Legends to Supplemental Figures and Tables

Fig S1: GTPase activity of purified recombinant RhoA, Cdc42, and Rac1 under different conditions. GTPase activity was determined by monitoring GTP to GDP ratios over time by HPLC measurements. GTP-loaded GTPases were incubated with either buffer (greyline) or cell lysates of Citrine (green line) or Citrine-GAP (blue line) transfected cells. Samples were collected at different time intervals, immediately treated with TCA for protein precipitation and neutralized with NaOH. Guanine nucleotide ratios were determined by HPLC and relative GTP ratios calculated from GTP/(GTP+GDP). Rate constants (k_{cat}) of intrinsic GTP hydrolysis were calculated from exponential fittings on the data. All three GTPases showed GTPase activity that was further stimulated by the respective GAP.

Fig S2: LF-qGAP from brain lysates from additional tissues for (A) Rac1 (Cerebellum), (B) RhoA (Cerebellum) and (C) Cdc42 (Hippocampus). Specific interactors (colored in orange) are distinguished from background proteins based on a combination of the log2 fold change and the t-test p-value. GTPγS-specific interactors are expected in the upper right corner, interactors of the GDP-form in the upper left corner.

Fig S3: Validation of loading state dependent interactions *in situ* with the proximity ligation assay (PLA) (details given in Fig. 5A). This figure is complementary to figure 5.

Fig S4: Fragment spectra of Rho GTPase interactors identified by only one peptide sequence. For each protein the best available spectrum of the corresponding peptide was selected. All spectra are shown in the mass to charge deconvoluted version as listed in the msms.txt as provided by MaxQuant. Red peaks belong to the y-ion series, blue peaks to the b-ion series and grey peaks are unidentified background peaks of each spectrum. Orange labels indicate a modification of the corresponding fragment.

Table legends

Supplemental Table 1: Interaction partners of Rho GTPases. For the six Rho GTPases RhoA, RhoB, RhoC, RhoD, Rac1 and Cdc42 a total number of 293 distinct interactions were identified with LF-qGAP. A number of interactions were identified repeatedly in different tissues.

Supplemental Table 2: List of antibodies used for the proximity ligation assays. This list gives additional information about suppliers, used dilutions and reference links.