Supplemental figure captions

Figure S1
(A) GFP cell fill in 3D view of SIM image depicted in Figure 1E.
(B) GFP cell fill in 3D view of SIM image depicted in Figure 1F.
(C) Immuno-EM images showing Bin1 labeling associated with various synaptic specializations
(D) Single plane SIM images showing colocalization between Bin1 (green) and PSD95 (red). Areas of overlap are subtracted in the bottom right image, visualized by white regions (zoomed in regions denoted by white boxes, blown up to right). Scale bar = 5 um
(E) Subcellular fractionation of mouse cortex homogenates showing Bin1 staining in various fractions. Definitions of each fraction located to the right of the image. PSD95 staining demonstrates successful PSD-enrichment in the S6 fraction.

Figure S2
(A) Representative single plane confocal images of mCherry-filled cortical neurons expressing scrambled (scr) or Bin1 knockdown (kd) plasmids immunostained for Bin1 and CaMKIIa (to identify pyramidal cells). Images used for analysis are outlined in white in the Bin1 staining image. Scale bar = 10 µm.
(B) Representative flattened confocal images of a GFP-filled cortical neuron expressing Bin1-mCherry.
(C) SDS-PAGE and Western blot of HEK cell lysates expressing Bin1-GFP together with scrambled (scr) or different Bin1 knockdown RNAi constructs. RNAi construct #1 was utilized for all experiments.
(D) Quantification of Bin1 knockdown based on Western blots for RNAi #1 (n = 4); *Welch's t-test* **** *P<0.0001*
(E) Quantification of Bin1 knockdown based on Bin1 immunostainings in cortical neurons for RNAi #1. Values are expressed as a ratio of mCherry positive to mCherry negative cell Bin1 staining in each imaging field (scr: n = 18 cell pairs, kd: n = 16 cell pairs); *Unpaired t-test* **** *P<0.0001*
(F) GFP-cell fill of representative confocal images of Rab11 staining depicted in Fig 2C for control (scr) and Bin1 knockdown (kd) neurons, scale bar = 10 µm.
(G) Left: GFP-cell fill of representative confocal images of Chc staining for control (scr) and Bin1 knockdown (kd) neurons, scale bar = 10 µm.
Right: Representative flattened confocal images of Chc staining in GFP filled control (scr) and Bin1 knockdown (kd) neurons. The outline indicates the GFP-cell fill (shown on left).
(H) Quantification of area occupied by and number of Chc positive puncta in scr and kd neurons. (n = 8 cells for each condition). *Area occupied: Mann-Whitney test, Puncta number: unpaired t-test.*
(I) Left: GFP-cell fill of representative confocal images of Chc staining for control (scr) and Bin1 overexpressing (ox) neurons, scale bar = 10 µm.
Right: Representative flattened confocal images of Chc staining in GFP filled control (ctrl) and Bin1 overexpressing (ox) neurons. The outline indicates the GFP-cell fill (shown on left).
(J) Quantification of area occupied by and number of Chc positive puncta in ctrl and ox neurons. (ctrl: n = 13 cells, ox: n = 9 cells). *Area occupied: Mann-Whitney test, Puncta number: unpaired t-test.*
(K) Left: GFP-cell fill of representative confocal images of Rab11 staining for control (scr) and Bin1 overexpressing (ox) neurons, scale bar = 10 µm.
Right: Representative flattened confocal images of Rab11 staining in GFP filled control and Bin1 overexpressing (ox) neurons. The outline indicates the GFP-cell fill (shown on left). Scale bar = 10 µm.
(L) Quantification of area occupied by and number of Rab11 positive puncta in ctrl and ox

neurons. (ctrl: n = 17 cells, ox: n = 16 cells). *Area occupied: Mann-Whitney test, Puncta number: unpaired t-test.*

Figure S3
(A) Additional images for the representative 3D reconstruction of a stack of SIM images showing the relative localization of Bin1 and Arf6 in spines; scale bar = 50 nm.
(B) Representative single plane SIM images of individual spines and line scans showing that Bin1 and Arf6 define distinct compartments that can be in contact with each other.
(C) GFP-cell fill of representative confocal images of Arf6 staining depicted in Figure 3A for control (scr) and Bin1 knockdown (kd) neurons, scale bar = 10 μm.
(D) GFP-cell fill of representative confocal images of Arf6 staining depicted in Figure S3E for control (ctrl) and Bin1 overexpressing (ox) neurons, scale bar = 10 μm.
(E) Left: Representative flattened confocal images of Arf6 staining in GFP filled control (ctrl) and Bin1 overexpressing (ox) neurons. The outline indicates the GFP-cell fill. Scale bar = 10 μm
Right: Quantification of area occupied by and number of Arf6 positive puncta in ctrl and ox neurons. (n = 15 cells for each condition). *Area occupied: Mann-Whitney test, Puncta number: unpaired t-test.*
(F) Quantification of area occupied by and number of Arf6 positive puncta in neurons expressing RNAi resistant Bin1 together with scrambled and Bin1 knockdown RNAi constructs. (n = 10 cells per condition). *Area occupied: Mann-Whitney test, Puncta number: unpaired t-test.*
(G) Quantification of total Arf6 staining in N2A cells used for experiments in Fig. 3B,C (n = 16 cells per condition). *Mann-Whitney test.*

Figure S4
(A) Quantification of AMPA mEPSCs in cortical neurons reveals no significant difference in average mEPSC inter event interval with Bin1 knockdown (scr: n = 14 cells, kd: n = 22 cells). The same is seen with Bin1 overexpression (ctrl: n = 24 cells, ox: n = 26 cells). *Mann-Whitney test.*
(B) Additional images for the representative 3D reconstruction of a stack of SIM images showing the relative localization of Bin1 and GluA1 in spines; scale bar = 50 nm.

Figure S5
(A) GFP-cell fill of representative confocal images of surface GluA1 staining of cultured rat neurons used in Fig. 5A.
(B) Representative flattened confocal images showing total GluA1 staining of cortical neurons expressing scrambled control (scr) or Bin1 knockdown (kd) RNAi (identified by GFP staining shown in white outline).
(C) Quantification of total GluA1 staining in soma of cortical neurons expressing scrambled control (scr, n=11) or Bin1 knockdown (kd, n=7) RNAi. *Unpaired t-test.*
(D) Total GluA1 staining of N2A cells for experiments shown in Fig. 5G,H (n = 16 for each condition). *Kruskal-Wallis with Dunn's post-hoc tests.*