Shank3 Binds to and Stabilizes the Active Form of Rap1 and HRas GTPases via Its NTD-ANK Tandem with Distinct Mechanisms

Highlights
- Shank3 binds to Rap1 with an unexpected mode and a 1:2 stoichiometry
- Shank3 binds to HRas via a canonical binding mode and a 1:1 stoichiometry
- Binding of Shank3 prevents Rap1 GTP hydrolysis by SynGAP
- Binding of Shank3 to HRas or Rap1 is promoted upon synapse activation

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In Brief
Cai et al. report that Shank3 N-terminal NTD-ANK tandem forms an integral structural supramodule, binds to two copies of Rap1 with distinct modes, and prevents Rap1 GTP hydrolysis catalyzed by SynGAP.
Shank3 Binds to and Stabilizes the Active Form of Rap1 and HRas GTPases via Its NTD-ANK Tandem with Distinct Mechanisms

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SUMMARY

Shank1/2/3, major scaffold proteins in excitatory synapses, are frequently mutated in patients with psychiatric disorders. Although the Shank N-terminal domain and ankyrin repeats domain tandem (NTD-ANK) is known to bind to Ras and Rap1, the molecular mechanism underlying and functional significance of the bindings in synapses are unknown. Here, we demonstrate that Shank3 NTD-ANK specifically binds to the guanosine triphosphate (GTP)-bound form of HRas and Rap1. In addition to the canonical site mediated by the Ras-association domain and common to both GTPases, Shank3 contains an unconventional Rap1 binding site formed by NTD and ANK together. Binding of Shank3 to the GTP-loaded Rap1 slows down its GTP hydrolysis by SynGAP. We further show that the interactions between Shank3 and HRas/Rap1 at excitatory synapses are promoted by synaptic activation. Thus, Shank3 may be able to modulate signaling of the Ras family proteins via direct binding to and stabilizing the GTP-bound form of the enzymes.

INTRODUCTION

SH3 and multiple ankyrin repeat domain (Shank) proteins (encoded by SHANK1-3) are major scaffolding proteins in excitatory postsynaptic densities (PSDs) (Naisbitt et al., 1999; Tu et al., 1999). All Shank proteins share similar domain organizations, each containing an N-terminal domain (NTD) with a “Ras-association domain” fold (Lilja et al., 2017; Mameza et al., 2013), an ankyrin repeats domain, an SH3 domain, a PDZ domain, a proline-rich sequence, and a C-terminal SAM domain (Figure 1A). Through direct and specific domain-domain interactions, such as binding to SAPAP (Zeng et al., 2016), Homer (Tu et al., 1999), and self-association by SAM (Baron et al., 2006) (Figure 1A), Shank proteins can form an extensive protein-protein interaction network in PSDs, which is critical for the formation, maintenance and plasticity of PSDs (Hayashi et al., 2009; Kim and Sheng, 2004; Sheng and Hoogenraad, 2007; Ting et al., 2012; Tu et al., 1999; Zhu et al., 2016). Mutations of genes encoding Shank proteins are highly penetrant in causing psychiatric disorders, such as autism and schizophrenia (Monteiro and Feng, 2017; Sala et al., 2015). For example, 22q13.3 deletions, leading to SHANK3 haploinsufficiency, are known to cause Phelan-McDermid syndrome (Bonaglia et al., 2006). Overexpression of Shank3 has been implicated in hyperkinetic neuropsychiatric disorders (Han et al., 2013). Many point mutations of SHANK3 have been identified in autism patients or individuals with intellectual disabilities (Durand et al., 2007; Gauthier et al., 2009; Moessner et al., 2007). Mutations of Shank genes in rodents often recapitulate many autism spectrum disorder (ASD) phenotypes, such as altered social behavior, anxiety-like phenotype, and obsessive-compulsive disorder-like repetitive behaviors (Peca et al., 2011; Schmeisser et al., 2012; Won et al., 2012; Zhou et al., 2019). Therefore, tremendous progress has been made in linking mutations in Shank genes and ASD via combined approaches of human genetics and animal model-based studies (Jiang and Ehlers, 2013; Leblond et al., 2014).

Structural studies showed that Shank NTD indeed couples with the ankyrin repeats to form an NTD-ANK supramodule (Lilja et al., 2017). Several proteins were identified to bind to the NTD-ANK tandem, and these proteins include Sharpin (Lim et al., 2001), α-fodrin (Bockers et al., 2001), HCN channel (Yi et al., 2016), and the guanosine triphosphate (GTP)-bound form of the Ras superfamily proteins of small GTPases (Lilja et al., 2017). However, the molecular basis governing the bindings of Shank NTD-ANK to any of these proteins is not known.

Small GTPases are critical regulators of synaptic plasticity. During long-term potentiation (LTP), small GTPases are activated by upstream Ca2+ signal and subsequently regulate diverse downstream signaling processes (Harvey et al., 2008; Hedrick et al., 2016; Murakoshi et al., 2011). There are several members of the Ras superfamily proteins, Ras and Rap, at excitatory synapses. These GTPases show a high amino acid sequence identity, adopt highly similar 3D structures, and share many common regulators/effectors or binding partners (Bos, 1994). Small GTPases are activated by guanine nucleotide exchange factors (GEFs) and inactivated by GTP-activating proteins (GAPs). Thus, GEFs and GAPs act as molecular switches...
of GTPases. SynGAP, a highly abundant protein in PSDs, is a Ras/Rap GAP functioning to “turn off” Ras and Rap activities and therefore acts as an inhibitory factor to the functions of the GTPases (Kim et al., 2003; Komiyama et al., 2002). LTP induction rapidly disperses SynGAP from dendritic spines to shift the balance of Ras and Rap toward their active forms (Araki

Figure 1. Specificity of the Interactions between Shank Proteins and Ras Superfamily Proteins
(A) Schematic diagram showing the domain organization of Shank proteins with selected binding partners for each domain indicated.
(B) Summary of ITC-based measurements of the binding affinities between the NTD-ANK tandems of Shank proteins and variant members of the Ras superfamily GTPases.
(C) Interaction between Shank3 and GMPPNP-bound Rap1 measured by ITC-based experiment. Left panel: 150 μM Shank3 NTD-ANK in the syringe was titrated into 30 μM GMPPNP-bound Rap1 in the cell. Right panel: 200 μM GMPPNP-bound Rap1 in the syringe was titrated into 10 μM Shank3 NTD-ANK in the cell. The fitted K_d and n value for the titration curve are indicated in red within the panel. We also report K_d and n values of the reaction in the format of mean ± SD (in blue) by averaging three independent titration experiments using different batches of proteins; same as in (E).
(D) Interaction between Shank3 and GDP-bound Rap1 measured by ITC-based experiment. Shank3 NTD-ANK (150 μM) in the syringe was titrated into 15 μM GDP-bound Rap1 in the cell.
(E) Interaction between Shank3 and GMPPNP-bound HRas measured by ITC. Shank3 NTD-ANK (150 μM) in the syringe was titrated into 15 μM GMPPNP-bound HRas in the cell.
(F) Interaction between Shank3 and GDP-bound HRas measured by ITC-based experiment. Shank3 NTD-ANK (150 μM) in the syringe was titrated into 15 μM GDP-bound HRas in the cell. See also Figure S1.
et al., 2015). It is not known whether Shank binding to Ras/Rap functions merely as a scaffold to anchor the GTPases in PSDs or also directly modulate the activities of the enzymes. Indeed, Shank3 has been shown to sequester active Rap1 from activating integrin-mediated cell migrations in heterologous cells, suggestive of a regulatory role of the interaction (Lilja et al., 2017).

Here, we systematically studied the interactions between Shank proteins and the Ras family proteins using biochemical approaches. To understand the molecular mechanism governing their interactions, we solved the crystal structures of Shank3 NTD-ANK in complex with Rap1 or HRas. Unexpectedly, Shank3 interacts with Rap1 with a 1:2 stoichiometry through a canonical binding mode via the Ras-association domain and a previously unknown binding site where both NTD and ANK are involved in the binding. We also discovered that binding of Shank3 to Rap1 prevents SynGAP from activating GTP hydrolysis of Rap1. Finally, we demonstrated that the binding of Shank3 to HRas or Rap1 at excitatory synapses is promoted by glutamate-mediated synapse activation. Thus, the Shank and Ras superfamily interactions may play a role in sustaining the Ras/Rap activities in synapses.

RESULTS

Specificity of the Interactions between Shank Proteins and the Ras Superfamily Proteins

The Shank NTD was proposed to be a Ras-association domain that can directly interact with active (i.e., the GTP-bound form) Ras and Rap GTPases (Lilja et al., 2017). Using isothermal titration calorimetry (ITC)-based binding assays, we studied the specificity of the interactions between the Shank proteins and the Ras superfamily proteins. The NTD-ANK tandems of three members of Shank (Shank1, residues 73–440; Shank2, residues 49–423; and Shank3, residues 1–376) were purified and subjected to ITC experiments with several members of the Ras family GTPases (HRas, RRas, MRas, Rap1, and Rap2). Most pairs of interactions were relatively weak, except that the active form of Rap1 (i.e., the GMPPNP-bound form) binds to the NTD-ANK tandems from Shank2 and Shank3 with submicromolar dissociation constants (Figures 1B and 1C). Importantly, the GDP-bound Rap1 had no detectable binding to Shank NTD-ANK (Figure 1D). Unexpectedly, the binding ratio between Rap1 and the NTD-ANK tandem of Shank2 or Shank3 is 2:1 (Figures 1B, 1C, and S1A). In contrast, the binding between the GMPPNP-bound form of HRas and Shank3 NTD-ANK, the next strongest interaction, displayed a 1:1 stoichiometry under the same condition by ITC (Figure 1E), and the binding between HRas and Shank3 NTD-ANK is also strictly limited to the GTP-bound form of HRas (Figure 1F). Thus, Rap1 and HRas, two highly similar members of the Ras superfamily GTPases, behave differently when binding to the Shank proteins.

Analytical gel filtration chromatography coupled with multi-angle light scattering assay was also used to evaluate the bindings of Shank3 NTD-ANK to Rap1 and HRas. For the Shank3/HRas interaction, increase the molar ratio of HRas to Shank3 from 1:1 to 2:1 did not further alter the elution volume of the complex (Figures S1B and S1C). In contrast, for the Shank3/Rap1 interaction, increase of the molar ratio of Rap1 to Shank3 from 1:1 to 2:1 further shifted the complex elution peak to a smaller volume. Further increase of Rap1 did not alter the elution volume of the complex anymore (Figures S1B and S1C), indicating that each Shank3 NTD-ANK binds to more than one Rap1.

The Structure of Shank3 NTD-ANK in Complex with Rap1 Reveals a Canonical and a Non-canonical Rap1 Binding Sites in Shank3

To elucidate the molecular basis underlying the interaction between Rap1 and Shank3, we solved the crystal structure of Shank3 NTD-ANK in complex with GMPPNP-bound Rap1 to the resolution of 2.81 Å (Table 1). Consistent with our ITC-based assay (Figure 2), two GMPPNP-bound Rap1 molecules bind to one molecule of Shank3 NTD-ANK with two distinct binding surfaces (Figures 2A and 2B).

The structure of Shank3 NTD-ANK in complex with Rap1 is essentially the same as that of the apo-form NTD-ANK (Lilja et al., 2017) (PDB: 5G4X, an overall root-mean-square deviation of 0.566 Å between the Cx atoms of two structures). Two Rap1 binding sites on Shank3 NTD-ANK are non-overlapping with each other. The first Rap1 binding site is via the canonical Ras-association domain of NTD as predicted (Lilja et al., 2017) (Figure 2C). Superposition of Rap1/Shank3-NTD complex with complex structures of Rap1 and Ras with other association proteins, such as cRaf1, KRIT1, and RIAM, revealed a nearly identical binding mode (Figure S3A). In this canonical binding, part of the “switch I” region of Rap1 form an antiparallel β sheet with the second β strand of Shank3 NTD, forming an extended inter-molecular β sheet (Figures 2A, right and S3A). In addition to the backbone hydrogen bonds formed by the inter-molecular β strand pairing, the binding between Rap1 and Shank3 NTD also involves a hydrogen bond formed between side chains of K22/shank3 and Y40/rap1 and a salt bridge between R12/shank3 and E37/rap1 (Figure 2C). Both K22 and R12 are highly conserved in all members of the Shank family (Figure S2), indicating that the canonical binding site is likely to be common for all Shank proteins. For the canonical interaction with Ras-association domains, Rap1 normally has two additional salt bridge interactions involving K31/rap1 and D33/rap1, binding to a negatively charged and a positively charged residues in Ras-association domains, respectively (Huang et al., 1998; Nassar et al., 1995). However, the corresponding residues in Shank3 NTD are C41/shank3 and A42/shank3, which cannot form electrostatic interactions with K31/rap1 and D33/rap1. A negatively charged residue corresponding to C41/shank3 in Ras-association domain is considered as the major determinant for their specific binding to HRas or Rap1 (Nassar et al., 1996), but this is apparently not the case for the interaction between Shank3 NTD and Rap1.

We next purified isolated Shank3 NTD to study its binding to Rap1 as a way to dissect the role of the NTD-ANK supramodule in Rap1 binding. GMPPNP-bound Rap1 was found to bind to Shank3 NTD with a Kd ~0.41 μM and a 1:1 stoichiometry (Figure S3B), indicating that the canonical form of the binding between Rap1 and Shank3 NTD is independent of ANK. Substitution of K22/shank3, the residue critical for the interaction between Shank3 NTD and Rap1 (Figure 2C), with either Glu or Ala completely disrupted the NTD/Rap1 interaction. Substitution
of R12Shank3 significantly weakened the NTD/Rap1 interaction (Figures S3C–S3E). Correspondingly, the K22A mutant of Shank3 NTD-ANK interacted with Rap1 with a 1:1 stoichiometry and with a $K_d$ of $\sim 3.4 \mu M$ (Figure 2E), which represents the binding affinity of the second binding site of Shank3 NTD-ANK to Rap1.

There are two possible interfaces for the second Rap1 binding site on Shank3 NTD-ANK, which correspond to the two crystallographic symmetry-equivalent positions for Rap1 molecules (Figure S3F). The buried surface area of interface 1 ($322 \AA^2$) is much smaller than that of interface 2 ($1,046 \AA^2$) (Figure S3F). In addition, both “switch I” and “switch II” regions of Rap1 are involved in interface 2 (Figures 2A, 2B, 2D, and S3F), fitting with the biochemical data showing that only active Rap1 can directly interact with Shank3. In interface 1, neither switch I nor switch II is involved in the binding (Figure S3F). Thus, the interface 2 should be the second Rap1 binding site on Shank3 NTD-ANK, and interface 1 is the crystal-packing interface.

The C-terminal extension of ANK folds back to couple with the ANK domain through a series of hydrophobic interactions (Figure S4A). Three continuous, positively charged and highly conserved Arg residues (R355, R356, and R357; Figure S2) are involved in the coupling of the C-terminal extension to NTD-ANK (Figure S4A). A stretch of C-terminal residues of ANK also directly participate in the binding to Rap1 in the second binding site (Figure 2D). This stretch of residues were not resolved in the apo-form structure of Shank3 NTD-ANK presumably due to its undefined structure (Figure S4B). We purified a C-terminal truncated Shank3 NTD-ANK (residues 1–348, termed ΔCT in Figure 2E), which is exactly the same with the construct used in a previous study (Lijia et al., 2017). The Shank3 NTD-ANK ΔCT binds to Rap1 with a 1:1 stoichiometry and with a $K_d$ of $\sim 0.85 \mu M$ (Figure 2E), indicating that the removal of part of the C-terminal extension of Shank3 NTD-ANK prevents Rap1 from binding to the second site of the tandem. The second binding site also contains two prominent pairs of salt bridges, K66Shank3-D38Rap1 and R72Shank3-E62Rap1, which are in the vicinity of the switch I and switch II regions, respectively (Figure 2D). Substitution of either K66Shank3 or R72Shank3 with Glu completely eliminated Rap1’s binding to the second site of Shank3 NTD-ANK, as either of the Shank3 mutants bound to Rap1 with a 1:1 stoichiometry and with a binding affinity comparable with the interaction between NTD and Rap1 (Figures S3B, S3G, and 2E). The residue corresponding to R72Shank3 is conserved in all Shank proteins (Figure S2), whereas the residue corresponding to K66Shank3 is only conserved in Shank2 and Shank3 (Figure S2). This analysis explains that Shank2, but not Shank1, also binds to Rap1 with a 1:2 stoichiometry (Figure 1B). Finally, a combination of the K22A and R72E mutations of Shank3 totally abolished the interaction between Shank3 and Rap1 (Figure 2E).

**HRas binds to Shank3 NTD-ANK with a Canonical Mode**

Shank3 binds to HRas with a 1:1 stoichiometry and a much lower affinity than to Rap1 (Figure 1E). We speculated that HRas only binds to the first binding site of Shank3 NTD. We were not able to obtain crystals of the Shank3 NTD-ANK/HRas complex, so we sought to introduce mutations to Shank3 NTD based on the binding mode observed in the NTD/Rap1 interaction (Figure 2C) to stabilize Shank3 NTD-ANK/HRas complexes. The A42K mutation of Shank3 NTD-ANK was designed to introduce

### Table 1. Crystallographic Data Collection and Refinement Statistics

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Numbers in parentheses represent the values for the highest-resolution shell.

$^aR_{merge} = \sum_i |I_i - <I>|/\sum_i |I_i|$, where $I_i$ is the intensity of measured reflection and $<I>$ is the mean intensity of all symmetry-related reflections.

$^bCC_{1/2}$ was defined in (Karplus and Diederichs, 2012).

$^cR_{work} = \sum_i |F_{obs}|-|F_{calc}|/\sum_i |F_{obs}|$, where $F_{obs}$ and $F_{calc}$ are observed and calculated structure factors. $W$ is working dataset of about 95% of the total unique reflections randomly chosen and used for refinement.

$^dR_{free} = \sum_i |F_{obs}| - |F_{calc}|/\sum_i |F_{obs}|$, where $T$ is a test dataset of about 5% of the total unique reflections randomly chosen and set aside before refinement.
a critical salt bridge with D33Ras, and a C41K/A42K double mutant was used to introduce two pairs of salt bridges with E31Ras and D33Ras (Figure 2C). ITC-based binding experiments showed that the A42K mutation could dramatically increase the binding affinity (Kd = 0.88 μM, Figure S5A). Further substitution of Cys41 with Lys did not enhance the interaction.
any further (Figure S5B). We were able to obtain crystals of Shank3 NTD-ANK A42K in complex with GMPPNP-bound HRas and determined the complex structure to the resolution of 3.30 Å (Table 1).

The A42K mutant of Shank3 NTD-ANK binds to active HRas with a 1:1 stoichiometry and with a canonical binding mode (Figures 3A–3C). In addition to the canonical K22 Shank3–Y40HRas and R12 Shank3–E37HRas interactions, the mutation introduced, K42 Shank3, forms an additional electrostatic interaction with D33 HRas and thus enhances the binding affinity of the complex (Figure 3C). The wild-type (WT) Shank3 should have the same binding mode albeit with a lower affinity, as substitution of K22 Shank3 with Ala also abolished the Shank3 NTD-ANK and HRas interaction (Figure 3D). Curiously, HRas contains essentially all critical residues as those in Rap1 for binding to the second site of Shank3 NTD-ANK, but HRas does not bind to the second Rap1 binding site of Shank3 NTD-ANK. We do not have an explanation for this odd observation. As expected, neither the K66E Shank3 nor the R72E Shank3 mutants of Shank3 NTD-ANK had any impact on the binding of the tandem to HRas as the canonical binding site is totally separated from the second Rap1 binding site (Figures S5C and S5D).

Shank3 Prevents SynGAP from Activating the GTPase Activity of Rap1

It has been reported that SynGAP predominantly activates Rap GTPase activity (Krapivinsky et al., 2004; Pena et al., 2008). We next investigated whether Shank3 might alter the SynGAP-mediated GTPase activation property of the Ras family enzymes. Consistent with previous reports, our in vitro GAP activity assay showed that SynGAP prominently stimulates the GTPase activity of Rap1 (Figure S6A). The structure of the Shank3 NTD-ANK in complex with GMPPNP-bound Rap1 revealed that the Shank3 NTD-ANK binding stabilizes the GTP-bound Rap1 conformation and should block SynGAP from stimulating the GTPase activity of Rap1. Indeed, addition of equimolar amounts of Shank3 NTD-ANK (e.g., 20 μM Shank3 NTD-ANK and 20 μM Rap1) near completely blocked the GTPase activity of Rap1 (Figure 4A). Addition of half-equivalent amounts of Shank3 NTD-ANK (e.g., 10 μM Shank3 and 20 μM Rap1) also greatly inhibited the GTPase activity of Rap1 (Figure 4A), because each Shank3 NTD-ANK has two Rap1 binding sites, and both can prevent SynGAP from stimulating the GTPase activity of Rap1. The K22A mutant (the canonical site 1 mutant) or the R72E mutant (the non-canonical site 2 mutant) of Shank3 NTD-ANK could still prevent SynGAP from stimulating the GTPase activity of Rap1, albeit requiring higher concentrations than the WT Shank3 NTD-ANK to reach the same level of inhibitions (Figures 4B and 4C). The K22A and R72E double mutant of Shank3 NTD-ANK is totally incapable of inhibiting SynGAP from activating Rap1 (Figure 4D), as the mutant has lost both Rap1 binding sites and has no detectable binding to Rap1 (Figure 2E). The apparent GTPase activities of the reaction mixtures are inversely proportional to the binding affinities of each form of Shank3 NTD-ANK to Rap1 (Figure 4E).

It is noted that Shank3 NTD-ANK binds to Rap1 with a moderately strong affinity (apparent $K_d \approx 0.47–0.84 \mu M$) (Figure 1C), and blocks SynGAP from binding to Rap1 (Figure S6B). On the other hand, Rap1 effectors bind to Rap1 with moderately strong or
strong affinities (e.g., $K_d \approx 0.007 \mu M$ for theRalGDS [Linnemann et al., 2002], $\sim 0.7 \mu M$ for the RIAM [Zhang et al., 2014], and $\sim 1.8 \mu M$ for the KRIT1 [Li et al., 2012]). Thus, the above GAP activity assays together with our structural analysis indicate that Shank3 can prevent SynGAP from activating its GTPase activity of Rap1 by directly competing with SynGAP in binding to the active form of Rap1. By doing so, Shank3 may be able to protect the active form of Rap1 from SynGAP-mediated inactivation before acting on its effectors.

Rap1 Can Be Incorporated into a Shank-Mediated Protein-Protein Interaction Network in PSDs

We next studied whether Rap1 can be incorporated into Shank3-organized PSD protein complexes. We were not able to prepare full-length recombinant Shank proteins likely due to their large sizes. Based on our previously reported, simplified Shank3 (Zeng et al., 2018), we further added the NTD-ANK tandem and generated an updated version of simplified Shank3 (termed NtosAM, Figure 4F). NtosAM consists of an NTD-ANK tandem,
an extended PDZ domain specifically binding to a SAPAP tail (Zeng et al., 2016), a Homer-binding sequence and a C-terminal SAM domain with M1718E mutation to improve protein solubility (Baron et al., 2006) (Figure 4F). The version of Shank3 used in the current study lacks most of the low complexity and predicted unstructured regions between these domains.

Using analytical gel filtration chromatography, we monitored the complex formation by adding three Shank3 binding partners one by one: first Rap1, then the C-terminal tail of SAPAP (termed SAPAP-CT), and finally Homer1a. The analytic gel filtration chromatographic experiments showed that the elution volumes of the mixtures were progressively shifting to smaller volumes upon mixing one (Rap1), or two (Rap1 and SAPAP-CT), or all three (Rap1, SAPAP-CT, and Homer1a) proteins with Shank3 NtoSAM (Figure 4G), indicating that all three target proteins can simultaneously bind to Shank3. SDS-PAGE analysis of the four-protein mixture showed that all binding partners were co-eluted with Shank3 NtoSAM on gel filtration chromatography (Figure 4H), demonstrating that Rap1 can be incorporated into Shank-mediated PSD protein interaction network.

**LTP Induction Promotes Shank3 Binding to Ras Superfamily Proteins in Postsynaptic Density**

We hypothesized that the interactions between Shank proteins and Ras superfamily proteins in dendritic spine might be modulated by synaptic plasticity. Under resting condition, SynGAP is highly enriched in dendritic spines to keep Ras superfamily proteins majorly in GDP form. Thus, there is little interaction between Shank and Ras superfamily proteins. LTP induction triggers dispersion of SynGAP (Araki et al., 2015), which would shift Ras superfamily proteins to the GTP-bound active form that is favorable for binding to Shank.

Thus we sought to investigate whether Shank3 could interact with HRas or Rap1 at the single spine level upon LTP induction. We monitored Shank3 and HRas or Rap1 interaction in dendritic spines of CA1 pyramidal neurons in hippocampal organotypic slice cultures using Förster resonance energy transfer-fluorescence lifetime imaging microscopy (FRET-FLIM) method (Saneyoshi et al., 2019). N-terminal enhanced GFP-tagged Shank3 and N-terminal mCherry-tagged HRas or Rap1 were co-transfected into neurons (Figure 5A). Several mutations of Shank3 identified from our biochemical experiments (the K22A and R72E double mutant) were also investigated in the FRET-FLIM experiments. We stimulated single dendritic spine with a protocol that typically induce structural LTP (Saneyoshi et al., 2019). However, overexpression of Shank3 in neurons induces spine enlargements (Sala et al., 2001) and further stimulation of synapse with glutamate uncaging do not induce additional enlargement of spines. Nevertheless, there was a dramatic increase in FRET following the LTP-inducing stimulus with glutamate uncaging in the WT Shank3- and HRas-expressing neurons that persisted at least 15 min after the stimulation, indicating that Shank3 and HRas interacted with each other persistently (Figures 5B and 5C). Neither the K22A mutant nor the K22A/R72E double mutant of Shank3 showed detectable LTP-induced binding to HRas (Figures 5B and 5C), as neither mutant is capable of binding to Ras proteins (Figure 3D). In contrast, the R72E mutant of Shank3 showed significant interaction with HRas (Figures 5B and 5C), as R72 is in the second Rap1 binding site and the R72E mutation does not affect Shank3’s binding to HRas (Figures 2 and S3H).

For Shank3 WT and Rap1, the induction of LTP also significantly triggered their interaction. Surprisingly, unlike HRas, the interaction between Shank3 and Rap1 was transient and the change of fluorescence lifetime quickly went back to the baseline (Figures 5D and 5E), suggesting different regulation mechanisms for Rap1 and HRas to dissociate from Shank3 in dendritic spines. Interestingly, neither the K22A mutant nor the R72E mutant could undergo LTP-induced binding to Rap1, indicating that both binding sites on the NTD-ANK tandem are required for Rap1 to bind to Shank3 in dendritic spines of living neurons (Figures 5D and 5E). As expected, the K22A/R72E double mutation of Shank3 showed no LTP-induced binding to Rap1 (Figures 5D and 5E).

Taken together, the interactions between Shank3 and HRas or Rap1 can be triggered by LTP induction. Although HRas and Rap1 show high amino acid sequence identity and share a similar Ras-association domain-mediated binding mode, HRas binding to Shank3 is long-lasting and Rap1 binding to Shank3 is transient. The distinct Shank3 binding kinetics for HRas and Rap1 may be correlated to different roles played by HRas and Rap1 in synaptic plasticity (Zhu et al., 2002).

**DISCUSSION**

In this study, we discovered that Rap1 and HRas, two conserved members of Ras superfamily, interact with the Shank NTD-ANK tandem with distinct binding modes. Rap1 interacts with Shank2 or Shank3 with a 2:1 stoichiometry, whereas HRas binds to Shank proteins with a 1:1 stoichiometry. Our crystal structures further revealed that Rap1, in addition to the canonical Ras-association domain (or NTD) binding site as predicted previously (Lilja et al., 2017), uses an unconventional binding site formed by both NTD and ANK of Shank3. Both Rap1 binding sites of Shank3 directly involve the specific conformations of switch I and switch II regions of the GTP-bound form of Rap1, explaining that only active (GTP bound) Ras proteins can bind to Shank.

Rap was initially identified as an antagonist of Ras (Cook et al., 1993; Kitayama et al., 1989), because Rap and HRas have high amino acid identity, and share many binding partners. Rap was then discovered to be involved in signaling pathways distinct from Ras and has its own specific effectors (Bos, 1998; Ohtsuka et al., 1996). In synapse, Ras and Rap mediate independent signaling pathways in activity-dependent synaptic plasticity (Zhang et al., 2018; Zhu et al., 2002). The distinct binding modes of Shank3 to Rap1 and HRas may contribute to their specific downstream signaling pathways.

We provided evidence that the interactions between Shank proteins and Ras superfamily proteins in dendritic spine are modulated by synaptic plasticity. Small GTPases usually are modulated by molecular switches during synaptic plasticity. LTP induction will “turn on” the molecular switches to convert small GTPases to GTP-bound form for Shank binding. Thus the interaction between Shank and Ras superfamily proteins are initiated by LTP induction. On the other hand, LTP needs a mechanism to convert short Ca2+ pulses into long-lasting...
structural and functional plasticity. Recently, the formation of reciprocally activating signaling complex between CaMKII and a small GTPase modulator, TIAM1, was reported to be responsible for maintaining the enlargement of dendritic spines during LTP (Saneyoshi et al., 2019). Here, our biochemical data suggest that Shank can compete with SynGAP for HRas/Rap1, and thus slow down GTP hydrolysis for HRas/Rap1. This will induce a sustained interaction between Shank and small GTPases, which may be a potential mechanism for storing the active form of small GTPases for the following long-lasting structural and functional LTP.

Our structural data revealed two distinct Rap1 binding sites on Shank3 with an order of magnitude differences in affinities (Kd ~0.41 μM for site 1 and Kd ~3.4 μM for site 2). Considering that Shank is one of the most abundant scaffolding protein in PSDs, highly compartmentalized and abundant Shank proteins can potentially recruit and “store” an active form of small GTPases in PSDs for its downstream signaling events. Two distinct Rap1 binding sites on Shank3 provide a potential mechanism for the fine-tune of Rap1 signaling pathway. For the Rap1 effectors with much lower binding affinities, Shank3 can function as a “barrier” to limit the weak effectors from accessing Rap1. For the Rap1 effectors with modest affinities, only part of Rap1, which binds to the second site, may be preferentially released to be accessible by the modest effectors. For the strong effectors, Rap1 bound to both sites on Shank3 would be released for binding to the strong effectors. At same time, Shank3 itself may be an effector of Rap1/HRas, but the hypothesis needs to be investigated in the future. Our FRET-FLIM data also suggest a distinct dissociation mechanism of Rap1 from the Shank scaffold in neuronal spines after LTP stimulation.

Figure 5. LTP Induction Promotes Shank3 and Ras Superfamily Proteins Interaction in Postsynaptic Density

(A) Schematic diagram showing experimental design of FRET-FLIM. N-terminal EGFP-tagged Shank3 as donor and N-terminal mCherry-tagged Rap1 or HRas as acceptor were co-transfected into hippocampal neurons. Single spine was stimulated by uncaging MNI-glutamate using a two-photon laser.

(B) Interaction between various forms of Shank3 and HRas as visualized by FRET-FLIM before and after glutamate uncaging. Warmer color hues indicate more interaction.

(C) Averaged time course of the interactions between various forms of Shank3 and HRas measured as a change of the lifetime of EGFP-Shank3 after glutamate uncaging. Data are expressed as the mean ± SEM.

(D) Interaction between various forms of Shank3 and Rap1 as visualized by FRET-FLIM before and after glutamate uncaging.

(E) Averaged time course of the interactions between various forms of Shank3 and Rap1 measured as a change of the lifetime of EGFP-Shank3 after glutamate uncaging. Data are expressed as the mean ± SEM.

Numbers of observation (n) indicated in (C and E) represent the number of spines that were imaged, and one spine was selected from one neuron for each assay. *p < 0.05, compared with a control group in which only EGFP-Shank3 was transfected using one-way ANOVA with Scheffé’s post hoc comparison. Control group presented in (C and E) are identical. Because both groups were tested at the same time, we pooled control groups together.

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STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.str.2019.11.018.

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AUTHOR CONTRIBUTIONS

Q.C., T.H., and M.Zeng performed the experiments. Q.C., T.H., M.Zeng, Y.H., and M.Zhang analyzed the data. Q.C., T.H., and M.Zhang designed the research. Q.C. and M.Zhang drafted the paper and all authors commented on the paper, and M.Zhang coordinated the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


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learning in the complex with postsynaptic density 95 and NMDA receptor. J. Neurosci. 22, 9721–9732.


STAR METHODS

KEY RESOURCES TABLE

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**Deposited Data**

Crystal structure of Shank3 NTD-ANK/Rap1 complex | This paper | PDB: 6KYK |
Crystal structure of Shank3 NTD-ANK/HRas complex | This paper | PDB: 6KYH |

**Experimental Models: Organisms/Strains**

| Rat: SD. | SLC | N/A |
| Escherichia coli: BL21-CodonPlus(DE3)-RIL | Agilent | Cat#230245 |

**Recombinant DNA**

| Plasmid: 32mTEV-Shank1-NTD-ANK (aa 73-440) | This paper | N/A |
| Plasmid: 32m3c-Shank2-NTD-ANK (aa 49-423) | This paper | N/A |
| Plasmid: 32m3c-Shank3-NTD-ANK (aa 1-376) | This paper | N/A |
| Plasmid: 32m3c-Shank3-NTD-ANK (aa 1-368) | This paper | N/A |
| Plasmid: 32m3c-Shank3-NTD-ANK (aa 1-362) | This paper | N/A |
| Plasmid: 32m3c-Shank3-NTD-ANK-A42K (aa 1-362) | This paper | N/A |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mingjie Zhang (mzhang@ust.hk). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All experimental protocols were approved by the Kyoto University Committee for Animal Care guidelines.
Animals
Rats from Sprague-Dawley strain (purchased Japan SLC, both males and females) were used for hippocampal slice culture and dissociated neuronal cultures.

Bacterial Strain
*Escherichia coli* BL21-CodonPlus(DE3)-RIL cells (Agilent) were used in this study for the production of recombinant proteins. Cells were cultured in LB medium supplemented with necessary antibiotics.

METHODS DETAILS

Constructs
The cDNA encoding mouse Shank1 (Genbank: NM_031751.3) NTD-ANK fragment (residues 73-440), HRas (Genbank: AY373386.1, residues 1-167) and SynGAP (Uniprot: J3QQ18) were PCR-amplified from a mouse cDNA library. The human Shank2 NTD-ANK fragment (residues: 49-423) was PCR-amplified from the full-length Shank2 gene (Genbank: NM_012309) purchased from YouBio Co. Various fragments of mouse Shank3 were PCR-amplified from the full-length Shank3 gene (Genbank: AB231013), provided Prof. Guoping Feng at Massachusetts Institute of Technology. Shank3 NTD-ANK fragment (residues 1-376, 533-665, 1294-1323, 1400-1426 and 1654-1730). Various fragments of mouse Shank3 were PCR-amplified from the full-length Shank3 gene (Genbank: AB231013), provided Prof. Guoping Feng at Massachusetts Institute of Technology. SynGAP C2-GAP fragment (residues 244-740) was PCR-amplified from the full-length rat SynGAP (Uniprot: Q9QUH6), provided by Prof. Richard Huganir at Johns Hopkins University School of Medicine. Human RRas (residues 22-201), Rap1b (residues 1-167), Rap2a (residues 1-167), p120GAP (residues 714-1047) and mouse MRAs (residues 10-178) genes were provided by Prof. Xuewu Zhang in UT Southwestern Medical Center.

Protein Expression and Purification
All proteins were expressed in *Escherichia coli* BL21-CodonPlus(DE3)-RIL cells (Agilent Technologies) in LB medium at 16°C. Reombinant proteins were firstly purified using Ni²⁺-NTA resin (GE Healthcare). Then Superdex 200 26/60 or Superdex 75 26/60 gel filtration columns were used for further purifications. The affinity tag of each protein was cleaved by HRV-3C protease or TEV protease at 4°C overnight and removed by another step of gel filtration chromatography in the buffer containing 50 mM Tris (pH 8.0), 50 mM NaCl, 50 mM ammonium tartrate, 2 mM DTT and 2 mM MgCl₂.

The active (GMPNNP-bound) form of small GTPases was generated according to the previous reported protocol (John et al., 1990). Briefly, each purified small GTPase was exchanged into the buffer containing 50 mM Tris (pH 8.0), 100 mM NaCl, 200 mM ammonium sulfate, 2 mM DTT, then concentrated to 100 μM. GMPNP and alkaline phosphatase (NEB) were added to reach final concentrations of 500 μM and 2 units/mg GTPase, respectively. After incubated at 4°C overnight, the protein was desalted into the buffer containing 50 mM Tris (pH 8.0), 50 mM NaCl, 50 mM ammonium tartrate, 2 mM DTT and 2 mM MgCl₂. The GDP-bound small GTPases was prepared by incubating purified small GTPases with corresponding GAP protein (SynGAP C2-GAP for Rap1 and p120GAP for the rest of small GTPases) for 24 h at 4°C in the buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM DTT and 2 mM MgCl₂. Then small GTPases were further purified by a step of gel filtration chromatography.

Protein Crystallization and Structure Determination
All crystals were obtained by hanging drop vapor-diffusion method at 16°C. Shank3 NTD-ANK (residues 1-368) and Rap1b (residues 1-167) were mixed at a molar ratio of 1:2 with the concentration of 15 mg/mL for crystallization. Crystals of Shank3 and Rap1 complex were grown in the condition containing 0.1 M bicine (pH8.5), 2 mM DTT, then concentrated to 100 μM. GMPNP and alkaline phosphatase (NEB) were added to reach final concentrations of 500 μM and 2 units/mg GTPase, respectively. After incubated at 4°C overnight, the protein was desalted into the buffer containing 50 mM Tris (pH 8.0), 50 mM NaCl, 50 mM ammonium tartrate, 2 mM DTT and 2 mM MgCl₂. The GDP-bound small GTPases was prepared by incubating purified small GTPases with corresponding GAP protein (SynGAP C2-GAP for Rap1 and p120GAP for the rest of small GTPases) for 24 h at 4°C in the buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 2 mM DTT and 2 mM MgCl₂. Then small GTPases were further purified by a step of gel filtration chromatography.

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molecules could be found in an asymmetric unit. Manual model building and refinement were carried out iteratively using Coot (Emsley et al., 2010) and Refmac5 (Murshudov et al., 2011). High resolution crystal structures of Shank3 NTD-ANK, Rap1b and HRas were used as references to generate external restraints during refinement. The final models were validated by MolProbity (Chen et al., 2010) and statistics were summarized in Table 1. The figures were produced using PyMOL (http://www.pymol.org/).

Isothermal Titration Calorimetry (ITC) Assay
ITC experiments were carried out using a VP-ITC calorimeter (Malvern) at 25°C. All proteins used in ITC experiments were in the buffer containing 50 mM Tris (pH 8.0), 50 mM NaCl, 50 mM ammonium tartrate, 2 mM DTT and 2 mM MgCl₂. Each titration point was performed by injecting a 10 μl aliquot of one protein in the syringe into its binding protein in the cell at a time interval of 120 s to ensure that the titration peak returned to the baseline. Titration data were fitted with the one-site binding model using Origin 7.0 to get the apparent Kₐ values.

GAP Activity Assay
The EnzChek phosphate assay kit (E6646, ThermoFisher) was employed to monitor the GAP activity. 20 μM small GTPases (including HRas, RRas, MRas, Rap1, Rap2) with 5 μM SynGAP C2-GAP were added into the reaction solution containing 50 mM Tris (pH8.0), 100 mM NaCl, 2 mM DTT, 5mM EDTA, 150 μM GTP, 1 U/ml purine nucleoside phosphorylase (PNP) and 200 μM 2-amino-6-mercapto-7-methylpurine riboside (MESG) to study the specificity of SynGAP. Then 20 μM Rap1 with or without 5 μM SynGAP-C2-GAP was added into the same condition with 10 μM or 20 μM Shank3 NTD-ANK mutants to study the influence of Shank3 on the GAP activity of SynGAP. The absorbance at 360 nm was measured every 5 s in a 1 cm path-length cuvette at 25°C for 40 min using a UV spectrophotometer (UV-1800, Shimadzu). The data were fitted with one phase association equation with shared plateau using GraphPad Prism to derive the Kₐ values.

Glutamate Uncaging and Fluorescent Lifetime Imaging
Glutamate uncaging and FRET-FLIM observation using hippocampal slice was described previously (Bosch et al., 2014; Saneyoshi et al., 2019). Briefly, 5-6 DIV hippocampal organotypic slice cultures were transfected by biolistic method (Gene-Gun, Bio-Rad, CA, USA) with GFP-Shank3, mCherry-Rap1 or mCherry-HRas and DsRed plasmids at a 2:3:1 ratio. At 10-12 DIV, imaging was carried out in Mg²⁺-free artificial cerebrospinal fluid (ACFS) containing 4 mM CaCl₂, 1 μM tetrodotoxin, 50 μM picrotoxin, and 2.5 mM MNI-caged-L-glutamate aerated with 95% O₂ and 5% CO₂ using two-photon microscope (FV1000-MPE, Olympus, Tokyo, Japan) and Ti-sapphire lasers (Spectra-Physics, CA, USA). Glutamate uncaging was performed on the dendritic spines at primary or secondary dendrites from the distal part of the main apical dendrite of CA1 pyramidal neurons with 2 ms 2-photon laser pulses (720 nm at 5 mW under objective lens) repeated at 0.5 Hz for 60 sec. Fluorescent lifetime of GFP-Shank3 was measured by time-correlated photon-counting technology (SPC-830, Becker and Hickl, Berlin, Germany; H7422P-40, Hamamatsu Photonics, Hamamatsu, Japan) at 910 nm excitation. Emission light was filtered with a 680 nm short-pass and 510/70 nm band-pass filters. The lifetime images were analyzed using SPC Image (Becker and Hickl, Berlin, Germany) and a custom written macro in Igor-Pro (Wavemetrics, OR, USA). Averaged fluorescence lifetime in the spine head was calculated and presented as the difference from baseline.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical parameters including the definitions and exact values of n are reported in the figures and corresponding figure legends. The statistical significance of the results was assessed using one-way ANOVA with Scheffe’s post hoc comparison. P < 0.05 was considered significant. Statistical analysis was performed in Real Statistics Resource Pack for Excel (Microsoft).

DATA AND CODE AVAILABILITY
The atomic coordinates of Shank3 NTD-ANK/Rap1 complex and Shank3 NTD-ANK/HRas complex have been deposited to the Protein Data Bank (PDB) with the accession numbers of 6KYK and 6KYH, respectively.