Supplementary information, Figure S4

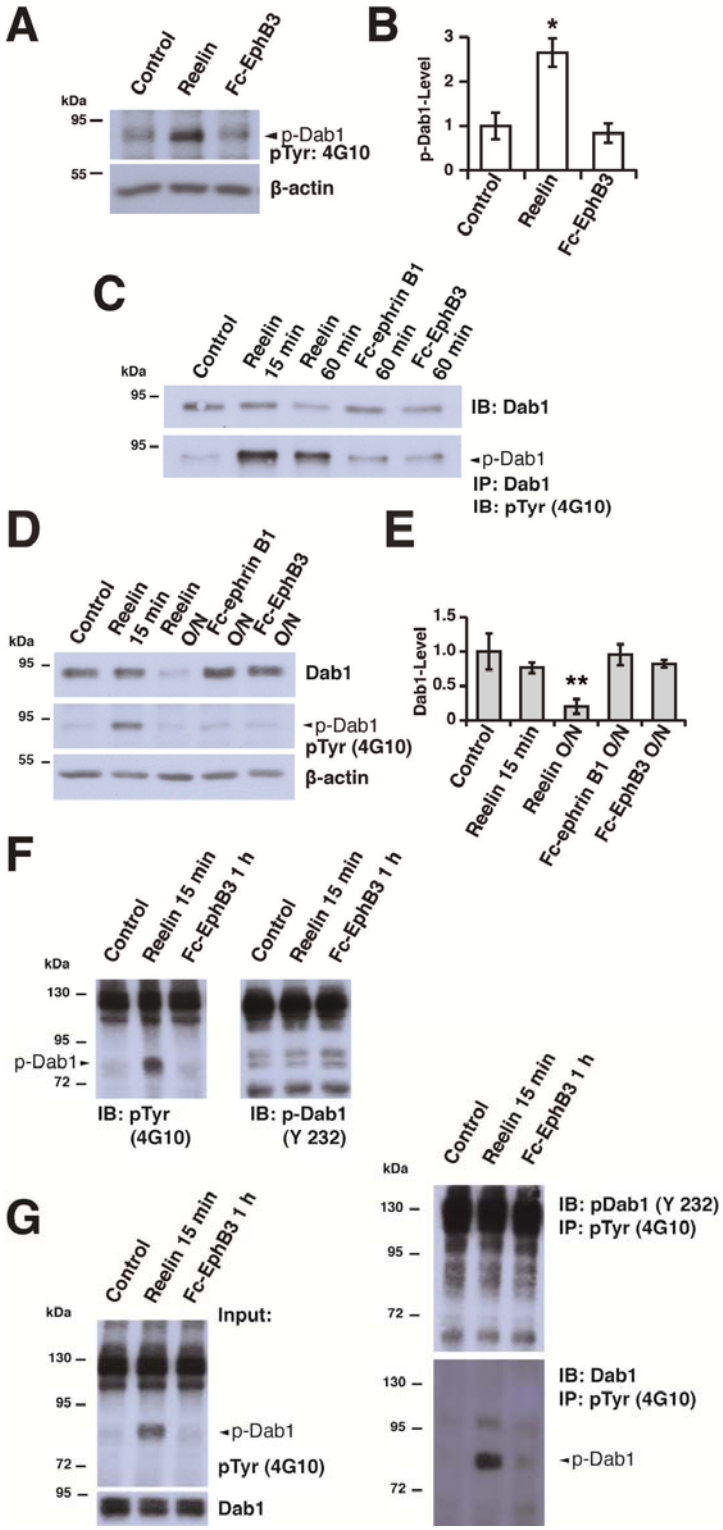

Figure S4 Effect of preclustered soluble EphB3 ectodomain on activation of Dab1 signaling. **(A)** Stimulation of cortical neurons with Reelin or preclustered Fc-EphB3 for 15 min. The lysates were separated by SDS-PAGE and blotted with the 4G10

phosphotyrosine antibody. Actin served as a loading control. **(B)** The p-Dab1 signal intensities were quantified (s.d., *$P < 0.05$ as compared to control, $n = 3$). Stimulation with clustered Fc-EphB3 did not induce Dab1 phosphorylation. **(C)** Primary neurons were treated with Reelin for 15 and 60 min and with preclustered Fc-ephrin B1 or Fc-EphB3 for 60 min, respectively. After lysis and immunprecipitation with a Dab1 antibody (IP: Dab1), tyrosine phosphorylation levels of Dab1 were assessed using the 4G10 antibody (IB: pTyr). Again, no increase in Dab1 phosphorylation was detected after treatment with preclustered soluble ephrin B1 or EphB3 ectodomain. **(D)** Neurons were treated with Reelin for 15 min or with Reelin, preclustered Fc-ephrin B1 or Fc-EphB3 overnight (O/N). **(E)** Quantification of the treatment shown in **C**. (s.d., **$P < 0.01$ as compared to control, $n = 3$). Degradation of full-length Dab1 was only seen in neurons treated with Reelin overnight. **(F)** Treatment of primary cortical neurons with recombinant Reelin for 15 min induced robust tyrosine phosphorylation of Dab1 as compared with control-treated neurons, whereas treatment with preclustered Fc-EphB3 (4 µg/ml) did not. The neurons were lysed and tyrosine phosphorylation was detected by immunoblotting using the 4G10 monoclonal antibody (left). The same cell lysates were probed with a rabbit antibody raised against a synthetic peptide corresponding to residues surrounding Tyr232 of human Dab1 (1:1000, Cell Signaling Technology). This antibody does not recognize tyrosine-phosphorylated Dab1 in primary neurons (right). **(G)** To further evaluate the specificity of the p(Y232)-Dab1 antibody, we immunoprecipitated tyrosine-phosphorylated proteins from Reelin- or Fc-EphB3-treated neurons with the 4G10 antibody and probed the immunoprecipitates with the p(Y232)-Dab1 antibody (right, top) or (after membrane stripping) with an antibody against total Dab1 (right, bottom). The p-Dab1 does not recognize tyrosine-phosphorylated Dab1, whereas the Dab1 antibody detects an increase in tyrosine-phosphorylated Dab1 only in immunoprecipitates from Reelin-treated neurons. The lysates (input) used for the immunoprecipitation were also immunoblotted with the 4G10 antibody (left) to proof that Reelin treatment induced tyrosine phosphorylation of Dab1 in this experiment (with total Dab1 serving as a loading control).