Hou et al., http://www.jcb.org/cgi/content/full/jcb.201303035/DC1

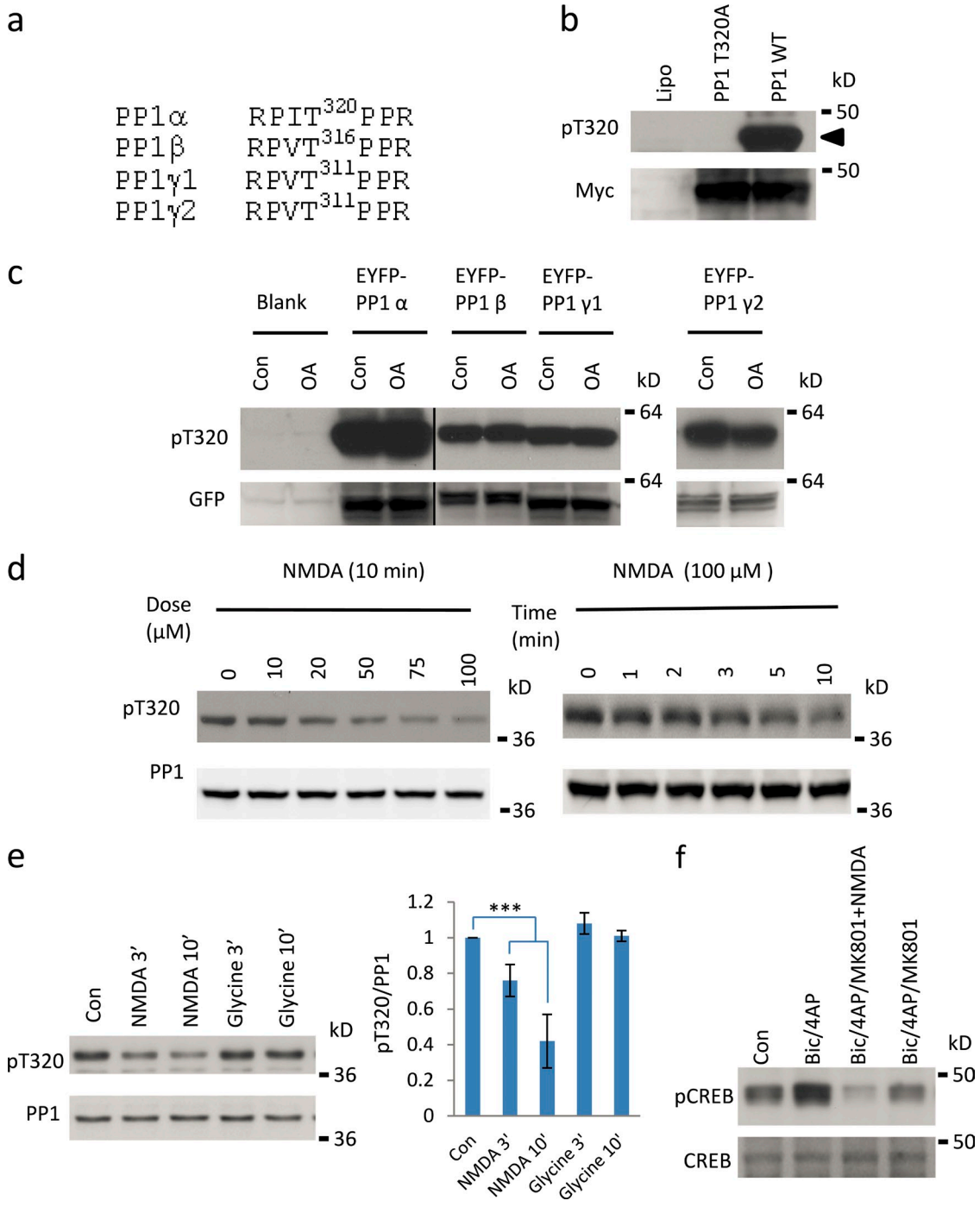

Figure S1. **PP1 phosphorylation site conservation and phospho-antibody pT320 characterization.** (a) Threonine phosphorylation sites in the C terminus of all PP1 isoforms are conserved. (b) The antibody recognizing phosphorylated PP1α at threonine 320 (T320), termed pT320, is specific because it only recognized expressed wild-type PP1 α-myc-HIS (PP1 WT), but not phosphorylation-resistant PP1 α-myc-HIS (T320A) (PP1 T320A) expressed in HEK293 cells, and analyzed by immunoblotting. (c) The pT320 recognized all phosphorylated recombinant PP1 isoforms, individually expressed as EYFP-chimeric proteins in HEK293 cells and analyzed by immunoblotting. (d) Experiments were performed using cultured cortical neurons (DIV21) as described in Fig. 1. NMDA application led to a decrease in PP1 phosphorylation at T320 in a dose- and time-dependent manner, as measured by immunoblotting using the pT320 antibody. (e) ChemLTP stimulus does not affect PP1 phosphorylation at T320. Cortical neurons were preconditioned in ACSF for 2 h before incubation with 20 μM NMDA/20 μM glycine (chemLTD stimulus), or 200 μM glycine only (chemLTP stimulus) for 3 or 10 min. (f) Cultured cortical neurons were exposed to NMDA in a bath application after synaptic NMDA receptor blockade. Phosphorylation of CREB at serine 133 (pCREB) was assayed by immunoblotting with a specific phospho-CREB antibody. Experiments were performed as in Fig. 1 f.

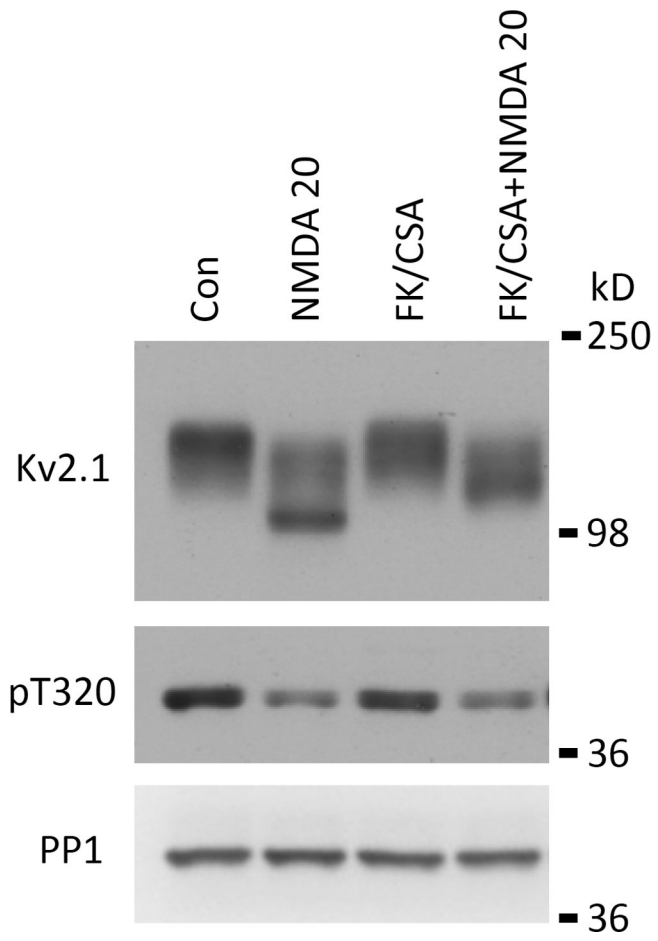

Figure S2. **Effectiveness of FK506 and cyclosporine A in cortical neurons.** Cultured cortical neurons were incubated without or with NMDA (20 µM for 10 min) in the absence or presence of FK506 (FK, 1 µM) plus cyclosporine A (CSA, 20 µM), as described in Fig. 2 a. The levels of PP1, pT320, and Kv2.1 were assayed by immunoblotting with specific antibodies. The combination of FK506 and cyclosporine A blocked the Kv2.1 gel mobility shift in response to bath NMDA application as shown previously (Misonou et al., 2004). Figure corresponds to Fig. 2 a.

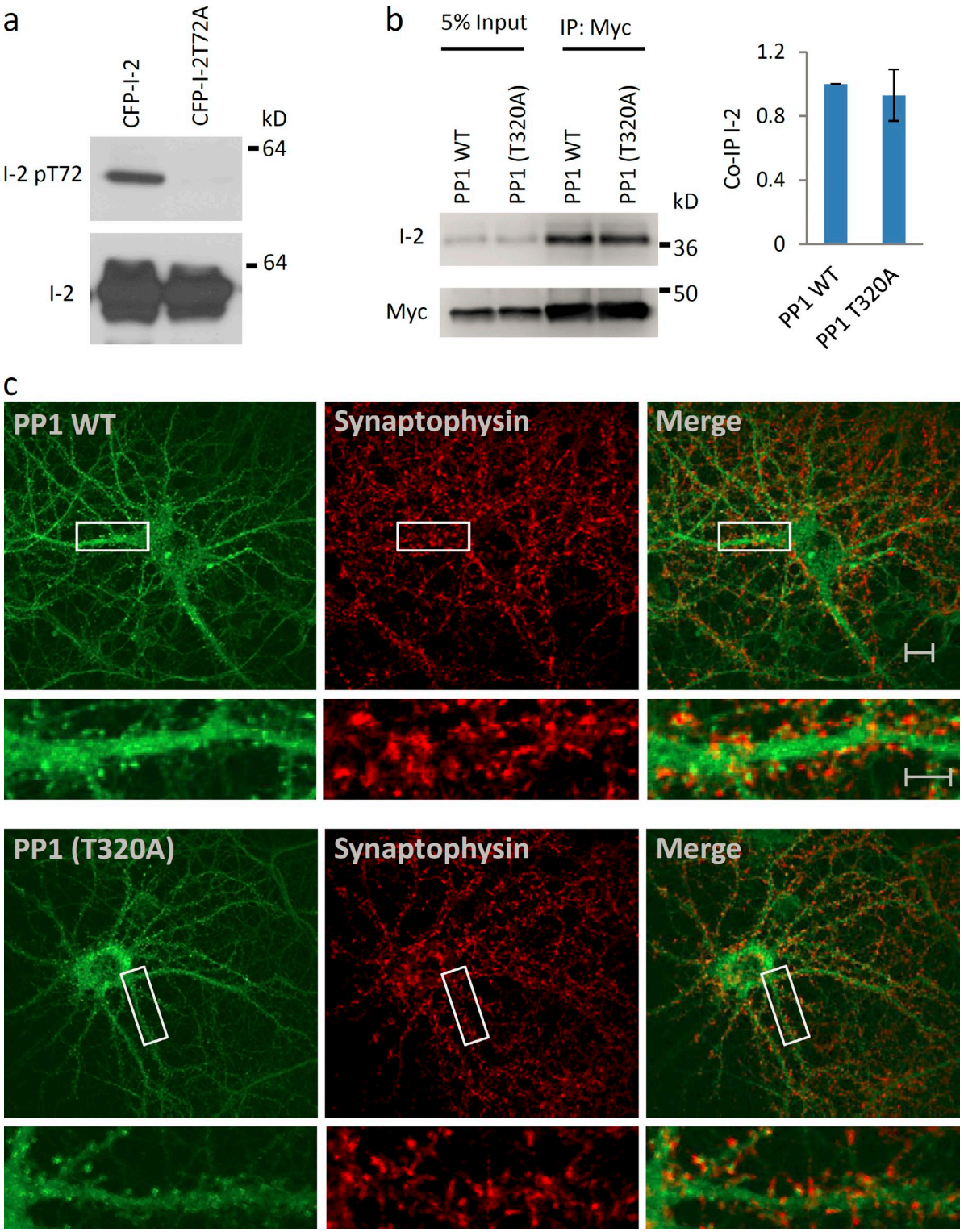

Figure S3. **Specificity of the pI-2T72 antibody and no effect of PP1 dephosphorylation at T320 on binding with I-2 and synaptic targeting.** (a) HEK 293cells were transfected with CFP-I-2 or CFP-I-2 (T72A) constructs for 48 h and I-2 and I-2 phosphorylation at T72 (by I-2pT72) was measured by immunoblotting. (b) PP1α-myc-His constructs (WT or T320A mutant) were transfected into HEK 293 cells, recombinant PP1 was immunoprecipitated using a Myc antibody, and total I-2 and PP1-myc-HIS were analyzed by immunoblotting. There was no difference in the amount of I-2 that was coimmunoprecipitated with WT or T320A PP1. (c) Sindbis viruses encoding PP1α-myc-HIS (WT or T320A mutant) were used to infect >3-wk-old cortical neurons. Localization of PP1 expression was assessed by staining for Myc. Co-staining for the presynaptic marker synaptophysin was used to assay the relative dendritic spine targeting of PP1 WT and T320A mutants. At both 24 h and 7 h after infection, there was no detectable difference between localization of the PP1 WT and T320A synaptic staining. Bar, 10 µm (inset, 5 µm). Figure corresponds to Fig. 3.

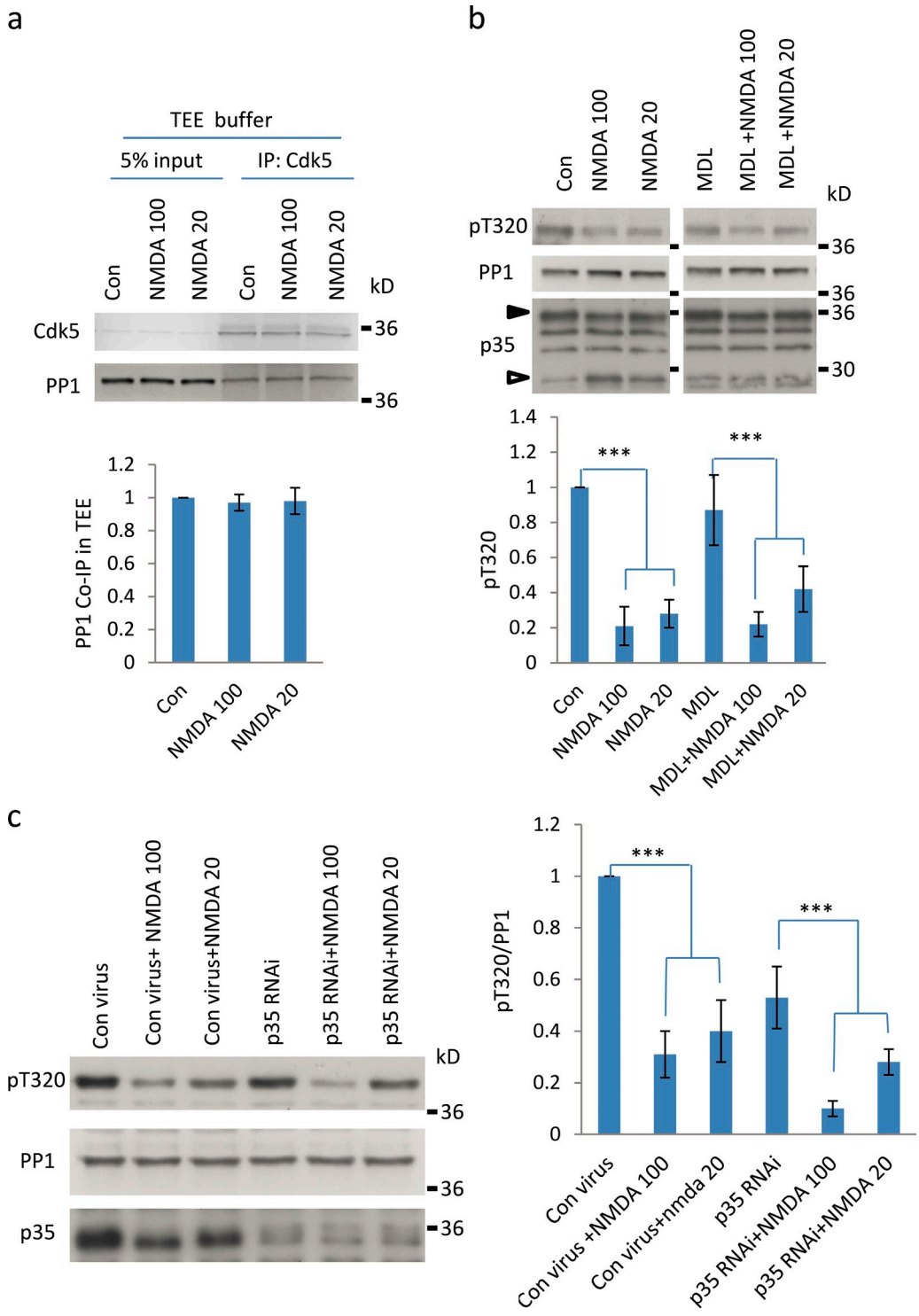

Figure S4. **NMDA application does not affect PP1–Cdk5 interaction, p35 to p25 conversion induced by NMDA application is not critical for PP1 dephosphorylation at T320, and NMDA application can cause further PP1 dephosphorylation at T320 in p35 KD neurons.** (a) Cultured cortical neurons were treated without (Con) or with NMDA (20 and 100 µM) for 10 min before the cells were harvested and soluble fractions were prepared using TEE buffers (see Materials and methods) before immunoprecipitation of Cdk5. Total Cdk5 and total PP1 were analyzed by immunoblotting. Cumulative results are shown in the bar graph; $n = 3$, standard error of the mean (SEM) was shown. (b) Cortical neurons were incubated for 30 min without or with the calpain inhibitor MDL28170 (100 µM), then incubated without (Con) or with NMDA (20 and 100 µM) for 10 min. Total PP1, pT320-PP1, and total p35 were measured by immunoblotting. The addition of MDL28170 blocked the cleavage of p35 (solid arrow) to p25 (hollow arrow), but did not block the reduction in pT320, in response to NMDA application. Cumulative results are shown in the bar graph; $n = 3$, SEM was shown. (c) NMDA application can induce further PP1 dephosphorylation at T320. P35 KD was performed as in Fig. 5 f. NMDA (20 or 100 µM) was applied to neurons for 10 min and then PP1 phosphorylation at T320 was determined by Western blotting. Figure corresponds to Fig. 5.

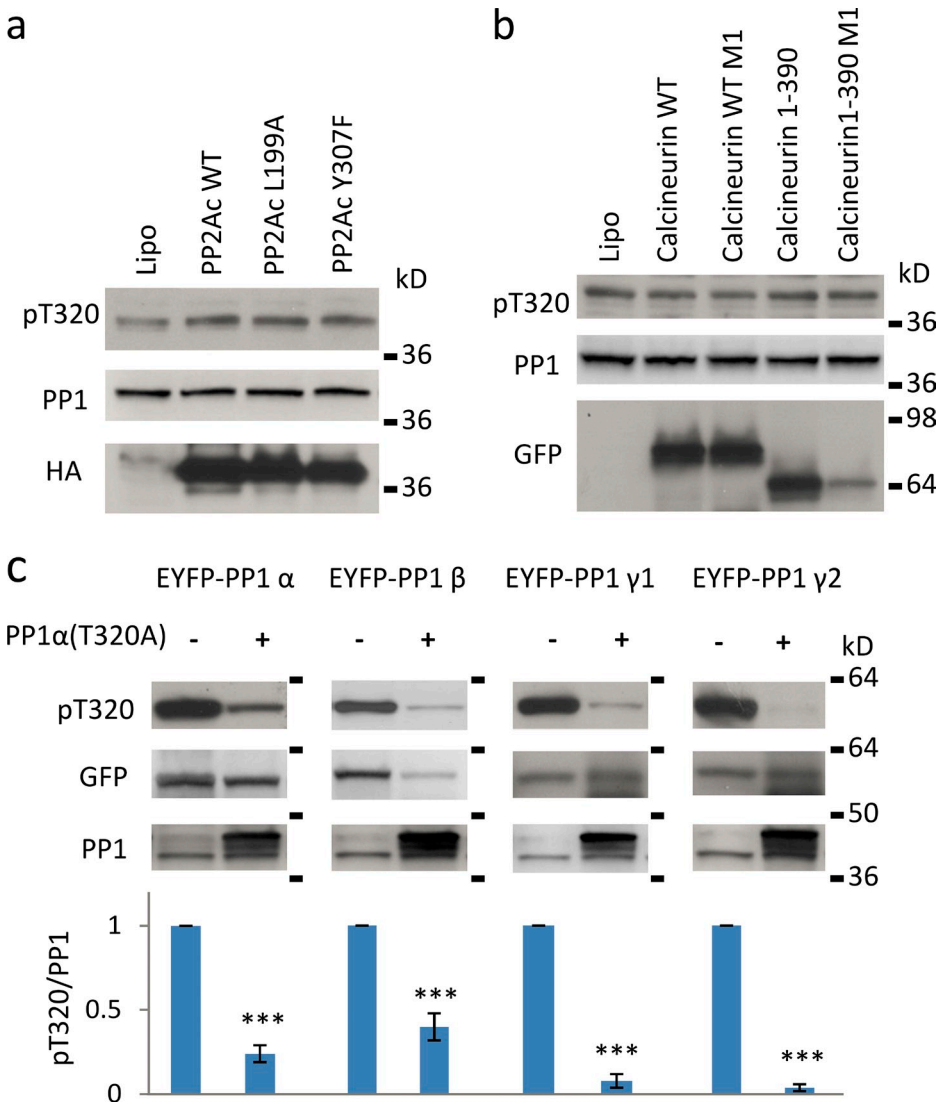

Figure S5. **Specificity of PP1 trans-dephosphorylation.** HEK293 cells were transfected with (a) various PP2A (HA tagged) or (b) calcineurin (GFP tagged) constructs. Total PP1 and p320 were analyzed by immunoblotting. PP2Ac is the catalytic subunit of the wild-type PP2A (PP2Ac); PP2Ac Y307F is the constitutively active PP2Ac mutant; PP2Ac L199A is the dominant-negative PP2Ac mutant; calcineurin WT M1 is the dominant-negative mutant for calcineurin wild type (calcineurin WT); calcineurin 1–390 is a constitutively active form of calcineurin; calcineurin 1–309 M1 is a dominant-negative mutant. Expression of HA- and GFP-tagged proteins was assessed by immunoblotting. (c) EYFP-tagged PP1α, β, γ1, or γ2 were singly transfected or doubly transfected with the constitutive active PP1 mutant PP1α (T320A)-myc-His in HEK 293 cells. Total PP1 and pT320 were analyzed by immunoblotting. EYFP-tagged PP1 isoforms were analyzed by immunoblotting using a GFP antibody. The PP1 antibody blots revealed both endogenous PP1 and overexpressed PP1 (myc-His tagged). Cumulative results are shown in the bar graph; $n = 3$, SEM was shown. *, $P < 0.001$, compared with the control. Figure corresponds to Fig. 6.

Reference

Misonou, H., D.P. Mohapatra, E.W. Park, V. Leung, D. Zhen, K. Misonou, A.E. Anderson, and J.S. Trimmer. 2004. Regulation of ion channel localization and phosphorylation by neuronal activity. *Nat. Neurosci.* 7:711–718. http://dx.doi.org/10.1038/nn1260