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Abstract

SynGAP, encoded by *SYNGAP1*, is a Ras/Rap GTPase activator specifically expressed in the nervous systems. SynGAP is one of the most abundant proteins in the postsynaptic densities (PSDs) of excitatory synapses and acts as a critical synaptic activity brake by tuning down synaptic GTPase activities. Mutations of *SYNGAP1* have been frequently linked to brain disorders including intellectual disability, autisms, and seizure. SynGAP has been shown to undergo fast dispersions from synapses in response to stimulations, a strategy that neurons use to control the specific activities of the enzyme within the tiny, semi-open compartments in dendritic spines. However, the mechanism governing the activity-dependent synaptic localization modulations of SynGAP is poorly understood. It has been shown recently that SynGAP α1, via specifically binding to PSD-95, can undergo liquid-liquid phase separation forming membraneless, condensed protein-rich sub-compartments. This phase transition-mediated, PSD-95-dependent synaptic enrichment of SynGAP α1 not only suggests a dynamic anchoring mechanism of the protein within the PSD, but also implies a new model for the PSD formation in living neurons.

Small G proteins regulate many aspects of cellular processes including cell adhesion and migration, growth and differentiation, vesicle trafficking and fusion by circling between their GTP-bound active forms and GDP-bound inactive forms. A wide variety of external and internal stimuli can activate small G proteins via converting GDP-bound form proteins to GTP-bound forms often aided by guanosine nucleotide exchange factors (GEFs). Conversely, GTPase activating proteins (GAPs) facilitate the reverse process via accelerating the GTP hydrolysis activity of the enzymes and converting them into inactive GDP-bound forms. Proper balance of the two opposite processes is of vital importance for physiological functions of small GTPase-mediated signaling events. Unlike many other enzymes that often contain intricate regulatory elements within their primary sequences, small G proteins contain minimal extra amino acid sequences other than the catalytic domains. The intricate enzymatic activities of small G proteins are, instead, largely regulated by a diverse collection of GEFs and GAPs, most of which are large proteins containing many regulatory elements in addition to catalytic GTPase exchanging or activating domains (Bos et al., 2007; Cherfils and Zeghouf, 2013). As such, proper understanding of a GTPase signaling requires integrated studies of its specific GTPase/GEF/GAP trio.

Neurons are extremely polarized and with massive peripheral processes (in terms of volumes, physical lengths, and excessive branching of axons and dendrites). Additionally, physiological functions of neurons require the physical structures of dendrites and axons as well as molecular assemblies within these processes to be dynamically regulated in a very broad time span (from seconds to hours and even longer) both in developing and mature neurons. Therefore, small GTPase signaling in the nervous systems perhaps requires even more elaborated spatial and temporal regulations when compared to GTPases in cells from other tissues. Consistent with this reasoning, neuronal processes and their sub-compartments (e.g. synapses) are often highly enriched in small GTPases and various GEFs and GAPs (Chua, 2014; Sheng and Hoogenraad, 2007). Mutations of many GEFs and GAPs are known to cause different forms of brain diseases ranging from brain mal-formations to psychiatric disorders in humans (Berryer et al., 2013; Hamdan et al., 2009; Shoubridge et al., 2010).

This brief review focuses on a brain-specific GAP protein called SynGAP (encoded by *SYNGAP1*) which is a major component of the excitatory postsynaptic density (PSD) protein

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assemblies. Mutations of *SYNGAP1* are frequently found in patients suffering brain disorders including intellectual disability (ID), autism spectrum disorders (ASDs), and epilepsy (Berryer et al., 2013; Carvill et al., 2013; Hamdan et al., 2011; Hamdan et al., 2009; Mignot et al., 2016; Parker et al., 2015; Rauch et al., 2012; Redin et al., 2014; Vissers et al., 2010). We first briefly summarize biochemical properties and cellular functions of SynGAP characterized to date. We then discuss a potentially novel spatial localization and concentration mechanism of SynGAP in synapses mediated by a physical process known as liquid-liquid phase separation (LLPS) that was discovered recently. Structural analysis indicates that some of the *SYNGAP1* mutations identified in psychiatric disorder patients do not directly alter the GAP activity of SynGAP. Instead, these mutations may affect its PSD localization via mutation-induced impairment of PSD-95 binding or mutation-induced SynGAP mRNA decay resulting loss of one of the encoding gene.

Biochemical features of SynGAP

SynGAP is a brain specific GAP with no detectable expressions in other tissues tested. In neuron, SynGAP predominantly localizes in the PSD regions of excitatory synapses (Chen et al., 1998; Kim et al., 1998). There are evidences showing that SynGAP is also expressed in inhibitory synapses (Moon et al., 2008) and even in growth cones of axons (Tomoda et al., 2004). Quantitative proteomic studies of purified PSDs revealed that SynGAP, as with its binding partner PSD-95, is among the most abundant PSD components that can reach a concentration of ~100 μM (calculated by detected copy numbers in molar terms and an average PSD volume) (Sheng and Hoogenraad, 2007). Its extremely high concentration suggests that, in addition to function as a GTPase activator, SynGAP may also function as a scaffold in PSDs.

The full-length SynGAP is ~140 kDa in its molecular mass and contains a PH (Pleckstrin homology) domain, a C2 domain and a GAP domain at its N-terminal half (Fig. 1A). It was originally identified as a RasGAP because its GAP domain shows high sequence similarities to canonical RasGAPs such as p120GAP (Chen et al., 1998). However, subsequent studies reported that SynGAP has higher catalytic activities towards Rap (another small G protein homologous to Ras but with different functions) than Ras (Krapivinsky et al., 2004; Pena et al., 2008). The RapGAP activity of SynGAP requires the direct coupling between the C2 and GAP domains, as

the isolated GAP domain has little detectable GAP activity towards Rap (Pena et al., 2008). In line with this study, we also confirmed that the C2 domain is essential for SynGAP's RapGAP activity towards Rap1B using highly purified recombinant proteins (Fig. 1B). RalGDS specifically recruits GTP-bound Rap. Using GST-RalGDS-mediated binding to Rap-GTP as a readout, we demonstrate that the C2-GAP tandem, instead of the isolated GAP domain, catalyzes GTP hydrolysis of Rap1B-GTP. However, no obvious Rap2A GAP activity could be detected (Fig. 1B). We also assayed Ras-GTP hydrolysis by the isolated SynGAP GAP domain using a spectrophotometry-based GAP assay (Wang et al., 2012), and failed to detect obvious RasGAP activity using both M-Ras-GTP and R-Ras-GTP as substrates. In our controls, p120GAP can efficiently catalyze the GTP hydrolysis of both M-Ras-GTP and R-Ras-GTP (data not shown). Interestingly, a recent study indicated that SynGAP phosphorylation by different protein kinases can differentially modulate GAP activities of SynGAP towards Rap and Ras (Walkup et al., 2015). SynGAP phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) preferentially promotes its RapGAP activity; cyclin-dependent kinase 5 (Cdk5)-mediated phosphorylation instead only stimulated RasGAP activity of SynGAP (Walkup et al., 2015). This study provides a nice example showing that, in addition to protein-protein or protein-lipid interactions mediated by the well-defined domains in GAPs (and GEFs as well), post-translational modifications can also exert different regulatory roles on the activities of the enzymes.

After the GAP domain, there is a long flexible region without defined domains or structures and followed by a coiled-coil domain near its C-terminus (Fig. 1A). Recently, we reported the atomic structure of the SynGAP coiled-coil domain, showing that it forms a parallel trimeric coiled-coil structure (Zeng et al., 2016). The coiled-coil-mediated trimer formation is critical for concentrating SynGAP at PSDs by efficiently and specifically binding to PSD-95. Additionally, the coiled-coil domain may also play a role for forming SynGAP hetero-trimers, as all isoforms of SynGAP contain the identical coiled-coil sequence.

SynGAP is encoded by a single gene called *SYNGAP1*. *SYNGAP1* is a complex gene that gives rise to multiple SynGAP isoforms via alternative promoter usage and alternative splicing (McMahon et al., 2012). Due to alternative promoter usage, SynGAP isoforms A, B and C are with distinct N-termini, in which the isoform C does not contain a PH domain. Alternative

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splicing generates additional SynGAP isoforms with variations in their C-termini. Among all isoforms, SynGAP α1 is abundant and also most studied. SynGAP α1 contains a PDZ binding motif (PBM, with "-QTRV" sequence) at its C-terminus (Fig. 1A), and this PBM is responsible for binding to PDZ domain-containing scaffold proteins in excitatory PSD such as PSD-95. Earlier studies suggested that SynGAP α1 binds to all three PDZ domains of PSD-95 as well as a few other PDZ proteins (Kim et al., 1998; Krapivinsky et al., 2004). By carefully comparing quantitative binding affinities using purified recombinant proteins, we have demonstrated that SynGAP α1 binds to PDZ3 of PSD-95 with significantly higher affinity than to PDZ1 or PDZ2. The specific interaction between SynGAP α1 and PSD-95 PDZ3 originates from a second target binding site formed by the C-terminal α-helix extension and the □B-, □C-strands of PDZ3 in addition to the canonical target recognition groove of the PDZ domain. This new binding site accommodates a stretch of conserved hydrophobic residues at the upstream of the canonical SynGAP α1 PBM (Zeng et al., 2016). Additionally, the formation of the PDZ3-SH3-GK supramodule further enhances the interaction between PSD-95 PDZ3 with SynGAP α1 by another 10-fold or more. As such, the binding of SynGAP α1 to PSD-95 PDZ3 is several hundred times stronger than to PSD-95 PDZ1 or PDZ2 or to a few other reported PDZ domains (Zeng et al., 2016). Differing from α1, SynGAP α2 isoform does not contain a C-terminal PBM. Nonetheless, both SynGAP α1 and α2 have been shown to localize predominantly in PSDs of excitatory synapses by electron microscopy and mass spectrometry studies (McMahon et al., 2012; Yang et al., 2013). As mentioned above, SynGAP α2 may localize to PSDs via forming hetero-trimer with the α1 isoform via the coiled-coil domain.

Cellular functions of SynGAP

Complete elimination of *SYNGAP1* in mice is lethal at early post-natal stages (Kim et al., 2003; Komiyama et al., 2002), indicating that SynGAP plays critical roles for proper neuronal development. Heterozygous *SYNGAP1*^{+/-} mice are viable but display obvious long-term potentiation (LTP) impairments as well as social and cognitive defects (Clement et al., 2012; Guo et al., 2009; Kim et al., 2003; Komiyama et al., 2002; Ozkan et al., 2014). Restored expression of *SYNGAP1* in young mutant, but not adult, mice can rescue social and cognitive defects caused by *SYNGAP1* haploinsufficiency, supporting the critical roles of SynGAP for brain development (Aceti et al., 2015; Clement et al., 2012). It is estimated that *SYNGAP1*

mutation is accounted for 0.7%-1% of sporadic ID cases (Mignot et al., 2016) and up to 1% of epileptic encephalopathies in humans (Carvill et al., 2013). Most of *SYNGAP1* mutations lead to loss of functions of one copy of the gene, therefore most of the clinical phenotypes observed in patients are likely resulted from the loss of half of SynGAP due to haploinsufficiency. It is noted that, in addition to social and cognitive defects, patients with *SYNGAP1* mutations can also have other symptoms including mild microcephaly, growth retardation, constipation, hirsutism, hypotonia, and limb problems (Berryer et al., 2013; Mignot et al., 2016; Parker et al., 2015; Redin et al., 2014) , suggesting that SynGAP also plays functional roles beyond synaptic development and plasticity. In the following, we limit our discussion on the mechanistic roles of SynGAP in excitatory synapses.

SynGAP acts as a synaptic activity "brake" in excitatory synapses by tuning down local Rap/Ras activities and inhibiting AMPA receptor (AMPAR) incorporation into synaptic membranes. Overexpression of SynGAP α1 in neuronal culture significantly reduces AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) and results in a decreased AMPAR surface expression (McMahon et al., 2012; Rumbaugh et al., 2006). Both its GAP activity and synaptic localization signal are necessary for SynGAP's function in regulating synaptic transmissions. Consistent with its inhibitory role in synaptic AMPAR expression, synaptic transmission is elevated in neurons derived from *SYNGAP1*^{-/-} mice (Rumbaugh et al., 2006; Vazquez et al., 2004).

It appears that modulating synaptic localization of SynGAP (and presumably the subsequent Rap or Ras activities in synapse) is a powerful means in regulating synaptic activities. Immunogold staining coupled with electron microscopic imaging as well as living imaging by fluorescence microscopy of rat hippocampal neuron cultures revealed that both SynGAP α1 and α2 are enriched at the PSD core under basal conditions, and the enzymes can move out of the PSD after NMDA receptor (NMDAR) activation (Araki et al., 2015; Walkup Iv et al., 2016; Yang et al., 2013). Mechanistically, CaMKII-mediated phosphorylation on SynGAP C-terminus may weaken its binding to PSD-95, and thus facilitate its dispersion from dendritic spines (Araki et al., 2015; Walkup Iv et al., 2016; Yang et al., 2013). SynGAP dispersion could have two possible effects on synapse activities. The most direct outcome is to release the negative "brake" from the synapse via overall GAP activity decrease in PSDs (i.e. via modulating the enzyme

activity of SynGAP at PSDs) (Araki et al., 2015). Alternatively, activity-dependent dispersion of SynGAP out of the PSD core may free its occupied PDZ "slots" on PSD-95 or other Dlgs for glutamate receptors and adhesion molecules (i.e. via regulating the scaffolding role of SynGAP at PSDs) (Walkup Iv et al., 2016). Whatever the mechanisms are, the outcomes following SynGAP dispersions from synapses are enlarged spine volumes, more synaptic AMPAR insertions, and higher synaptic activities (Araki et al., 2015). It is noted that the two mechanisms are not mutually exclusive, instead may act synergistically to modulate synaptic activities.

Phase transition of SynGAP and PSD-95

PSDs of excitatory synapses are one of the most complicated protein-rich assemblies in neurons. Thousands of proteins are enriched in this tiny semi-open compartment with plasma membrane on one side and dendritic spine cytoplasm on the other, forming detergent insoluble protein assemblies responsible for synaptic signal processing and storages. Yet, numerous studies have demonstrated that proteins in PSDs can undergo activity-dependent exchanges with those in cytoplasm. The disc-shaped, protein-rich assembly of PSD was discovered more than 60 years ago (Palay, 1956). However, how such semi-open PSD compartments can autonomously form beneath the synaptic plasma membranes remains largely unknown. The activity-dependent SynGAP dispersions from PSDs offer a good opportunity for studying the mechanisms governing the PSD enrichment as well as dynamic exchanges of SynGAP between PSD and cytoplasm.

We recently discovered that the SynGAP (the SynGAP α1 C-terminal CC-PBM fragment to be precise) and PSD-95 (the PDZ3-SH3-GK tandem to be exact) complex mixture can undergo a protein concentration-dependent liquid-liquid phase separation, forming a dynamic, membrane-lacking protein-rich phase both in vitro and in living cells (Zeng et al., 2016). Both components are highly enriched in the condensed liquid phase comparing to the surrounding aqueous solutions in vitro or in cytoplasm of living cells (i.e. a large concentration gradient of the SynGAP/PSD-95 complex is maintained between the protein-rich condensed phase and the low concentration aqueous phase). Importantly, proteins in the two phases constantly undergo rapid exchange. The phase transition of the SynGAP/PSD-95 complex requires the multivalent interaction between SynGAP and PSD-95. Both formation of the SynGAP trimer and weak,

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inter-molecular PDZ3-SH3-GK-mediated multimerization capacity of PSD-95 are required for the SynGAP/PSD-95 complex to undergo phase transition. Additionally, specific SynGAP CC-PBM and PSD-95 PDZ3-SH3-GK interaction is also essential for phase transition to occur in living cells. Using hippocampal neuronal cultures as the model, we have demonstrated that the SynGAP α1 mutants (e.g. converting the SynGAP trimer into a monomer or weakening the SynGAP/PSD-95 interaction) that are defective in the SynGAP/PSD-95 phase transitions in vitro have been observed to be less well integrated into PSDs as well as to undergo more obvious chemical LTP-induced dispersions. Such mutant SynGAP expressing synapses contain higher amount of AMPA receptors and can be activated by low amplitude stimulations that are not sufficient to activate the wild type SynGAP containing synapses. These observations are consistent with the role of SynGAP as a synaptic activity "brake" in neurons, and mutations of SynGAP can lead to over-activation of synapses (Araki et al., 2015; Berryer et al., 2013; Clement et al., 2012).

The phase transition of the SynGAP/PSD-95 complex is highly protein concentration-dependent. Only when protein concentrations pass certain thresholds can phase transition occur. Regulating local SynGAP α1 concentration through protein synthesis/degradation or by isoform replacements might be other ways to modulate phase transitions in a longer timescale. This hypothesis coincides with the observation that overexpression of SynGAP α1 and α2 isoforms in hippocampal neurons have opposing effects on synaptic transmissions. Overexpression of PSD-95 binding SynGAP α1 isoform weakens synaptic strength, possibly by promoting more SynGAP α1 in the protein-rich PSD phase (McMahon et al., 2012; Rumbaugh et al., 2006). Oppositely, overexpression PBM-lacking SynGAP α2 leads to elevated mean mEPSCs amplitudes, presumably by lowering its PSD localization due to lowering the concentration of the effective PSD-95 binding population of SynGAP (McMahon et al., 2012).

The protein concentration dependence of the SynGAP/PSD-95 complex phase transition may also shed light on a long-lasting puzzle to us as to why SynGAP as an enzyme exists at such a high concentration in PSDs and why mammalian brain is so sensitive to the dosage of SynGAP. In rodents, SynGAP expression reaches its peak level around 14 days after birth, a period coinciding with the time window of substantial synaptogenesis (Clement et al., 2012). Loss of both copies of *SYNGAP1* in mice (*SYNGAP1$^{-/-}$*) leads to postnatal death, indicating that the gene

is indispensable for normal brain development. Loss of 50% of SynGAP in mice (i.e. *SYNGAP1*^{+/-}) is sufficient to cause premature dendritic spine maturation, impaired LTP, exaggerated signal propagation and shifts of the balance of neural circuits towards hyper-excitable states (Clement et al., 2012; Kim et al., 2003; Komiyama et al., 2002; Vazquez et al., 2004). Altered dendritic spine development and neuronal circuits function are believed to be closely related to cognitive dysfunctions and behavioral disorders (Volk et al., 2015). In agreement with this theory, lost one copy of *SYNGAP1* during early development leads to impaired cognition and reduced seizure threshold in mice (Aceti et al., 2015; Berryer et al., 2016; Clement et al., 2012; Ozkan et al., 2014). Theoretically, the enzymatic activity decrease caused by a 50% reduction of SynGAP in *SYNGAP1*^{+/-} animals should be easily buffered by the remaining high concentrations of the enzyme and by longer reaction time. However, under the scenario of phase transition-mediated incorporation of SynGAP into the PSD via binding to PSD-95, the concentration decrease of SynGAP in the PSD caused by loss of one copy of *SYNGAP1* may be much more severe. It is possible that a 50% reduction of total enzyme expression in *SYNGAP1*^{+/-} animals may lower the final concentration of SynGAP in the PSD by many folds due to the sharp concentration-dependent property of the SynGAP/PSD-95 complex phase transition (Zeng et al., 2016).

The liquid-liquid phase separation of the SynGAP/PSD-95 complex in aqueous solution is reminiscent of the dynamic PSD assembly in living neurons in the following ways: protein assemblies in the PSD are highly concentrated compared to the same proteins in the cytoplasm of dendritic spines; proteins in the PSD and in dendritic spines can coexist without needing of physical membrane-like structure for separations; proteins within and outside the PSD can undergo dynamic exchanges in response to neuronal activity changes; PSD protein assemblies can undergo size changes in responding to neuronal activity. Therefore, there is a possibility that formation and dynamic assembly/disassembly of PSDs can also occur via the liquid-liquid phase separation. This is certainly a bold hypothesis that requires further experimental tests. For example, the excitatory PSDs contain many abundant scaffold proteins and enzymes including PSD-95 (and other Dlgs), SAPAPs, Shank, Homer, CaMKII, and among others. These proteins can form a highly complicated protein-protein interaction network with many multivalent interactions. It remains to be tested whether these protein complexes can undergo liquid-liquid

phase separation. If these protein complexes can also undergo phase transition, it will be important to characterize both the thermodynamic (e.g. the threshold concentrations, stoichiometric compositions, stabilities of the assemblies, etc.) and kinetic (e.g. the assembly and exchange rates of the components, possible enzyme-mediated regulations of the assemblies) properties of the phase transitions and to investigate functional implications of forming such self-organized PSD assemblies not delimited by membrane bilayers.

SYNGAP1 mutations in patients with neurodevelopmental disorders

Human genetic studies in the past have identified many mutations of *SYNGAP1* in ASD and ID patients (Berryer et al., 2013; Carvill et al., 2013; Hamdan et al., 2011; Hamdan et al., 2009; Mignot et al., 2016; Parker et al., 2015; Rauch et al., 2012; Redin et al., 2014; Vissers et al., 2010). We have curated these mutations (Fig. 2A) and propose to divide these mutations into two major categories: one with mutations occur within or before the catalytic C2-GAP domain tandem, and these mutations are expected to impair the GAP activity of SynGAP; the mutations in the second category occur in the C-terminal half of the protein, and interestingly all these identified mutations are truncation mutations caused either by direct gain of stop codons or introduction of premature stop codons due to frameshifts.

The resulting SynGAP mutant proteins in the second category are defective in PSD-95 binding due to the loss of the coiled-coil domain and/or the extreme C-terminal PDZ binding motif, although these mutations may not directly impair the catalytic activity of SynGAP (Fig. 2A). Since the PSD-95 binding-mediated PSD localization is critical for SynGAP's functions in synapses, the SynGAP mutations in the second category are expected to have major impacts on brains functions in these patients if the mutant transcripts are stably transcribed. It should be noted that mutations leading to premature truncations of *SYNGAP1* can also cause nonsense-mediated RNA decay. Under such situations, the mutation will be more or less equivalent to the loss of one copy of *SYNGAP1* and the phenotype can be attributed to the haploinsufficiency of the gene. The exact outcomes of the truncation mutations are difficult to predict, and should be experimentally investigated. For example, it was demonstrated recently that two different mutations in the middle region of Shank3 have very different impacts on the mRNA stability and consequent mutant Shank3 protein levels (Zhou et al., 2016). One leads to very unstable mRNA

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transcripts and thus very little mutant protein expression, and the other with stable expression of truncated protein likely acting as a dominant negative. As the results, the two Shank3 mutations display distinct phenotypes in patients (Durand et al., 2007; Gauthier et al., 2010) and in mice models (Zhou et al., 2016).

For the first category, we selected three missense mutations occurring in the SynGAP C2-GAP tandem (L327P, W362R, and P562L; Fig. 2A) to test whether these mutations may alter the GAP activity of the enzyme. L327P localizes in the C2 domain and was found in a pair of monozygotic twins both with severe ID, autism and seizure (Parker et al., 2015). W362R is another mutation in the C2 domain and was identified in an ID patient with both autism and seizure (Berryer et al., 2013). P562L is in the GAP domain and was identified in an ID patient with autism but no seizure (Berryer et al., 2013). We purified the wild type SynGAP C2-GAP or its mutants each bearing one of the above three mutations, and compared their GAP activities towards Rap1B using a spectrophotometry-based GAP assay (Fig. 2B). Rap1B without addition of SynGAP shows very low intrinsic GAP activity. Comparing to the WT C2-GAP, all three mutants show decreased catalytic activities. L327P and W362R display moderate decreases in their GAP activities, whereas P562L has a more obvious decrease of the GAP activity of the enzyme. The different degrees of GAP activity changes caused by the three mutants observed in Fig. 2B was also confirmed by a GST-RalGDS pull-down assay (data not shown). The results shown in Fig. 2 suggest that many forms of mutations throughout *SYNGAP1,* either due to direct alterations of its GAP activities or because of disruptions of its synaptic localizations, can all lead to brain function impairments.

Methods

Plasmids, protein expression and purification

The full length rat SynGAP construct is a gift from Dr. Richard L. Huganir at Johns Hopkins University School of Medicine, Baltimore, MD, USA (reference sequence UniProt: Q9QUH6, 1308aa). The fragments encoding C2-GAP (aa 244E-740R) and GAP (aa 408T-740R) were PCR amplified and cloned into an in-house modified pETm3c vector with N-terminal His_6 tag. Note that, the amplified C2-GAP sequence does not contain the residues corresponding to 375G-389S of the reference sequence. Mutations of SynGAP C2-GAP were made by standard

PCR method and confirmed by DNA sequencing. Constructs encoding His$_6$-tagged GAP domain of p120GAP and small GTPases including Rap1B, Rap2A, M-Ras and R-Ras as well as GST-tagged RalGDS are gifts from Dr. Xuewu Zhang at University of Texas Southwestern Medical Center, Dallas, TX, USA (Wang et al., 2012). Recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) cells in LB medium at 16°C and purified using Ni^{2+}-NTA agarose (for His$_6$-tagged proteins) or glutathione sepharose (for GST-tagged protein) affinity chromatography followed by size-exclusion chromatography with a column buffer containing 50mM Tris pH7.8, 200mM NaCl, 1mM EDTA, 1mM DTT (for C2-GAP) or 50mM Tris pH7.8, 100mM NaCl, 1mM EDTA, 1mM DTT (for GAP proteins or GST-tagged proteins). MgCl$_2$ at a concentration of 4mM was kept in all purification buffers for small GTPases.

RalGDS pull-down assay

GTP-bound Rap was prepared by incubating purified Rap with 50-fold excess fresh GTP in the presence of 5mM EDTA on ice for 1 hour. The reaction was terminated by adding 10mM MgCl$_2$ and excess nucleotide was removed by a size-exclusion column. GTP-bound Rap (7.5µM) was initially incubated with 15µM SynGAP GAP or C2-GAP at room temperature for 30min. Pull down assay was performed by adding GST or GST-RalGDS (10µM) and incubating in 4℃ for 30min, and followed by adding glutathione-sepharose beads for another 30min incubation in 4℃. After extensive wash by PBS, Rap-GTP was eluted and detected by SDS-PAGE with Coomassie blue staining.

Spectrophotometry-based GAP activity assay

Rap1B (40µM) with or without C2-GAP (WT or mutants, 1µM) were added to the reaction system containing 50mM Tris pH7.8, 120mM NaCl, 4mM EDTA, 100µM GTP, 1 U purine nucleoside phosphorylase (PNP), and 200µM 2-amino-6-mercapto-7-methylpurine riboside (MESG). PNP and MESG were from EnzChek Phosphate Assay Kit (E6646, ThermoFisher). Directly after incubation, the absorbance at 360nm was monitored continuously in a 1-cm path-length cuvette at room temperature and each GAP-catalyzed reaction was monitored for 40 min.

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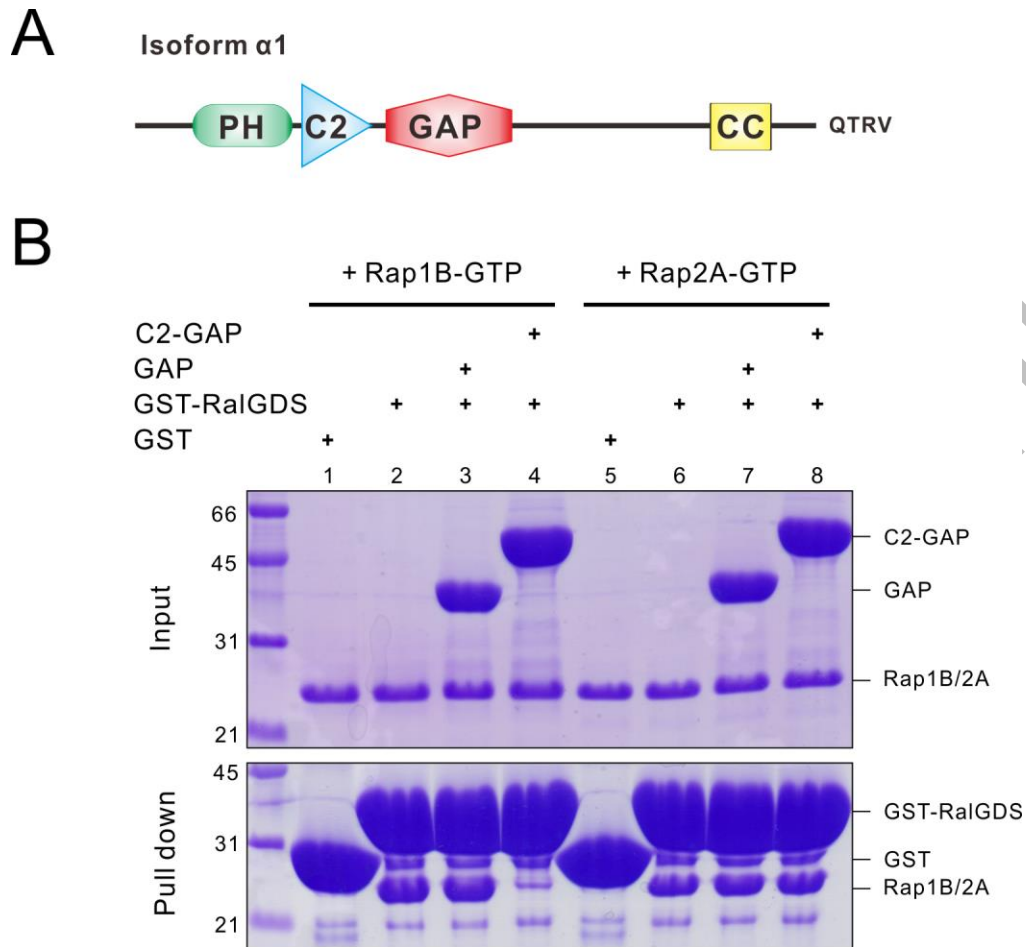
Figure 1: The C2-GAP tandem is required for SynGAP's GAP activity towards Rap1B.

(A)   Schematic diagram showing the domain organization of SynGAP α1.

(B)   GST-RalGDS pull-down assay showing the C2-GAP tandem, instead of the isolated GAP domain, of SynGAP is required for its Rap1B GAP activity. It is noted that neither the GAP domain nor the C2-GAP tandem of SynGAP had detectable GAP activity towards Rap2A.
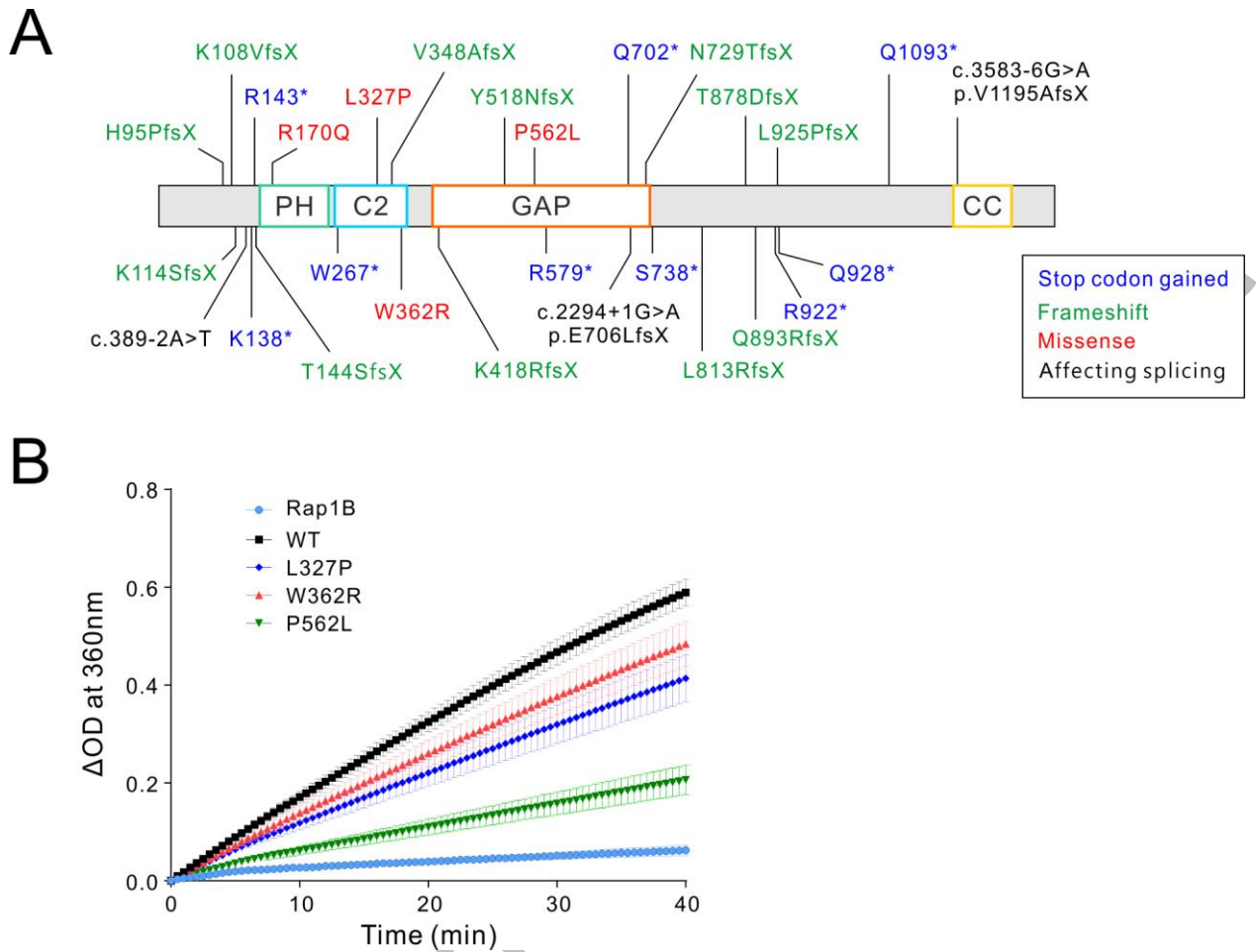
Figure 2: Mutations of SynGAP1 identified in human patients

(A)   Mapping of *SYNGAP1* mutations found in ID or ASD human patients onto the amino acid sequence of the protein with accession code of NP_006763. K138*, R579* and L813RfsX are reported in (Hamdan et al., 2009); V348AfsX is reported in (Vissers et al., 2010); K108VfsX, c.2294+1G>A (p.E706LfsX) and Q893RfsX are reported in (Hamdan et al., 2011); K418RfsX and T878DfsX are reported in (Rauch et al., 2012); H95PfsX, W362R, P562L, N729TfsX and S738* are reported in (Berryer et al., 2013); K114SfsX, c.389-2A>T, R143*, W267* and Q702* are reported in (Carvill et al., 2013); c.3583-6G>A (p.V1195AfsX) is reported in (Redin et al., 2014); T144SfsX, R170Q, L327P, Y518NfsX, R922*, L925PfsX, Q928* and Q1093* are reported in (Parker et al., 2015). Domains of SynGAP are defined as the PH domain (aa 150-253), C2 domain (aa 263-370), GAP domain (aa 408-734) and coiled-coil or CC domain (1188-1277).

(B)   Comparison of the Rap1B GAP activities of the WT or mutant SynGAP C2-GAP tandem.
Data represent three batches of independent experiments and are presented as mean± SEM.
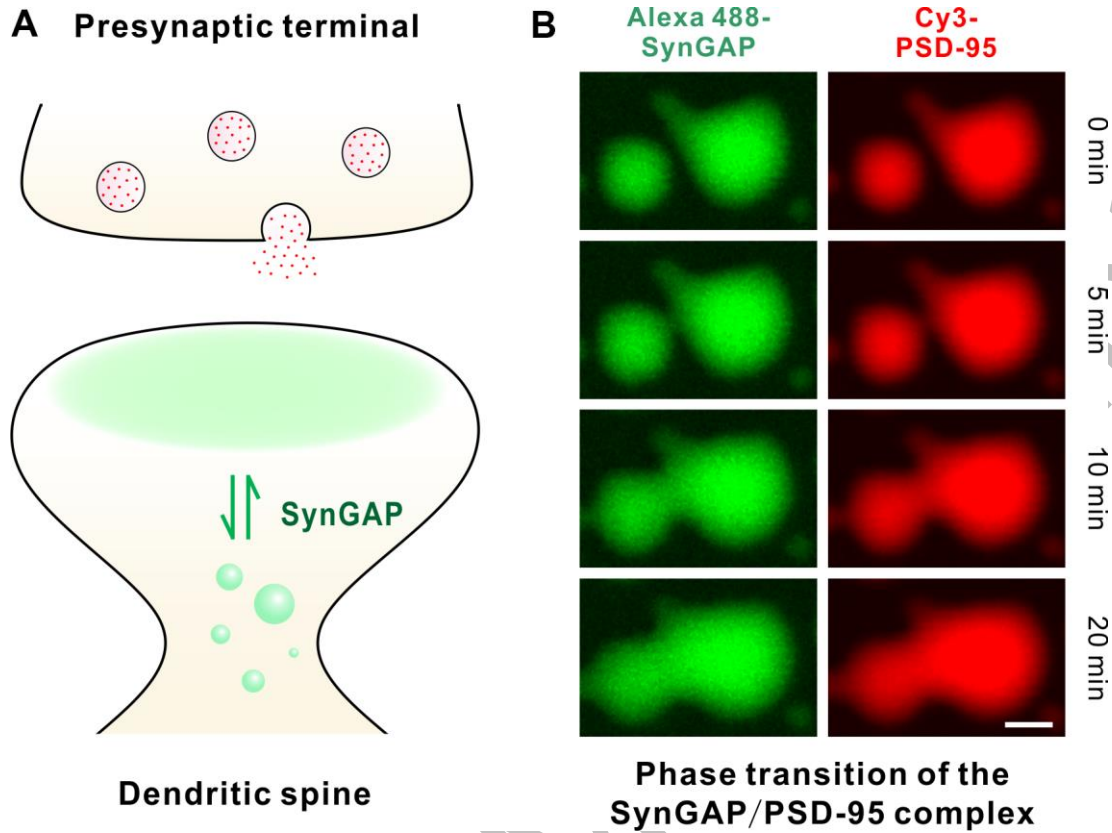
Figure 3: A schematic diagram of Phase transition-mediated SynGAP enrichment in PSD.

(A)   A Schematic diagram showing SynGAP enriched in PSD (shaded in pale green) can undergo dynamic exchange with SynGAP in dendritic shafts.

(B)   Co-localization of SynGAP and PSD-95 in the droplets with enriched concentrations. PSD-95 PSG and SynGAP CC-PBM labeled with Alexa488 and Cy3, respectively. A 1:1 mixture of Alexa488-SynGAP and Cy3-PSD-95 at a concentration of 50 μM. The time-lapse images are to demonstrate that small droplets can grow and fuse into larger ones.