated by MolProbity (6). The final refinement statistics are summarized in Table S2. All structure figures were prepared by PyMOL (http://www.pymol.org). The structure factors and coordinates of the structure have been deposited to PDB under the access code of 6KZJ.

Hippocampal neuronal culture and transfection

Primary neurons were cultured from newborn WT C57bl/6 mice hippocampus. The fresh hippocampal tissues were dissociated with 0.25% trypsin (Life Technologies), which was then inactivated by 10% decomplemented FBS (HyClone). The mixture was titrated through a pipette to make a homogenous mixture. After filtering through a 70-μm sterilized filter, the flow through was centrifuged. The pellet was then washed once with PBS (0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, and 0.002 M KH_2PO_4, pH 7.2) and once with DMEM in Earle's balanced salt solution containing 0.225% sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1% dextrose, and 1× Pen Strep (all from Life Technologies) with 5% FBS. Cells were then plated on poly-L-lysine (Sigma-Aldrich)-coated plates or glass coverslips at a density of 5×10^4 cells/mL. Neurons were incubated at 37 °C in Neurobasal medium (NB) supplemented with B27, 0.5 mM glutamine, 12.5 μM glutamate and 1× Pen Strep and with 5% circulating CO_2. The medium was changed every 48 h. The shRNA of AnkG was cloned into a BFP-pll3.7 vector with the sequence GCGTCTCCTATTAGATCTTTC, targeting a serine-rich region shared by both the 270 kDa and 480 kDa isoforms of mouse AnkG but not the rescuing rat AnkG. The shRNA of Ndel1 was cloned into a PLKO-BFP vector with the sequence CTTTCCTTGAAGTATAAGCAA, targeting the mouse Ndel1. The full-length TfR, CAR and GluR1 was PCR amplified from a human cDNA library and subcloned into the pEGFP-N1 vector to express GFP fusion proteins. Hippocampal neurons were cultured until day 4 before co-transfected with the shRNA and different versions of rescue vectors containing rat AnkG or co-transfected with the TfR-GFP/GluR1-GFP/CAR-GFP and different versions of AnkG peptide using the calcium phosphate-DNA co-precipitation method. On day 7, the neurons were fixed and processed for immunostaining.

Immunostaining

Cells were permeabilized in PBS-Triton at 4 °C and blocked with 10% donkey serum at room temperature, followed by incubation with anti-AnkG antibody (4G3F8; Life Technologies, 1:500), anti-Nde1 antibody (proteintech, 1:1000), anti-Ndel1 antibody (proteintech, 1:1000), or anti-GFP antibody (Abcam, 1:1000) at 4 °C for 24 h. 568-conjugated donkey anti-mouse and 488-conjugated donkey anti-rabbit antibodies or 488-conjugated goat anti-chicken antibody were applied as the secondary antibody. The nuclei were then stained with Hoechst 33258 (1 μg/mL; Sigma-Aldrich) for 15 min in the dark. The coverslips were mounted with Immunon mounting medium (Shandon) onto glass slides.

Microscopy and data analysis

All the images in this study were captured using a Zeiss LSM 710 laser-scanning confocal microscope. The hippocampal neurons were captured using 40 × 1.4 oil or 63 × 1.4 oil objective with pinhole setting to 1 Airy unit. The AIS is defined by the ankyrin-G staining when endogenous ankyrin-G was stained using anti-AnkG antibody. In the ankyrin-G-depleted cells, the axon is considered as the thinnest and longest neurite with less branching; and the AIS is defined within the first 50 μm length of the axon beginning from the axon hillock. In the ankyrin-G-depleted neurons co-transfected with WT or mutant of ankyrin-G-GFP, the overexpressed ankyrin-G also enriched at the proximal of the axon and defined the AIS region. Fluorescence intensity analyses were processed using ImageJ software. The intensity ratios in neurons were quantified and analyzed using GraphPad Prism 7. For the statistical analysis, the neuronal data were compared using one-way ANOVA followed by a Tukey post hoc test. A P value < 0.05 was considered to indicate statistical significance. ***$p < 0.001$. ****$p < 0.0001$. ns; not significant.

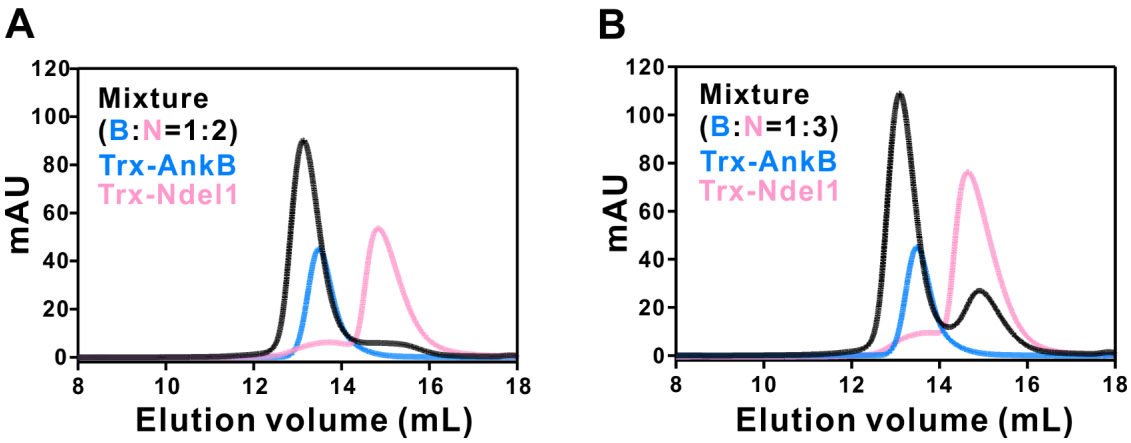

Fig. S1. Analytical gel filtration chromatography analyses of the interaction between AnkB-NIR and Ndel1 CT-CC.

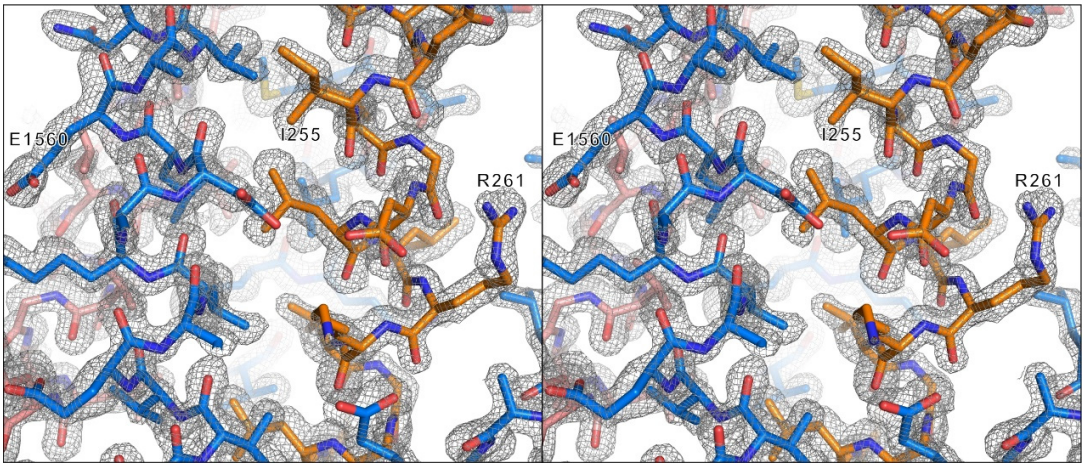

Fig. S2. A stereo view of the composition omit electron density map of the AnkB/Ndel1 complex contoured at 1.5 σ, showing the overall quality of the structure determination.

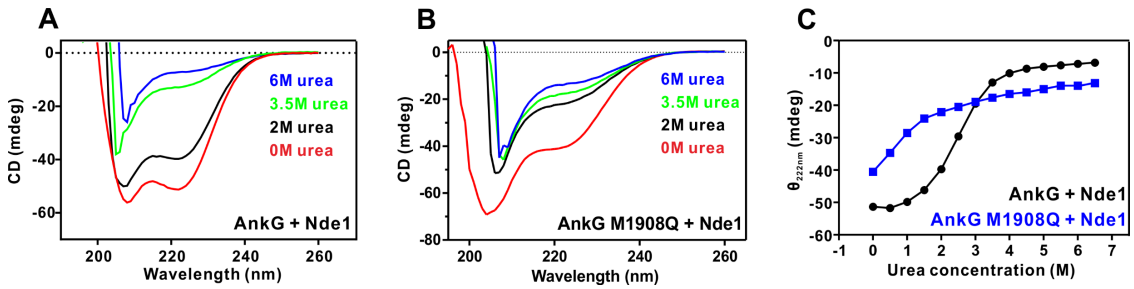

Fig. S3. CD spectra-based urea-induced denaturation assay showing that the AnkG/Nde1 complex with a single point mutation M1908Q of AnkG are less stable than WT AnkG/Nde1 complex.

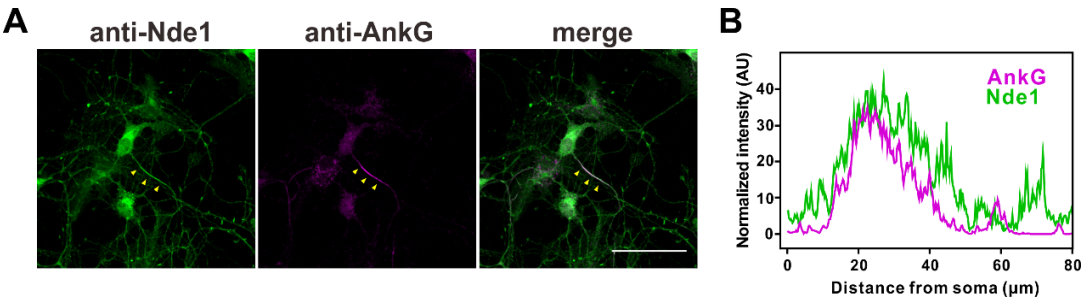

Fig. S4. (A) Hippocampal neurons at DIV7 stained for Nde1 (green) and the AIS marker AnkG (magenta) (scale bars, 50 µm). (B) Fluorescence intensity plots provide a comparison of immunosignal strength of AnkG (AIS, magenta) and Nde1 (green).

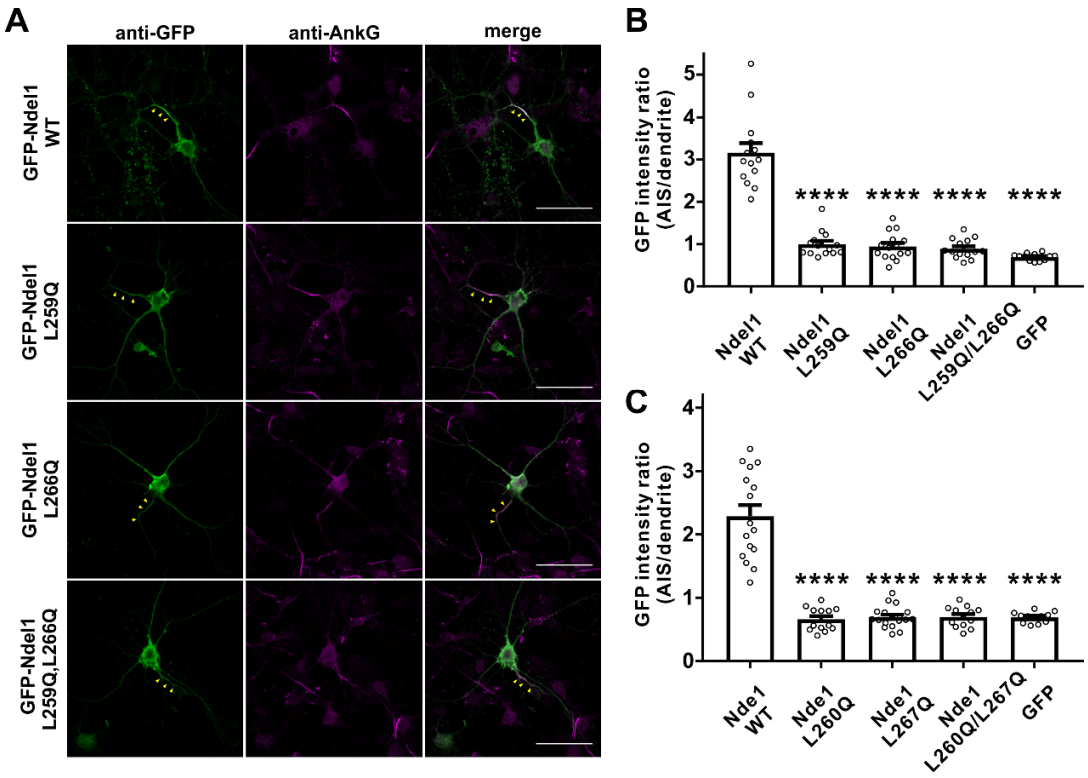

Fig. S5. (A) Neurons transfected with GFP-tagged Ndel1 WT and various mutants and stained for AnkG (magenta). Yellow arrowheads indicate the AIS (scale bars, 50 μm). (B) Quantification of the anti-GFP fluorescence intensity ratio of axons to dendrites in neurons transfected with WT (n = 14), L259Q (n = 14), L266Q (n = 15), L259Q/L266Q (n = 14) GFP-Ndel1 and GFP alone (n = 12). ****p<0.0001. Error bars, S.E. (C) Quantification of the anti-GFP fluorescence intensity ratio of axons to dendrites in neurons transfected with WT (n = 16), L260Q (n = 13), L267Q (n = 13), L260Q/L267Q (n = 18) GFP-Nde1 and GFP alone (n = 13). ****p<0.0001. Error bars, S.E.

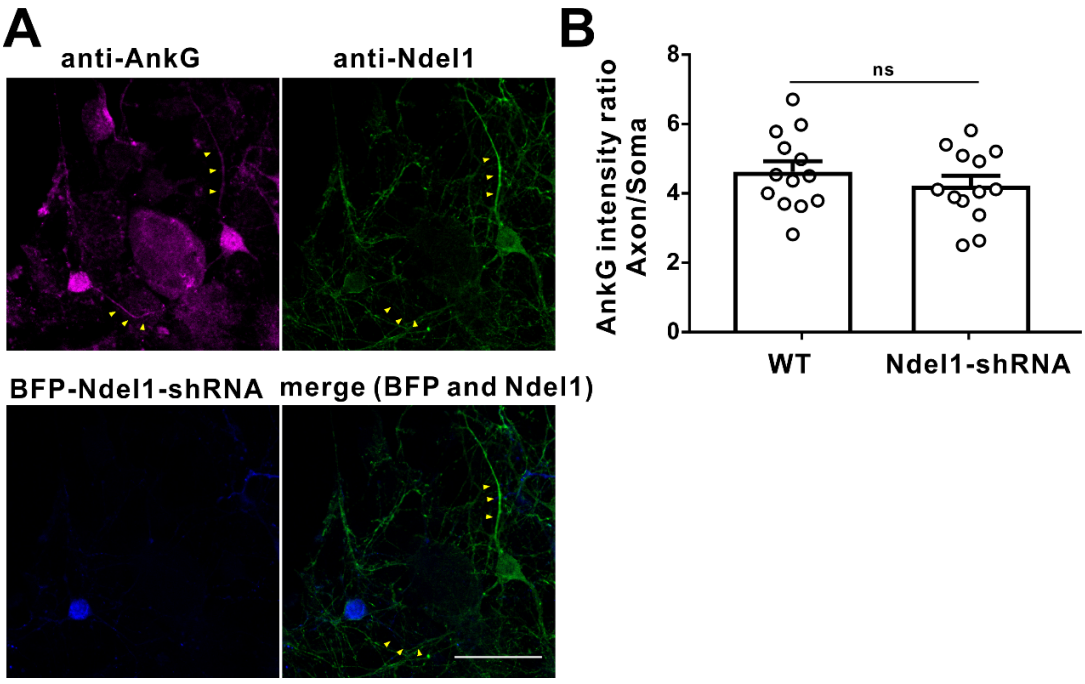

Fig. S6. (A) Neurons transfected with BFP -Ndel1-shRNA (untransfected neurons in the same field of view) and stained for AnkG (magenta) and Ndel1 (green) (scale bars, 50 μm). (B) Quantification of the anti-endogenous AnkG fluorescence intensity ratio of axons to somatodendrites in control neurons (n = 13) and neurons depleted of endogenous Ndel1 (n = 13).

Table S1. ITC-based mapping of the minimal Nde1 binding region in the exon 37 of AnkG and the mapping of the minimal AnkG-NIR binding region in Ndel. 'N.D.' indicates that no binding was detected.

AnkG exon 37	Nde1	K_d (nM)
1820-1989	**1-190**	**N.D.**
1820-1989	**191-335**	**83 ± 8.5**
1875-1933	**191-335**	**5000 ± 700**
1897-1966	**191-335**	**75 ± 9.5**
1897-1966	**239-286**	**73 ± 7.8**
1897-1940	**239-286**	**300 ± 45**
1940-1966	**239-286**	**N.D.**

Table S2. Statistics of X-ray Crystallographic Data Collection and Model refinement

Data collection	
Data sets	AnkB/Ndel1
Space group	$P2_12_12_1$
Wavelength (Å)	0.9785
Unit Cell Parameters (Å)	a=40.45, b=44.69, c=77.88 α=β=γ=90°
Resolution range (Å)	50-1.50 (1.53-1.50)
No. of unique reflections	22182 (1135)
Redundancy	10.7 (11.0)
I/σ	32.4 (4.6)
Completeness (%)	99.9 (95.0)
R_{merge} [a] (%)	5.9 (43.5)
$CC_{1/2}$ (last resolution shell) [b]	0.966
Structure refinement	
Resolution (Å)	10-1.50 (1.54-1.50)
R_{cryst} [c]/R_{free} [d] (%)	16.05/21.08 (15.90/20.70)
rmsd bonds (Å) / angles (°)	0.012 / 1.633
Average B factor (Å^2) [e]	17.2
No. of atoms	
Protein atoms	1114
Water	58
Ligands	0
No. of reflections	
Working set	20976 (1560)
Test set	1063 (71)
Ramachandran plot regions [d]	
Favored (%)	99.3
Allowed (%)	0.7
Outliers (%)	0

Numbers in parentheses represent the value for the highest resolution shell.

a. $R_{merge} = \Sigma |I_i - \langle I \rangle| / \Sigma I_i$, where I_i is the intensity of measured reflection and $\langle I \rangle$ is the mean intensity of all symmetry-related reflections.

b. $CC_{1/2}$ were defined by Karplus and Diederichs.

c. $R_{cryst}=\Sigma||F_{calc}| - |F_{obs}||/\Sigma F_{obs}$, where F_{obs} and F_{calc} are observed and calculated structure factors.

d. $R_{free}= \Sigma_T||F_{calc}| - |F_{obs}||/\Sigma F_{obs}$, where T is a test data set of about 5% of the total unique reflections randomly chosen and set aside prior to refinement.

e. B factors and Ramachandran plot statistics are calculated using MOLPROBITY.

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