Protein Phosphatase 4 and Smek complex negatively regulate Par3 and promote neuronal differentiation of neural stem/progenitor cells

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EXTENDED EXPERIMENTAL PROCEDURES

Construction of Plasmids and Viruses

To create doxycycline-inducible shRNA in a lentiviral expression system, the mir30 cassette was amplified by PCR from pCAG-mir30 (provided by Dr. Constance L. Cepko) and subcloned into pFTREW (Lyu et al., 2009). pFUIPW-rtTA2S-M2 was previously described (Lyu et al., 2009). shRNA targeting sequences were generated as described (Paddison et al., 2004) and inserted into the pFTREW-mir30 vector. Those sequences were: for non-coding regions of *Smek1* (#1 and #2), 5'-GCTCATCAAGTGCCAATTCAA-3' and 5'-GCAGTTTCTTTGTATAAAGAG-3'; for the *Smek1* coding region (#3), 5'-GCACAACAGAATGATGATGAT-3'; for *PP4c*, 5'-CGGTCTGACCCTGAAGACACAA-3'; and for *Par3*, 5'-AATGGTTGATGATGACGACGAA-3'. shRNA sequences against the *Smek1* coding region or from the pGIPZ control vector (Open Biosystems) served as non-silencing controls (see Figure S1E). To generate plasmids encoding Flag-tagged wild-type Smek1, Flag-tagged Smek1 deletion mutants (ΔRanBD, ΔDUF625, ΔArm, or ΔNLS) or Myc-tagged Par3 cDNA, fragments were generated by PCR and subcloned into pFTREW and pFUW. To generate the Notch reporter plasmid (pFCBFRE-lu), the ubiquitin promoter of pFUW was replaced with a CBF1-binding element (CBFRE) containing a basal SV40 promoter and a luciferase fragment was inserted downstream of CBFRE. All constructs were confirmed by DNA sequencing. Lentiviruses were generated as described (Lyu et al., 2008).

Antibodies

For immunoprecipitation and Western blotting, antibodies to Smek1 (Sigma and Bethyl Laboratories), PP4c (Bethyl Laboratories), Pax6 (Covance), TUJ1 (Covance), Par3 (Millipore), Flag (Sigma), Myc (Santa Cruz Biotechnology), Phospho-serine/threonine (BD; Cell Signaling), Phospho-serine (Sigma), and Actin (Santa Cruz Biotechnology) were used. For immunostaining, Smek1 (Sigma) Pax6 (Abcam), TUJ1 (Covance), Tbr1 (Abcam), Nestin (BD), Ki67 (Vector), Ser10-phospho-histone H3 (Millipore), Par3 (Millipore), or Flag (Sigma). To detect the Smek1 C-terminus, rabbits were immunized with peptides corresponding to residues 671-681 of mouse Smek1 in the form of a GST fusion protein and collected antisera were affinity-purified.

Preparation of Recombinant Proteins and Protein Extraction

For bacterial expression constructs, the Flag-Smek1 fragment was inserted into the pET11d vector (Novagen), and fragments encoding Par3 amino acids 1-338, 343-733, 711-1054, and 1055-1334 were generated by PCR and inserted into the same vector but containing an N-terminal His tag (Novagen). Flag- and His-tagged fragments were expressed in E.coli Rosetta 2 and purified, respectively, with Flag affinity agarose beads (Sigma) and Ni-NTA resin (Novagen), according to the manufacturers' protocol.

For immunoprecipitation and immunoblotting, protein samples were prepared in buffer containing 25mM Tris-HCl, pH 7.4, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 10mM β-glycerophosphate, 1mM sodium orthovanadate, 10% glycerol, and protease inhibitors (Roche).

To prepare chromosomal and cytosolic fractions, synchronized cells were chemically cross-linked in 1% formaldehyde and the reaction terminated by adding 2 M glycine. Cells were suspended in hypotonic buffer (50m M HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, plus a protease inhibitor mixture) containing 0.1% Triton X-100 on ice for 30 min and passaged through a 26-gauge needle. The cytoplasmic fraction was obtained by centrifugation at 100,000 × g for 90 min, and the nuclear and membrane pellets were resuspended in hypotonic buffer containing 1% Triton X-100 and 150 mM NaCl. The insoluble fraction (pellet),

designated the chromosomal fraction, was obtained after centrifugation at 16,000 × g and resuspended in SDS sample buffer.

To identify Smek1-interacting proteins, NPCs infected with lentivirus expressing Flag-Smek1 were lysed in a lysis buffer containing 25mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM EDTA, 0.5% Nonidet P-40, 10% glycerol, and protease inhibitors (Roche). Samples containing 10mg protein were incubated with Flag affinity agarose beads and, after extensive washing, bound proteins were separated by SDS-PAGE and detected with SYPRO Ruby protein staining. Gel bands were excised and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Generation of *Smek1* Mutant Mice

Embryonic stem cells containing a *Smek1* gene trap allele were obtained from the Sanger Institute Gene Trap Resource and injected into C57BL/6J-derived blastocysts. Chimeras were bred with wild-type C57BL/6J albino mice, and germline transmission of the targeted allele in pups was confirmed. Mice were genotyped using tail genomic DNA as a template for PCR. The following primers were used in a multiplex reaction (Figure S2A and B): for pairs flanking the gene-trap cassette insertion site (primer P1 and P2), 5'-TGCTTGACTTACTGGGCTGA-3' and 5'- TACATCATGCGCTTCTAACA-3'; for the gene-trap cassette (primer P3); 5'-CTTCACATCCATGCTGAGGA-3'.

Quantitative Real-time PCR and Luciferase Assay

Total RNA was prepared using an RNeasy kit (Qiagen). Quantitative real-time PCR was carried out using SYBR Green I (BD), and samples were quantified by amplification of *GAPDH*. PCR amplification was performed using specific primer pairs: for *Hes1*, 5'-GAGAGGCTGCCAAGGTTTT-3' and 5'-AGCCACTGGAAGGTGACACT-3'; for *Hes5*, 5'-ATGCTCAGTCCCAAGGAGAA-3' and 5'-TAGTCCTGGTGCAGGCTCTT-3'; and for *Gapdh*, 5'-ACGGCAAATTCAACGGCACAG-3' and 5'-GGTCATGAGCCCTTCCACAAT-3'. All experiments were performed in duplicate at least three separate times.

For luciferase reporter assays, NPCs were infected with lentivirus expressing CBFRE-luc and then transiently transfected with the internal control pRL-TK using lipofectamine 2000 (Invitrogen). Luciferase assays were performed using the Dual-Luciferase Kit (Promega).

SUPPLEMENTAL REFERENCES

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Supplemental Figures and Legends

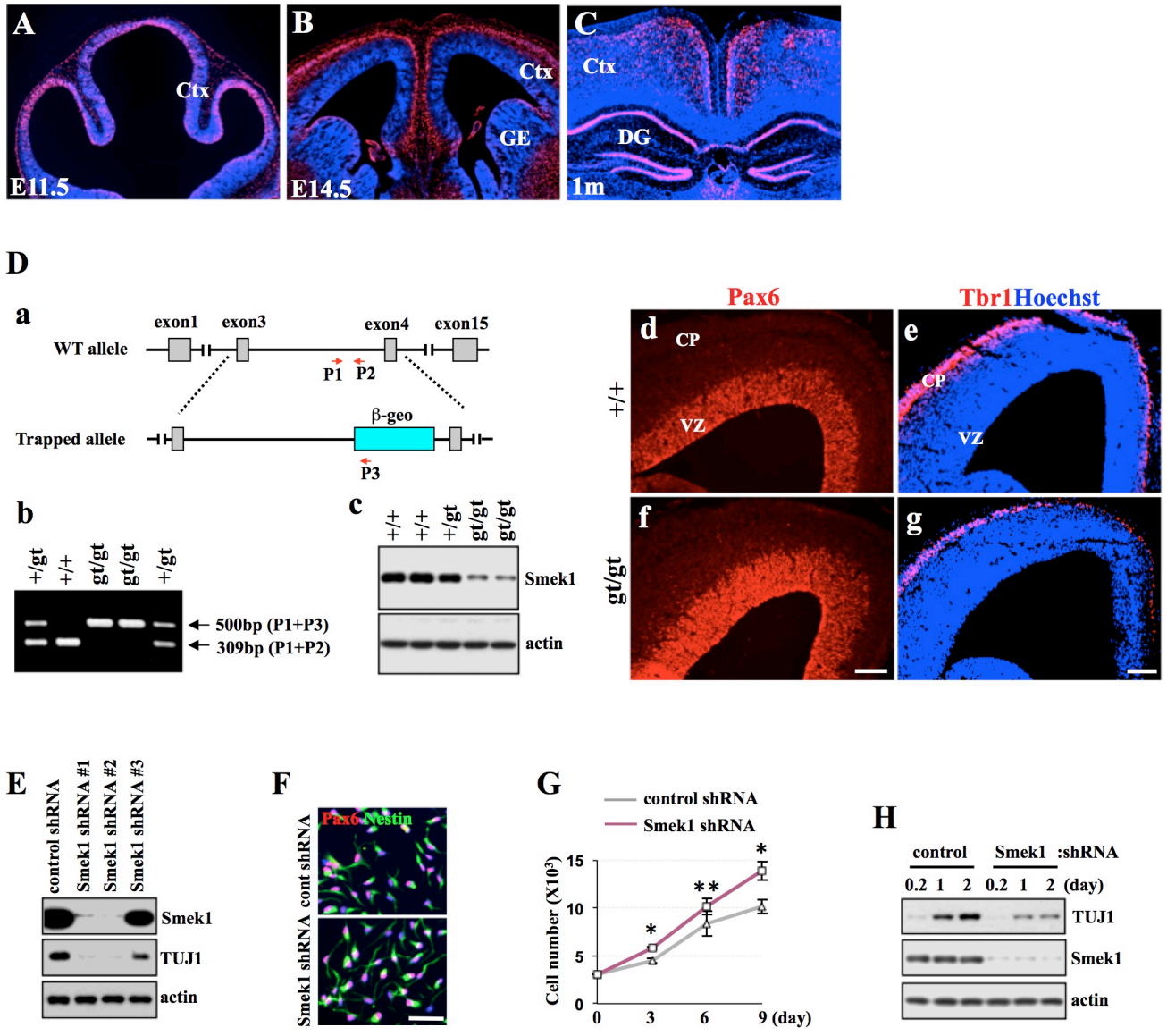

Figure S1. **Depletion of Smek1 leads to defective neuronal differentiation and hyperproliferation of NPCs, Relate to Figure 1**

(A-C) Smek1 expression in mouse forebrain cortex. Coronal sections of E11.5 (A), E14.5 (B), and 1-month (m) postnatal (C) mouse forebrains were immunostained with Smek1 antibody. Nuclei are revealed by Hoechst staining. The E11.5 section shows a strong Smek1 signal at the VZ, whereas E14.5 and postnatal cortex shows staining in a region of the CP. Postnatal cortex also shows prominent expression of Smek1 protein in the dentate gyrus (DG). Ctx, cortex; GE, ganglionic eminence.

(D) Hypomorphic *Smek1* mice show neurogenesis defects. Schematic of the *Smek1* locus and trapped allele with the β-geo cassette (a). PCR-based genotype analysis using PCR primer pairs indicated in a (red arrows) (b). Western blot analysis of brain extracts for Smek1 (c). Actin served as an internal control. Mice harboring a hypomorphic allele of *Smek1* (*Smek1*$^{gt/gt}$) show reduced Smek1expression. Immunostaining analysis of Pax6 (d, f) and Tbr1 (e, g) in E14.5 wild-type *Smek*$^{+/+}$ (d, e) and hypomorphic mutant *Smek*$^{gt/gt}$ mouse embryos (f, g) counterstained with Hoechst dye. Scale bars, 200 μm.

(E) NPCs were transduced with individual lentiviruses expressing three independent shRNAs for Smek1 or a non-specific control shRNA and then cultured in doxycycline for 6 days under differentiating conditions. Western blotting analysis with indicated antibodies shows a similar effect of number 1 and 2 shRNAs on Smek1 suppression and reduction of TUJ1 expression.

(F, G) Smek1 depletion leads to NPC hyperproliferation. NPCs expressing Smek1 shRNA or control were cultured for 9 days under proliferation conditions. Cells were counted at the indicated times. Purity of NPCs was confirmed by immunostaining for Pax6 and Nestin in cultures after 9 days. Hoechst dye served as a counterstain. Scale bar, 100 μm. NPCs positive for both Pax6 and Nestin represent 95 percent of the total cells. Quantitative analysis shows an increase in the number of Smek1 knockdown cells compared with controls (F). *$p < 0.001$; **$p < 0.05$.

(H) Western blot analysis using Smek1 and TUJ1 antibody at the early phase of NPC differentiation. Two days prior to differentiation, expression of *Smek1* shRNA or control shRNA was induced by addition of doxycycline. Cells were then cultured under differentiating conditions for indicated times. Smek1 or actin antibodies served as controls. Western analysis shows that TUJ1 protein levels decrease in Smek1 knockdown cells.

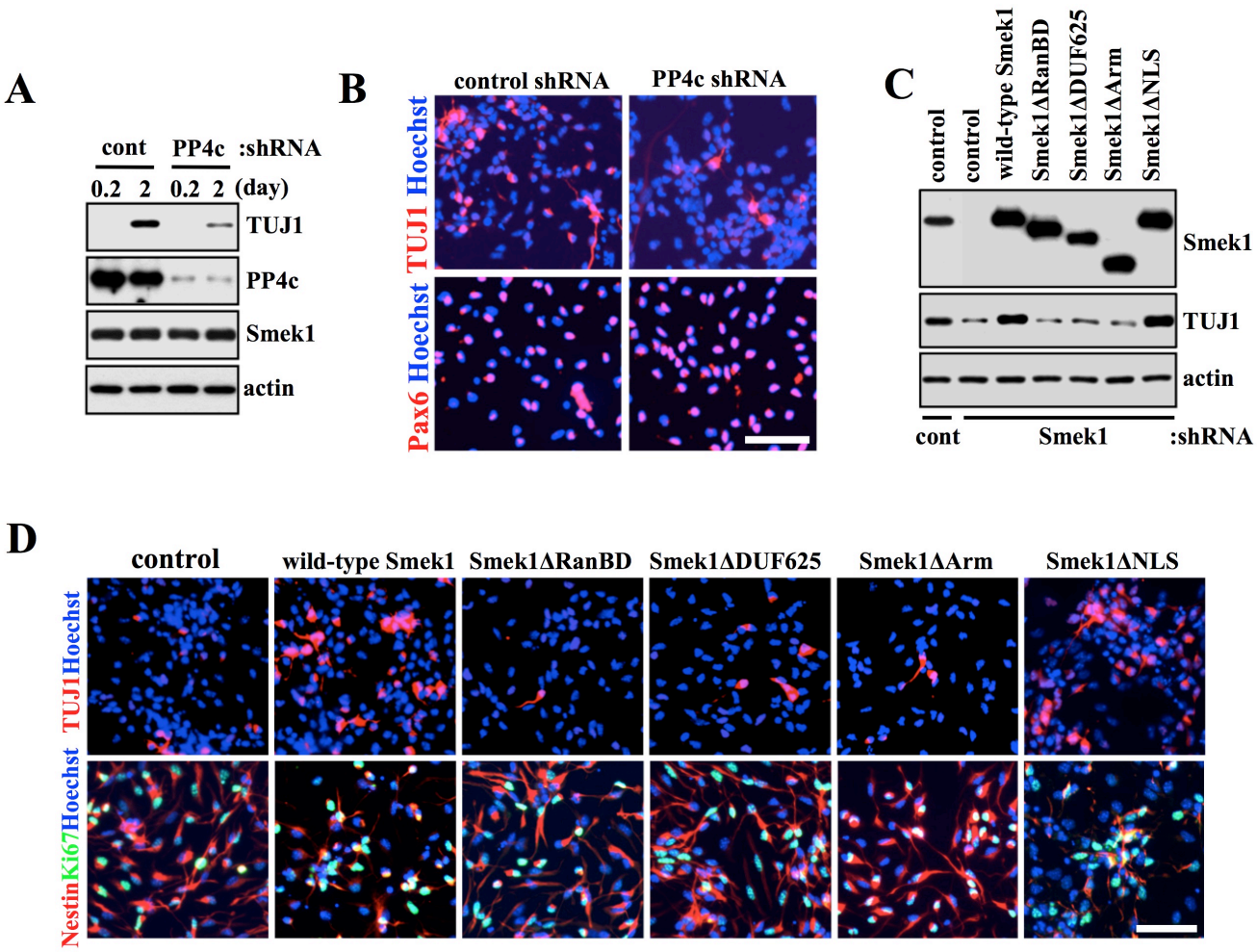

Figure S2. PP4c is important for Smek1-mediated neuronal differentiation, Related to Figure 2

(A, B) *PP4C* knockdown in NPCs blocks their neuronal differentiation. Western analysis of NPCs transduced with lentiviruses expressing *PP4c* shRNA or control shRNA under control of a doxycycline-inducible promoter. Cells were exposed to doxycycline for 2 days prior to differentiation and cultured for 2 more days under differentiating conditions (A). Cells in A were cultured for 1 day and then immunostained with TUJ1 or Pax6 antibodies (B). Scale bar, 100 μm.

(C, D) Neurogenesis defects seen in *Smek1* knockdown NPCs are rescued by re-expression of wild-type or ΔNLS mutant Smek1 but not by Smek1ΔRanBD, ΔDUF625, and ΔArm mutants. *Smek1* knockdown NPCs were infected with lentiviruses expressing wild-type or mutant Smek1 constructs prior to differentiation. These cells were then cultured under differentiating conditions for 2 days and subjected to Western blotting with anti-Smek1 and -TUJ1 antibodies (C). Actin expression served as a

loading control. At day 1, cultures were immunostained with TUJ1 or Nestin plus Ki67 antibodies (D). Hoechst dye served as a counterstain. Scale bar, 100 μm.

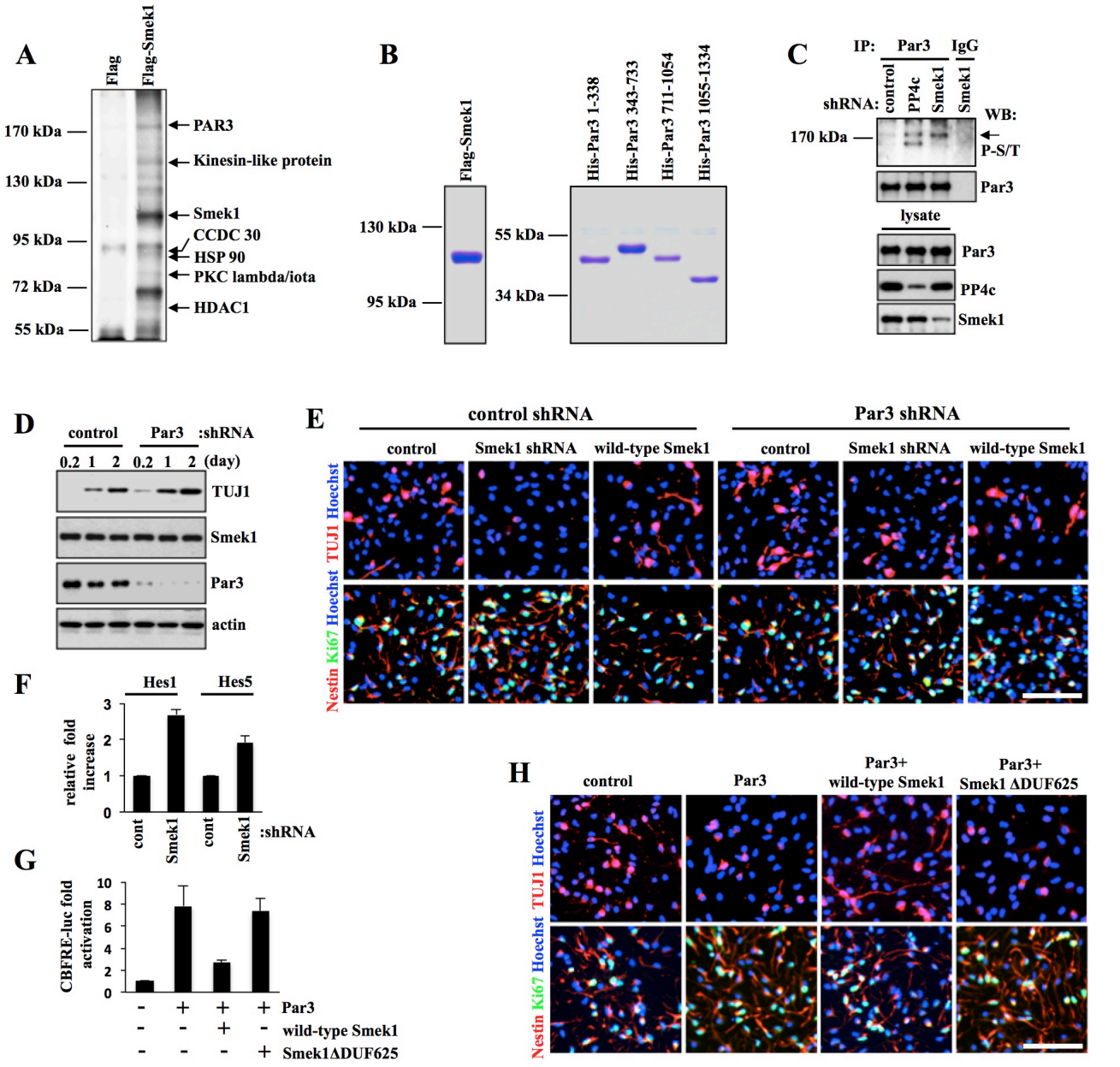

Figure S3. Smek1 binds to Par3 and negatively regulate its activity in neurogenesis, Related to Figure 3

(A) Lysates from NPCs infected with lentivirus expressing Flag-Smek1 or Flag alone were immunoprecipitated using anti-Flag affinity agarose. Immunoprecipitates were separated by SDS/PAGE and gel bands were cut and sequenced. An asterisk indicates IgG light chain.

(B) Indicated Flag- and His-tagged fragments produced in *E. coli* were purified and analyzed using SDS-PAGE stained with Coomassie blue.

(C) Depletion of Smek1 or PP4c increases Par3 phosphorylation. NPCs expressing *Smek1* shRNA, *PP4c* shRNA, or control shRNA were cultured for 1 day under differentiating conditions and then immunoprecipitated followed by Western blot analysis using anti-Par3 and -phospho-serine/threonine antibodies.

(D) shRNA-mediated depletion of Par3 causes an increase in TUJ1 protein level. NPCs transduced with lentivirus expressing Par3 or control shRNA under control of a doxycycline-inducible promoter were cultured for indicated times under differentiating conditions and then subjected to Western blotting with indicated antibodies.

(E) NPCs transduced with lentiviruses expressing Smek1 shRNA, wild-type Smek1, Par3 shRNA, or control shRNA under control of a doxycycline-inducible promoter were exposed to doxycycline for 3 days prior to differentiation. Cells were further cultured for 1 day under differentiating conditions and immunostained with TUJ1, Nestin, or Ki67 antibodies. Hoechst dye served as a counterstain. Scale bar, 100 μm.

(F) Smek1 depletion increases the levels of *Hes1* and *Hes5* mRNAs. Expression of *Hes1* and *Hes5* in control and *Smek1* knockdown cells cultured under differentiation conditions for 1 day, as determined by quantitative real-time PCR.

(G) A luciferase reporter (CBFRE-luc) driven by the canonical Notch effector C-promoter binding factor 1 (CBF1) response element (Hsieh et al., 1996) was introduced via lentivirus into NPCs together with Myc-tagged Par3, wild-type Flag-Smek1 or Flag-Smek1ΔDUF625 and luciferase activity was determined. Error bars represent the mean ± S.D. of four independent experiments. Overexpression of

wild-type Smek1 inhibited reporter gene activity induced by Par3 overexpression, while Smek1ΔDUF625 overexpression did not.

(H) Immunostaining of NPCs expressing Par3, wild-type Smek1, the Smek1ΔDUF625 mutant, or a control vector. Cells were cultured for 1 day under differentiating conditions and then immunostained with TUJ1, Nestin, or Ki67 antibodies. Hoechst dye served as a counterstain. Scale bar, 100 μm. Par3 overexpression decreases the number of TUJ1-positive cells and increases the number of Nestin/Ki67 double-positive cells. This effect is inhibited by expression of wild-type Smek1 but not by expression of Smek1ΔDUF625.

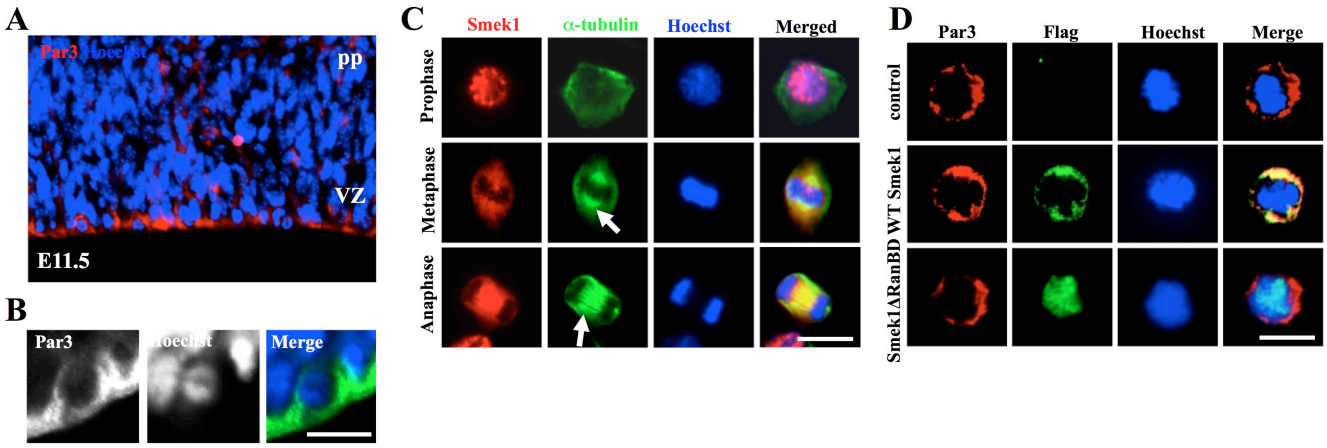

Figure S4. **Expression and subcellular localization of Smek1 and Par3, Related to Figure 4**

(A, B) Coronal sections of E11.5 mouse forebrains were immunostained with Par3 antibody. Nuclei are revealed by Hoechst staining. Par3 is expressed predominantly in cells at the ventricular surface (A). Cells undergoing mitosis in that surface region show cytoplasmic/cortical Par3 (B). Scale bar, 10 μm.

(C) Subcellular localization of Smek1 during mitosis. NPCs were cultured under differentiating conditions for 4-12 hrs and then immunostained with Smek1 and α-tubulin antibodies. In prophase cells, Smek1 fluorescence is localized to the nucleus while metaphase and anaphase cells show florescence in the cytoplasm as well as enrichment at spindle microtubules. Hoechst dye was used for DNA staining. Arrows denote the mitotic spindle labeled with α-tubulin antibody in each phase. Scale bar, 10 μm.

(D) Smek1 does not alter Par3 localization. *Smek1* knockdown NPCs were transduced with wild-type Flag-Smek1, Flag-Smek1ΔRanBD, or a control construct and immunostained with anti-Flag and -Par3 antibodies. DNA was stained by Hoechst dye. Scale bar, 10 μm.