Supplementary Figures and Methods for:
Cullin 5 regulates Dab1 protein levels and neuron positioning during cortical development
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Supplementary Methods

Plasmids and Constructs pCMXGFP-Dab1
, pCMXGFP-Dab1ab, pCMXGFP-Dab1cd, pCMXCFP-Dab1a, pCMXGFP-Dab1b and pCMXGFP-Dab1SF expression plasmids were constructed by cutting out fragments containing the corresponding mutants from pBSK HA-Dab1 (Howell et al., 2000) with EcoRI/XbaI and subcloning into pCMXGFP-p1k1 with EcoRI/Nhel. The pCAG-Dab1GFP was described before (Xu et al., 2005). Different phosphorylation site mutants were generated by swapping in BstXI/BgIII fragments from the corresponding pCMXGFP-Dab1 mutants. The following constructs were kind gifts from the following individuals: pME18S-Fyn(B) WT and KM(kinase dead), from Tohru Tezuka; pEF-Flag-mSOCS1/3, Neil Nathanson; pEF-Flag-mSOCS2, Chris Greenhalgh (Nicholson et al., 1999); pCEFL-hCbl-b-HA and corresponding Cbl, Cbl-3 and myc-E6AP constructs, Stanley Lipkowitz (Ettenberg et al., 1999; Hatakeyama et al., 1997; Keane et al., 1999); pCMX Flag-mLNX, Jane McGlade (Nie et al., 2002); Rnf5, Ze'ev Ronai (Didier et al., 2003); and Hakai, Yasu Fujita (Fujita et al., 2002).

Pulse-Chase Experiment COS cells, transfected with Dab1-GFP and Fyn in the presence or absence of SOCS1, were washed 24 h after transfection and incubated in Cys- and Met-free DMEM (Gibco) to deplete the intracellular pools of Met and Cys. After 15 min, the medium was replaced with Cys-, Met-free DMEM containing 100 Ci/ml L-[35S] in vitro Cell Labeling Mix (Amersham Bioscience) to label the newly synthesized proteins. After 45 min, the dishes were
washed once in PBS and incubated in chase medium (DMEM containing 10% fetal bovine serum and Cys and Met). At various time, the cells were lysed and immunoprecipitated with anti-GFP antibody followed by protein G-Sepharose, and separated by SDS-PAGE. Immune complexes were detected by Phosphorimager and quantified using ImageQuant TL.

**In situ hybridization** E17.5 embryonic mouse brains were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, postfixed overnight with the same solution, cryoprotected with 30% sucrose, and sectioned sagitally at 50 µm. Cul5 and Cul2 antisense riboprobes were prepared by cloning cDNA fragments containing bases 314 to 2030 of mouse Cul5 mRNA (gi: 142343675) and bases 843 to 2864 of mouse Cul2 mRNA (gi: 20073293) into pBluescript. Antisense probes were transcribed with T3 polymerase and sense probes with T7 polymerase (Ambion), in the presence of digoxigenin-d-UTP (Roche). **In situ** hybridization was performed on floating sections essentially as described elsewhere (Alcantara et al., 1998). Bound probe was detected by alkaline phosphatase reaction. Sense and antisense images were captured at same exposure time.


Xu, M., Arnaud, L., and Cooper, J. A. (2005). Both the phosphoinositide and receptor binding activities of Dab1 are required for Reelin-stimulated Dab1 tyrosine phosphorylation. Brain Res Mol Brain Res 139, 300-305.
Fig. S1. Effect of Reelin on levels of Dab1-GFP with mutations at the a and b sites.

Neurons were co-electroporated with MAP2-GFP and wild-type or mutant forms of Dab1-GFP. Duplicate dishes were treated with Control (C, containing VLDLR ectodomain to sequester endogenous Reelin) or Reelin (R) for 4 days. Lysates were analyzed by Western blotting.

Fig. S2. Effects of different E3 ligases on levels of Dab1 protein.

COS7 cells were co-transfected with plasmids expressing Dab1-GFP, Fyn, various E3 ubiquitin ligases, and GFP as a control for transfection efficiency. Dab1-GFP and GFP were detected using GFP antibody, and the E3 ligases with either Flag or HA antibodies. Western blots were quantified using ImageJ, and the ratio of Dab1-GFP to GFP calculated. Only SOCS1 specifically reduced the level of Dab1-GFP.

Fig. S3. Expression of Cul5 and Cul2 in developing cortex.

Cul5 (B) and Cul2 (D) transcripts were localized in sections of E17.5 embryo brain using in situ hybridization with antisense probes. Controls with the corresponding sense probes show less signal (A and C). All panels show the lateral cortex from the region of the hippocampus, pial surface at top and ventricle at bottom. MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; VZ, ventricular zone. Scale bar equals 50 µm.
COS7 cells were transfected with the indicated quantities of DNA and lysed 24 hr later. Western blots show that Cul5 and GFP expression from pCAGIG Cul5wt, which expresses wildtype Cul5 and GFP from the same mRNA, was inhibited by Cul5 shRNA but not by vector. Expression of Cul5* and GFP from pCAGIG Cul5*, which contains mutations in the target region for Cul5 shRNA, or from pCAGIG, was not inhibited by Cul5 shRNA.

**Fig. S4** Cul5* rescue construct resists Cul5 shRNA

**Fig. S5.** Cul5 shRNA affects Dab1 protein but not RNA levels.

A. Embryonic cortical neurons were electroporated with shRNA constructs against Cul5 or Cul2. Parallel cultures were prepared for RNA or protein analysis after 4 days incubation in the presence of Reelin. Western blotting showed that each shRNA reduced the level of its target protein, and that Cul5 but not Cul2 shRNA protected Dab1 from Reelin-induced degradation. RNA samples were analyzed by RT-PCR with primers to Dab1 mRNA and GAPDH mRNA. Samples were removed after different numbers of PCR cycles. Dab1 mRNA level decreased slightly but not significantly under conditions where Dab1 protein level increased.

B. In utero microinjection of Cul5 shRNA does not increase Dab1 mRNA levels. Embryos were electroporated with 1 ug pCAG-GFP and 2 ug Cul5 shRNA at E14.5. Sections from E17.5 cortex were subjected to in situ hybridization with antisense probe to Dab1 mRNA, and immunofluorescent localization of GFP-expressing cells. Neurons expressing GFP (red arrowheads) do not have increased Dab1 mRNA.
Fig. S6. Effect of Cul5 shRNA on neuron position at P2.

In utero electroporation of 2 mg/ml Cul5 shRNA and 0.5 mg/ml pCAGIG at E14.5. Sections prepared at P2, 7 days after electroporation.

Fig. S7. Effects of Cul5 shRNA on positioning of neurons with E14.5 birthdate. Embryos were electroporated on E14.5 with 2 mg/ml Cul5 or Cul2 shRNAs or vector and 0.5 mg/ml pCAGIG, and labeled with BrdU. Sections were prepared on E19.5 and stained for BrdU and GFP.

A. Distributions of GFP-labeled Cul2 shRNA and control neurons are similar, and deeper than Cul5 shRNA neurons.
B. The distributions electroporated neurons born on E14.5, identified by double label with GFP and BrdU, resembles the distribution of total electroporated neurons. This means that the displacement of Cul5 shRNA neurons to upper layers is not due to selective loss of the first-born progeny of the electroporated precursors. Mean±SEM of Cul5 shRNA (n=2 embryos) and Cul2 shRNA (n=3 embryos).
C. Representative images of BrdU-labeled Cul5 shRNA and vector control cortex. Note the relative positions of BrdU-labeled and GFP-labeled neurons. Bright BrdU+ cells, born on E14.5, are mixed in with the brightest GFP+ control neurons, but are mostly below the GFP+ Cul5shRNA neurons.
Fig. S8. Cul5 shRNA does not prevent inhibition of new Dab1 protein synthesis by Dab1 shRNA.

Cul5 shRNA and Dab1 shRNA, or their corresponding empty vectors, were mixed in the ratio used for in utero electroporation (Fig. 8C). 5 µg of each mixture was added to 1 µg of pCAG-Dab1GFP and 0.5 µg of pCAG-MAP2GFP, and introduced into cortical neurons by electroporation. 4 days later, the synthesis of Dab1-GFP was measured relative to the MAP2-GFP control by Western blotting with GFP antibodies. Dab1 shRNA inhibited new synthesis of Dab1-GFP protein whether or not Cul5 shRNA was present.