SUPPLEMENTAL MATERIAL

Hur et al., GSK3 controls axon growth via CLASP-mediated regulation of growth cone microtubules

ITEMIZED LIST OF SUPPLEMENTAL MATERIAL.

Supp legend Fig. 1 – Representative images from rescue experiments to confirm the specificity of CLASP2 depletion in the regulation of axon and dendrite growth.

Supp legend Fig. 2 – Detailed analysis of the time-lapse experiments presented in Fig. 2C-E.

Supp legend Fig. 3. – Possible role of CLASP2 and GSK3 in the regulation of axonal branching.

Supp legend Fig. 4 – Representative images of +TIPs and CLASP2 mutants in a neuronal cell line, CAD.

Supp legend Fig. 5 – Effects of CLASP2 mutants on growth cone microtubule (MT) structures.

Supp legend Fig. 6 – Representative images from a GSK3-depleted growth cone showing increased lattice-binding activity of CLASP2.

Supp legend Fig. 7 – Effect of the CLASP2-5xS/D mutant on axon growth.

Supp legend Fig. 8 – Blockade of myosin II activity reversed both the prevention of MT protrusion into the growth cone periphery and impaired axon growth induced by CLASP overexpression or GSK3 inhibition.

Supp legend Fig. 9 – Regulation of CLASP-MT association and differential regulation of CLASP and GSK3 in embryonic cortical and adult DRG neurons.
Supp legend Fig. 10 – Further validation of the model presented in Fig. 7D.

Supp Movies – Time-lapse movies for Fig. 2C.

Supp Materials and Methods – Detailed methods of cell culture and transfection, time-lapse video microscopy, and immunocytochemistry.

Supp Table 1 – Sources of antibodies, materials, and cDNA constructs used in the present study.

Supp References – References within Supplemental Material.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Both the promotion of axon growth and the impaired dendrite development induced by CLASP2 depletion are rescued by adding back resCLASP2-FL. E15 cortical neurons were transfected with tdTomato (control), siCLASP2, and/or siRNA-resistant form of CLASP2 (resCLASP2-FL), as indicated. Neurons were fixed at DIV4 and immunostained for a dendritic marker, MAP2. Note that the enhanced axon growth (yellow arrows) and the impaired dendrite growth (white arrowheads) phenotypes induced by knocking down CLASP2 were rescued by expressing an siRNA resistant form of full length CLASP2 (resCLASP2-FL). Representative images are presented.

Supplemental Figure 2. Possible role of CLASP2 and GSK3 in the regulation of axonal branching. E15 cortical neurons were transfected with siCLASP2 and/or shGSK3, as indicated, and the number of primary axonal branches in each condition was analyzed. Knocking down GSK3 increased the number of primary branches to similar extent in the presence or absence of siCLASP2.

Supplemental Figure 3. CLASP2 regulates axon growth and mediates axon growth inhibition induced by GSK3 depletion via controlling the rate of axon extension. E15 cortical neurons were transfected with the indicated siRNAs together with tdTomato to visualize transfected cells, as in Figure 2. Time-lapse images were acquired at 1 hr intervals at
DIV3-4, when neurons were fully polarized. For the histogram (A), two consecutive images were used to calculate the changes in axon length. Representative axon growth profiles of individual neurons depleted with GSK3 and/or CLASP2 are presented in B. Initial axon length was subtracted from each image to plot changes in axon length.

Supplemental Figure 4. MT-binding activities of EB1, APC, CLASP2, and CLASP2 mutants.

(A-C) MT-binding activities of +TIPs in CAD cells. CAD cells expressing EGFP-CLASP2-FL (A), EGFP-EB1 (B) or EGFP-APC (C) were fixed and immunostained for tubulin. Note that MT protrusion towards the cell periphery is inhibited when EGFP-CLASP2-FL displays prominent lattice-binding (arrows). By contrast, when CLASP2 tracks MT plus ends, MTs splay apart and extend into the cell periphery (upper and lower right in A) in a manner similar to those of untransfected cells (arrowheads in lower left). Expression of EGFP-EB1 (B) or EGFP-APC (C) had little effect on MT structures.

(D, E) MT-binding activities of CLASP2-ΔL and CLASP2-5xS/D mutants. EGFP-CLASP2-ΔL (D) or EGFP-CLASP2-5xS/D (E) expressed in CAD cells displayed prominent plus end-binding activities. Indicated areas (arrows in D and E) are enlarged at right.

Supplemental Figure 5. Effect of CLASP2-ΔM and CLASP2-5xS/D on growth cone MT structures.

(A) Representative images of MT structures in growth cones expressing CLASP2-ΔM and
CLASP2-5xS/D.

(B) Quantification of MT structures in growth cones. The number of free MT ends in the growth cone P domain expressing the indicated constructs was quantified and normalized against the size of the growth cone as described in Materials and Methods. The numbers of growth cones analyzed are indicated.

Supplemental Figure 6. Depletion of GSK3 increases the lattice-binding activity of CLASP2.

Neurons were transfected with siGSK3 together with tdTomato to identify transfected neurons. Representative images of a GSK3-depleted growth cone immunostained for CLASP and tubulin. Note the greatly increased lattice-binding activity of CLASP (white arrowheads) along microtubules. The four lower right panels show enlarged images of CLASP staining indicated by the white arrowheads.

Supplemental Figure 7. Effect of CLASP2-5xS/D on axon growth.

E15 cortical neurons were transfected as indicated and axon growth was analyzed. Note that in contrast to CLASP2-FL, introducing CLASP2-5xS/D into CLASP2-depleted neurons failed to reverse the enhanced axon growth induced by siCLASP2. The axon growth promoting effect of siCLASP2 was completely reversed by co-expression of CLASP2-5xS/D and CLASP2-FL. The bar that shows the effect of adding back CLASP2-FL alone in CLASP2-depleted neurons (from Figure 5H) is placed adjacent to the bar that shows the effect of CLASP2-5xS/D for comparison. Representative images (A) and quantification of axon length (B) are shown. Scale bar, 50 µm.
Supplemental Figure 8. Inhibition of myosin II activity promotes MT protrusion into the growth cone periphery and rescues the axon growth defect induced by CLASP2 overexpression or suppression of GSK3 activity.

(A, B) Neurons were transfected with EGFP-CLASP2-FL or control vector, and treated with blebbistatin or DMSO (as a vehicle control). For GSK3 inhibition, neurons were treated with an inhibitor of GSK3 (GSKi, 6-bromoindirubin-3′-acetoxime) in the presence or absence of blebbistatin, as indicated. Representative images of growth cones (A) and quantification of axon length (B) are shown. *** p < 0.001; n.s., statistically not significant.

Supplemental Figure 9. Regulation of CLASP-MT interactions by GSK3 activity and CLASP protein levels.

(A, B) CLASP-MT association is regulated by the expression level of CLASP and GSK3 activity. COS cells were co-transfected with EGFP-CLASP2 and GSK3β mutants or RFP (as a control), as indicated, and were divided into three categories depending on the expression level of EGFP-CLASP2. CLASP-MT association, especially the lattice-binding was significantly increased with increased CLASP2 levels (A). The MT lattice-binding activity of CLASP2 was also increased by inhibiting GSK3 (B), which was achieved either by treating the cells with an inhibitor of GSK3 (GSKi, 6-bromoindirubin-3′-acetoxime) or expressing a kinase-dead mutant of GSK3β (GSK3β-KM). Conversely, GSK3β-S9A completely disrupted CLASP-MT association, even at the highest expression level of EGFP-CLASP2, consistent with the role of GSK3 as a crucial
regulator of CLASP-MT association. Note that GSK3β-R96A specifically reduced the lattice-binding activity without inhibiting the plus end-binding activity of CLASP2. † p < 0.05; ‡ p < 0.01; # p < 0.001 compared with lattice-binding of control cells transfected with EGFP-CLASP2 and RFP.

(C, D) E15 cortical and adult DRG extracts were subjected to Western blot analysis and probed with anti-phospho-GSK3β Ser9 and anti-total GSK3β antibodies. Representative blots (C) and quantification of the immunoblots (D) are presented. For quantification, level of phospho-GSK3β Ser9 was normalized by that of total GSK3β. ** p < 0.01.

(E) E15 cortical and adult DRG neurons were immunostained side-by-side with anti-pan-CLASP and anti-tubulin antibodies. Total fluorescence intensity of CLASP immunostaining was measured in the growth cone and was normalized by the size of growth cone as describe in Materials and Methods. * p < 0.05.

Supplemental Figure 10. CLASP functionality is altered by manipulating GSK3 activity.

(A) Adult DRG neurons were transfected with shGSK3 and/or CLASP2 siRNA (ON-TARGETplus siRNA pools), as indicated, and replated at DIV3 to allow axon growth anew. Axon length was quantified at 18 hr after replating. Note that knocking down CLASP2 completely prevents shGSK3 from inhibiting axon growth.

(B, C) E15 cortical neurons (B) or adult DRG neurons (C) were transfected as indicated, and axon length was measured. Note that GSK3β-R96A enhances axon growth in cortical neurons (B) but not in adult DRG neurons (C). GSK3β-R96A, however, fails to further enhance axon
growth of cortical neurons when CLASP2 is depleted. *** p < 0.001.
Supplemental Movies S1-4. Time-lapse movies of representative neurons depleted with GSK3 and/or CLASP2. Embryonic cortical neurons were transfected with siGSK3 (Movie S2), siCLASP2 (Movie S3), or both (Movie S4), together with tdTomato to visualize transfected cells. For control, neurons were transfected with tdTomato only (Movies S1). Axon growth was monitored by time-lapse microscopy between DIV3-4 after the neurons were fully polarized.
SUPPLEMENTAL MATERIALS AND METHODS

Antibodies, Materials, and cDNA Constructs

A full list of reagents, cDNA constructs, and antibodies is provided in the Supplemental Data document (Table S1).

Cell Culture

DRG and cortical neurons were prepared from mice according to Institutional Animal Care and Use Committee regulations. Dissection and culture of mouse cortical and DRG neurons were performed as described previously (Hur et al., 2011). In brief, for adult DRG neurons, DRGs were dissected from 8- to 12-week-old adult CF-1 mice and digested with collagenase A (1 mg/ml) for 2 hr, followed by trypsin-EDTA for 20 min at 37°C. For embryonic DRG neurons, DRGs were dissected from E14-15 mice and digested with collagenase A (1 mg/ml) for 15 min, followed by trypsin (0.05%) for 5 min at 37°C. The DRGs were then washed three times with MEM and dissociated with plating medium. For overexpression experiments, dissociated DRGs were transfected and plated directly on glass coverslips coated with poly-D-lysine (100 µg/ml) and laminin (5 and 10 µg/ml for embryonic and adult DRGs, respectively). For knocking down experiments, transfected neurons were first plated on culture dishes coated with poly-D-lysine and laminin and then were replated after 3 to 5 days on glass coverslips coated with poly-D-lysine and laminin. Embryonic DRGs were cultured in MEM supplemented with 5% fetal bovine serum (HyClone, Logan, UT) and antimitotics (20 µM 5-fluoro-2-deoxyuridine, 20 µM uridine).
Adult DRGs were cultured in serum-free MEM. For blebbistatin experiments, cells were transfected and plated on coverslips coated with poly-D-lysine only.

Transfection

ShRNA/siRNAs or other DNA constructs were transfected with Nucleofector™ from Lonza (Cologne, Germany) as previously described (Hur et al., 2011). Briefly, dissociated neurons were centrifuged to remove the supernatant and resuspended in 80 µl of specified Amaxa electroporation buffer with plasmid DNA (10 - 20 µg and 2 - 3 µg for DRG and cortical neurons, respectively). Suspended cells were then transferred to a 2.0-mm cuvette and electroporated with an Amaxa Nucleofector™ apparatus. After electroporation, cells were immediately transferred to the desired volume of prewarmed culture medium and plated. After neurons fully attached to the substrates (2 - 4 hr), the medium was changed to remove the remnant transfection buffer.

Time-lapse Video Microscopy

E15 cortical neurons were transfected with control, siCLASP2, and/or siGSK3 together with tdTomado to visualize the transfected neurons. Transfected neurons were plated on polylysine-coated culture slides (BD Biosciences, Bedford, MA). Time lapse imaging was initiated after the neurons were fully polarized, and neurons were imaged between DIV3 and DIV4. Live cell imaging was performed in an environmental chamber (37°C, 5% CO₂) mounted onto the stage of a motorized inverted microscope (Zeiss Axiovert 200M) equipped with a Cascade 512B II CCD camera at 1 hr intervals. Axon length of a neuron was measured from each image, and two
consecutive images were used to calculate the changes in axon length.

Immunostaining and Fluorescence Microscopy

To stain endogenous CLASPs in neurons and to visualize MT-binding of ectopically expressed EGFP-CLASP2, cells were fixed with methanol at -20°C for 5 min and rehydrated with PBS. For staining MTs and actin in growth cones, neurons were fixed in prewarmed cytoskeleton stabilization buffer [60 mM 1,4-piperazinediethanesulfonic acid, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM ethylene glycol tetraacetic acid (EGTA), and 2 mM MgCl₂, pH 6.9] containing 4% PFA, 0.25% glutaraldehyde, and 10 µM taxol at 37°C for 20 min. For all other staining, neurons were fixed with 4% PFA at room temperature for 20 min. Fixed neurons were washed with PBS and blocked in blocking solution (2% BSA, 0.1% Triton X-100, and 0.1% sodium azide in PBS). Primary and secondary antibodies were diluted with the blocking buffer. All secondary antibodies (1:400 - 500) were incubated for 1 hr at room temperature. After extensive rinsing with PBS and distilled water, coverslips were mounted onto glass slides for observation. Neurons were viewed with an inverted light microscope (Zeiss Axiovert 200, Carl Zeiss MicroImaging, Inc.) equipped with epifluorescence optics. Images were captured with a CCD camera controlled by Axiovision software (Carl Zeiss MicroImaging, Inc.). A 10 X or 20 X objective (0.45 NA) was used to record whole neurons, and a 100 X (1.3 NA) oil objective was used for high-resolution imaging of the growth cones. For higher resolution imaging, images were taken with Apotome (Carl Zeiss MicroImaging, Inc.), a grid-based optical sectioning imaging system.
**Supplemental Table S1. Sources for antibodies, materials, and cDNA constructs**

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<td>Antibodies and materials</td>
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<tr>
<td>Rabbit polyclonal anti-pan-CLASP antibodies</td>
<td>VU-83 from Dr. I. Kaverina (Vanderbilt University Medical Center, Nashville, TN).</td>
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<td>Rabbit polyclonal anti-CLASP1 antibody</td>
<td>Dr. F. Severin (Moscow State University, Moscow, Russia).</td>
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<td>Mouse anti-pan-GSK3 antibodies</td>
<td>Clone 4G-1E from Millipore (Temecula, CA).</td>
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<td>Mouse anti-Tau1 and anti-MAP2 antibodies</td>
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<td>Blebbistatin and GSK3β inhibitor I</td>
<td>Calbiochem (San Diego, CA).</td>
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<td>Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).</td>
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<td>Mouse anti-EB1 antibody</td>
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<td>Mouse anti-β-tubulin and polylysine</td>
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<td>NGF</td>
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<td>Laminin, B27 supplement, and all fluorescence-tagged secondary antibodies</td>
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<td>cDNA constructs</td>
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<td>Full-length CLASP2 and its deletion mutants</td>
<td>Drs. A. Akhmanova (Erasmus Medical Center, Rotterdam, Netherlands) and T. Wittmann</td>
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<td>(University of California, San Francisco, CA).</td>
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<td>EGFP-CLASP2-ΔL</td>
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<td>EGFP-CLASP2 obtained from Dr. A. Akhmanova.</td>
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<td>shGSK3</td>
<td>A previously described (Kim et al., 2006) construct from Dr. W. Snider (University of North Carolina at Chapel Hill).</td>
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<td>shCLASP1 and shCLASP2.</td>
<td>Generated by using the pSUPER system (Oligoengine, Seattle, WA) with insertion of the previously verified sequences for siRNAs against CLASP1 and 2 (Mimori-Kiyosue et al., 2005).</td>
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SUPPLEMENTAL REFERENCES


Control siCLASP2 siCLASP2 + resCLASP2-FL

MAP2
Hoechst

Hur et al., 170159_Supplemental Fig. 1
Hur et al., 170159_Supplemental Fig. 2

![Graph showing the number of primary branches (per 100 µm axon) for different conditions.

- EGFP
- shGSK

The graph indicates the following p-values:
- p=0.012
- p=0.024
- p=0.047

Legend:
- Control
- eCLASP2

Mean ± SD]
Hur et al., 170159_Supplemental Fig. 3

A

B

Percentage of events (%)

Change in axon length (μm/hr)

Ctrl
siGSK3
siCLASP2
siGSK3 + siCLASP2

Time (hr)

Changes in axon length (μm)

0 5 10 15 20 25

0 5 10 15 20 25

0 5 10 15 20 25
Hur et al., 170159_Supplemental Fig. 5

A

Control

CLASP2-ΔM

CLASP2-5xS/D

B

# of free MT ends in growth cone periphery (% of Ctrl)

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Hur et al., 170159_Supplemental Fig. 7

**A**

- Control
- CLASP2-res5xS/D
- siCLASP2 + CLASP2-resFL

- siCLASP2
- siCLASP2 + CLASP2-res5xS/D
- siCLASP2 + CLASP2-resFL

**B**

Axon length (% of control)

- Control
- CLASP2-res5xS/D
- siCLASP2 + CLASP2-res5xS/D
- siCLASP2 + CLASP2-resFL

- p=0.02
- p=0.026

*** ***
Hur et al., 170159_Supplemental Fig. 8

A

Control
CLASP2
CLASP2 Blebbistatin

B

Axon length (% of DMSO Control)

DMSO
Blebbistatin

**p = 0.002

n.s.

Hur et al., 170159_Supplemental Fig. 8
Hur et al., 170159_Supplemental Fig. 9

**A**

- **Plus end-binding**
- **Lattice-binding**
- **Both**
- **No binding**

**B**

- **Plus end-binding**
- **Lattice-binding**
- **Both**
- **No binding**

**C**

- E15 Cortical
- Adult DRG

- α-GSK3β pSer9
- α-GSK3β

**D**

- Adult DRG
- E15 Cortical

- pGSK3/total GSK3 (a.u.)

**E**

- Adult DRG
- E15 Cortical

- CLASP level in the growth cone (fluorescence intensity, a.u.)