REGULATION OF DENDRITIC SPINE DEVELOPMENT AND CELL MIGRATION THROUGH ASEF2-MEDIATED SIGNALING

By

Joseph Corey Evans

Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

December, 2014

Nashville, Tennessee

Approved:

Terry Page, Ph.D.

Bruce Carter, Ph.D.

Douglas McMahon, Ph.D.

Alissa Weaver, M.D., Ph.D.

Donna Webb, Ph.D.

ACKNOWLEDGEMENTS

First, I would like to thank the members of my committee – Dr. Terry Page (Chair), Dr. Bruce Carter, Dr. Douglas McMahon, and Dr. Alissa Weaver – for their thoughtful questions and suggestions during the course of my project. I would also like to thank my advisor, Dr. Donna Webb, for providing invaluable advice about my project and for fostering my interest in writing and editing. I would like to thank all of the past and present members of the Webb lab for their support and friendliness. I would also like to thank the National Institutes of Health for funding my work, as well as the Department of Biological Sciences for generously providing me with funding to attend multiple conferences to present my research. Lastly, I would like to thank my family for their love and support.

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ABR	APC-binding region
Abr	Active BCR-related
ADP	Adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
AP5	DL-2-amino-5-phosphonovaleric acid
APC	Adenomatous polyposis coli
APC^{ARM}	Armadillo repeat domain of APC
Arp2/3	Actin-related protein 2/3
Asef	APC-stimulated guanine nucleotide exchange factor
ATP	Adenosine triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
Bcr	Breakpoint cluster region
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
Ca^{2+}	Calcium ion
CaMKII	Calcium / calmodulin-dependent protein kinase II
CP	Capping protein
Dbl	Diffuse B cell lymphoma
DCX	Doublecortin
DH	Dbl homology
DIV	Day *in vitro*
DLG	Discs large
DTT	Dithiothreitol
ECM	Extracellular matrix

EGF	Epidermal growth factor
EM	Electron microscopy
FAB	F-actin binding
F-actin	Filamentous actin
FRET	Fluorescence resonance energy transfer
G-actin	Globular actin
$GABA_AR$	GABA type A receptor
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HEK293	Human embryonic kidney 293
HGF	Hepatocyte growth factor
IAM	Iodoacetamide
IRSp53	Insulin-receptor substrate p53
K^+	Potassium ion
Lbc	Lymphoid blast crisis
LC	Liquid chromatography
Lfc	Lbc's first cousin
LIM	Lin11, Isl-1, and Mec-3
LTD	Long-term depression
LTP	Long-term potentiation
MAP1B	Microtubule-associated protein 1B

MDCK	Madin Darby canine kidney
MEF	Mouse embryonic fibroblast
Mg^{2+}	Magnesium ion
mGluR	Metabotropic glutamate receptor
miRNA	Micro RNA
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
Na^{+}	Sodium ion
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-beno(F)quinoxaline
Neurabin	Neural tissue-specific F-actin binding protein
NMDA	N-methyl-D-aspartate
NPF	Nucleation promoting factor
PAK	p21-activated kinase
PBD	p21-binding domain
PDZ	PSD95 / DLG / ZO-1
PFA	Paraformaldehyde
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PH	Pleckstrin homology
PP1	Protein phosphatase 1
PSD	Postsynaptic density
PSD95	Postsynaptic density protein 95
R2F	Rat 2 fibroblast
RNAi	RNA interference
ROCK	Rho-associated kinase
SAM	Sterile α motif
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SH3	Src homology 3
SV2	Synaptic vesicle protein 2
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
Tiam1	T lymphoma invasion and metastasis 1
TrkB	Tropomyosin-related kinase B
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin homologous protein
ZO-1	Zona occludens 1

CHAPTER I

INTRODUCTION

Memory Formation and Storage

The ability of the brain to process novel experiences (learning) and to encode these experiences into recoverable mental representations (memory) are two processes that are essential for survival. In particular, the hippocampus has emerged as a central mediator of the formation of declarative memories (i.e. factual information and personal experiences) (Eichenbaum 2004). Ever since pioneering work by Scoville and others (Scoville and Milner 1957; Penfield and Milner 1958) demonstrated that removal of the hippocampus caused both loss of declarative memory and defects in new memory formation, researchers have investigated the regions of the brain responsible for memory formation and storage. These studies led to significant advances in our understanding of the communication between interconnected brain regions, as well as the underlying molecular mechanisms that mediate the establishment and maintenance of memories.

The hippocampus and long-term memory – Case studies involving patients with damaged or surgically removed areas of the brain were instrumental in linking memory formation to discrete brain regions. For example, patients with amnesia (Ribot 1882) or encephalitis (Rose and Symonds 1960) were unable to remember recent experiences or facts but could recall information from a much earlier time; this suggested a temporal component of memory storage, with newer memories being more susceptible to loss (Wiltgen et al. 2004). The link between memory formation and the hippocampus was solidified by studies of a patient

known as H.M., who was treated for epilepsy by removal of the medial temporal lobe (including the hippocampus) (Scoville and Milner 1957). H.M.'s seizures were reduced, but he began to display defects in the formation of new memories without disruption to older memories or to overall intellectual ability (Scoville and Milner 1957). Numerous studies, including those using rodent (Anagnostaras et al. 1999), primate (Zola-Morgan and Squire 1990), and human (Victor and Agamanolis 1990) subjects, have shown that damage to the hippocampus alone is responsible for defects in new memory formation. Collectively, these results suggest that the region of the brain responsible for memory formation is distinct from the one responsible for long-term memory storage. To identify the part of the brain that facilitates memory storage, researchers once again relied on patients with injuries to specific brain areas. In several cases, it was noted that the ability to recall old memories is disrupted in patients who have received damage to the neocortical regions of the brain (Graham and Hodges 1997; Bayley et al. 2003). Therefore, the neocortex has been hypothesized to mediate long-term memory storage (Takehara et al. 2003; Maviel et al. 2004; Wiltgen et al. 2004). This was demonstrated by Bontempi et al., who measured brain metabolism in mice that had been trained in a maze then required to recall the training after a short or a long time interval; the researchers observed that the hippocampus is more active when the mice recall newer memories, while the neocortex is more active when the mice have to recall older memories (Bontempi et al. 1999). Furthermore, gene expression is enhanced in the neocortex when old memories are recalled (Frankland et al. 2004; Maviel et al. 2004), while gene expression increases in the hippocampus during recall of new memories (Frankland et al. 2004). The study of hippocampal and neocortical functions, therefore, have provided much insight into the physically separated, yet interconnected, roles of these brain regions during memory formation and storage.

The plasticity of memory – The discovery that new memories are formed in one region of the brain, while older memories are stored in another, implied that there is a neural

mechanism for memory transfer, as well as a mechanism for ensuring that these memories are resistant to perturbation over time (Marr 1970; Marr 1971). The process of memory stabilization is known as consolidation (Frankland and Bontempi 2005; McKenzie and Eichenbaum 2011). Currently, there are several models describing how learned information is transmitted between the hippocampus and the neocortex during consolidation. In the most common model, learning causes multiple cortical regions to send signals to the hippocampus, which then processes this information into a distinct memory (Eichenbaum 2004; Frankland and Bontempi 2005). At this time, the memory is susceptible to alteration (Duncan 1949; Flexner et al. 1965; McGaugh and Krivanek 1970; Gordon and Spear 1973). Over time, however, repeated communication between the hippocampus and the neocortex leads to the strengthening of the neocortical connections that initially encoded the information; eventually, the memory trace is stored via these enhanced connections without influence from hippocampal signaling. This model is supported by an observed loss of hippocampal-neocortical communication, and a subsequent increase in intracortical signaling, following learning (Takashima et al. 2009). Another model of consolidation posits that related memories in the neocortex are grouped into so-called "schemas," and that new memories originating from the hippocampus are incorporated into these schemas via network alteration (McClelland et al. 1995). This was demonstrated experimentally in a study showing that rats could create a schema of memories associated with the location of food; learning this schema allowed the rats to quickly establish novel food-location associations, which were not present when the rats were placed in a different environment (Tse et al. 2007). A third model of consolidation, called the multiple trace theory (Nadel and Moscovitch 1997), states that, upon memory reactivation, the hippocampus and neocortex create new memory traces that are integrated with pre-existing information, eventually leading to the separation of context-independent factual information (stored in the neocortex) and context-specific information (stored in the hippocampus) (Nadel and Moscovitch 1997; Dudai 2012). This model is supported by functional imaging studies showing that the

hippocampus is active during retrieval of autobiographical (i.e. context-specific) information, and this activity is independent of the length of time between memory formation and recall (Fink et al. 1996; Conway et al. 1999). While these models differ in the specific mechanisms of memory stabilization, they all stress the importance of hippocampal-neocortical communication during this process.

The models of consolidation initially suggested that memories are no longer susceptible to alteration once stored in the neocortex (McKenzie and Eichenbaum 2011). The emergence of a related process called reconsolidation, however, provided evidence for the malleability of memories post-consolidation (Misanin et al. 1968; Nader et al. 2000; Nader and Hardt 2009). When a consolidated memory is recalled, it becomes less stable; after the memory is altered, it undergoes a second round of stabilization (i.e. reconsolidation) (Tronson and Taylor 2007). The process of reconsolidation was demonstrated in rats that had been trained using a fear conditioning protocol (Nader et al. 2000). Recall of the memory, followed by treatment with the protein synthesis inhibitor anisomycin (to block consolidation), caused a disruption in long-term memory (Nader et al. 2000). When the anisomycin treatment was delayed by several hours, however, long-term memory was no longer impaired (Nader et al. 2000). This suggested that a retrieved memory can undergo a transient period of destabilization, followed by a stabilization process similar to consolidation (Nader and Hardt 2009). Although consolidation and reconsolidation are functionally identical (i.e. both stabilize memories), they are not exactly identical; for example, consolidation – but not reconsolidation – is impaired by reduced expression of brain-derived neurotrophic factor (BDNF), while reconsolidation – but not consolidation – is disrupted by reduced expression of the transcription factor Zif268 (Lee et al. 2004). Nevertheless, the existence of consolidation and reconsolidation mechanisms reinforces the fact that memories are not permanently stable. Recalled memories can be altered in response to novel experiences (Rodriguez-Ortiz et al. 2005; Morris et al. 2006), thus implying a

role for consolidation and reconsolidation in continuously responding to new information (Tronson and Taylor 2007).

Dendritic Spines and Synapses

The changes in neuronal communication between the hippocampus and the neocortex during memory consolidation, as well as the ability of memories to be altered prior to reconsolidation, supports the existence of a structural mechanism for encoding memory traces that can be rapidly remodeled in response to new information (Lamprecht and LeDoux 2004). An abundant collection of evidence has demonstrated that memories are encoded by modulating cell-cell communication at specific sites called synapses (Mayford et al. 2012). Neurotransmitters that bind to receptors embedded in the postsynaptic membrane can either promote (excitatory) or impede (inhibitory) neuronal activity (Smart and Paoletti 2012). Excitatory synapses have been shown to form on dendritic spines, which are actin-rich protrusions from the dendrite that contact the axons of neighboring neurons (Sala and Segal 2014). Therefore, spines serve as physical loci for neuronal communication, and alterations in spine density and morphology are linked to changes in the efficacy of synaptic signaling (Nikonenko et al. 2002; Hofer et al. 2009). This so-called synaptic plasticity is hypothesized to underlie the stabilization (or destabilization) of memories (Caroni et al. 2012). As a result, studies of the molecular mechanisms that regulate spine and synapse formation are crucial for elucidating how memories are shaped.

Dendritic spine formation – The most widely accepted model of spine development proposes that spines originate from dynamic, filopodia-like precursors that actively search the neighboring environment for axonal interaction partners (Papa et al. 1995) (Figure 1). Validation for this model includes the observation that immature neurons possess many filopodia and few spines; gradually, the number of filopodia decreases, and the density of stable spines increases

(Dailey and Smith 1996; Ziv and Smith 1996; Dunaevsky et al. 1999). Furthermore, live imaging of dendritic filopodia showed that they form transient contacts with axons, but only contacts that lead to excitatory synapse formation are stabilized (Lohmann and Bonhoeffer 2008). A second model states that synapses can form on the dendrite itself, followed by a process of spine outgrowth from the dendrite (Miller and Peters 1981) (Figure 1). Immature neurons form synapses predominantly on dendrites, as visualized by electron microscopy (EM) (Harris et al. 1992). Also, time-lapse imaging of hippocampal neurons showed the transport of assembled postsynaptic components from the dendrite into a developing spine (Marrs et al. 2001), and rapid spine formation has been observed in the absence of a filopodial intermediate (Dailey and Smith 1996). A third spine development model has been demonstrated in Purkinje neurons of the cerebellum (Figure 1): these cells can still form numerous dendritic spines even in the absence of presynaptic neurons (Sotelo 1990). For example, inhibition of presynaptic signaling actually leads to an increase in spine density (Cesa et al. 2007), thus emphasizing the synapse-independent nature of spine formation in these cells. While the filopodia precursor model has been best characterized, the possibility that spines can form via multiple mechanisms is also possible (Ethell and Pasquale 2005).

The postsynaptic density and synaptic signaling – EM images of synapses in rat neurons revealed an electron-dense thickening of the postsynaptic membrane at the synaptic site (Palay 1956; Gray 1959); this structure was termed the postsynaptic density (PSD). In-depth characterization of the protein components that define the PSD, including mass spectrometry-based analyses of purified PSDs (Walikonis et al. 2000; Li et al. 2004; Peng et al. 2004; Cheng et al. 2006), has reiterated the importance of this structure for neuronal communication. The PSD houses glutamatergic (i.e. excitatory) neurotransmitter receptors, cytoskeletal proteins, cell-cell adhesion molecules, signaling molecules such as kinases and phosphatases, and numerous types of protein scaffolds that locally stabilize the other PSD

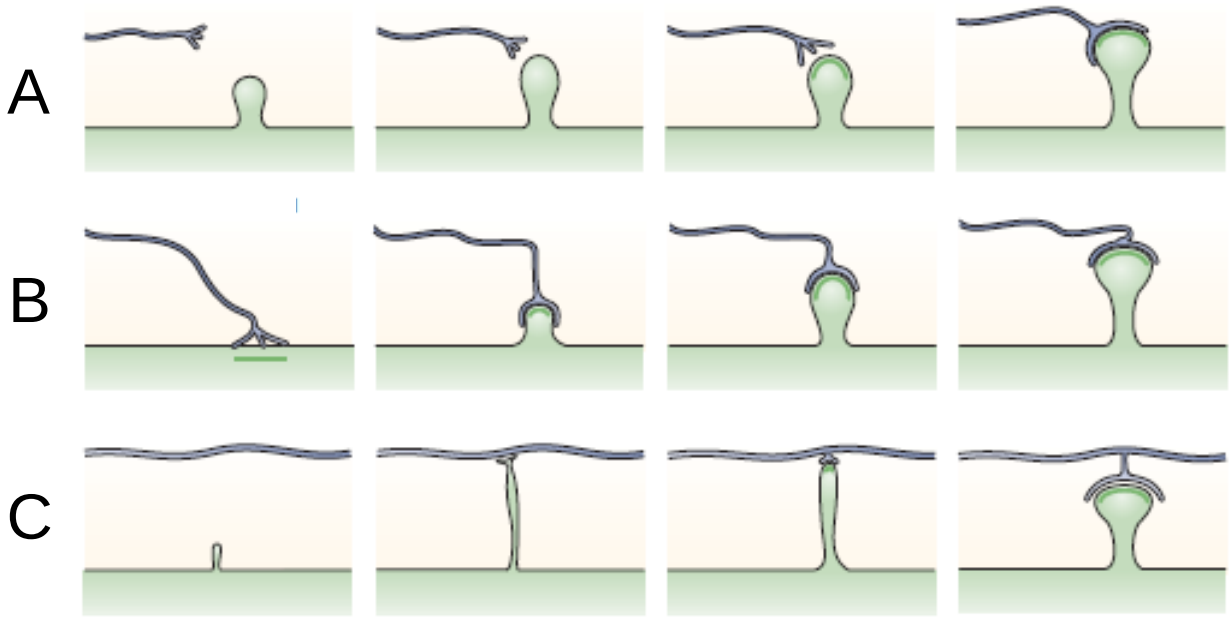

Figure 1. Spine formation models. Schematic showing three models of spine formation. Row (A) shows a model in which spine formation is independent of synaptic contact; the spine grows from the dendrite and eventually contacts another neuron. Row (B) shows spine formation that is initiated by axonal contact with a dendritic shaft synapse. This contact promotes outgrowth and maturation of a dendritic spine. Row (C) shows the most common model of spine formation: an immature filopodial protrusion makes contact with an axon and matures into a spine. Figure reprinted from (Yuste and Bonhoeffer 2004).

constituents (Boeckers 2006; Sheng and Hoogenraad 2007) (Figure 2). Analysis of the relative amounts of PSD proteins revealed that the neurotransmitter receptors are in relatively low abundance compared to scaffold proteins and other signaling molecules (Cheng et al. 2006; Sheng and Hoogenraad 2007). The higher proportion of signaling molecules could aid in the amplification of incoming signals from the neurotransmitter receptors, so that relatively small changes in synaptic activity could rapidly alter downstream signaling (Kennedy et al. 2005; Sheng and Hoogenraad 2007). For example, calcium / calmodulin-dependent protein kinase II (CaMKII) – the most abundant protein in the PSD (Kennedy et al. 1983; Cheng et al. 2006) – phosphorylates numerous effector proteins and is crucial for calcium-dependent synaptic plasticity (Colbran and Brown 2004). The interplay between multiple signaling networks in each PSD is responsible for modulating synaptic activity (Kennedy et al. 2005); therefore, the biochemical activity in dendritic spines underlies memory formation.

The signaling event that initiates excitatory synaptic transmission is the binding of glutamate to receptors embedded in the plasma membranes of spines. The mammalian central nervous system contains two families of glutamate receptors: the ionotropic receptors (N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate receptors) signal via cation flux, and the metabotropic receptors (mGluRs) signal via G-protein-dependent mechanisms (Ozawa et al. 1998). The NMDA and AMPA receptors, in particular, are indispensable for the modulation of synaptic plasticity, since pharmacological blockage of these receptors inhibits the signaling pathways that regulate the efficacy of synaptic transmission (Harris et al. 1984; Kauer et al. 1988). These receptors possess differences in subunit composition: NMDA receptors are composed of heteromeric NR1 and NR2A-NR2D subunits, and AMPA receptors are composed of GluR1 – GluR4 subunits (Ozawa et al. 1998). As a result, these receptors have unique ion permeability characteristics. While both receptor types are permeable to potassium (K^+) and sodium (Na^+) ions (Anwyl 1977), NMDA receptors are permeable to calcium ions (Ca^{2+}) (MacDermott et al. 1986) and are inhibited by magnesium

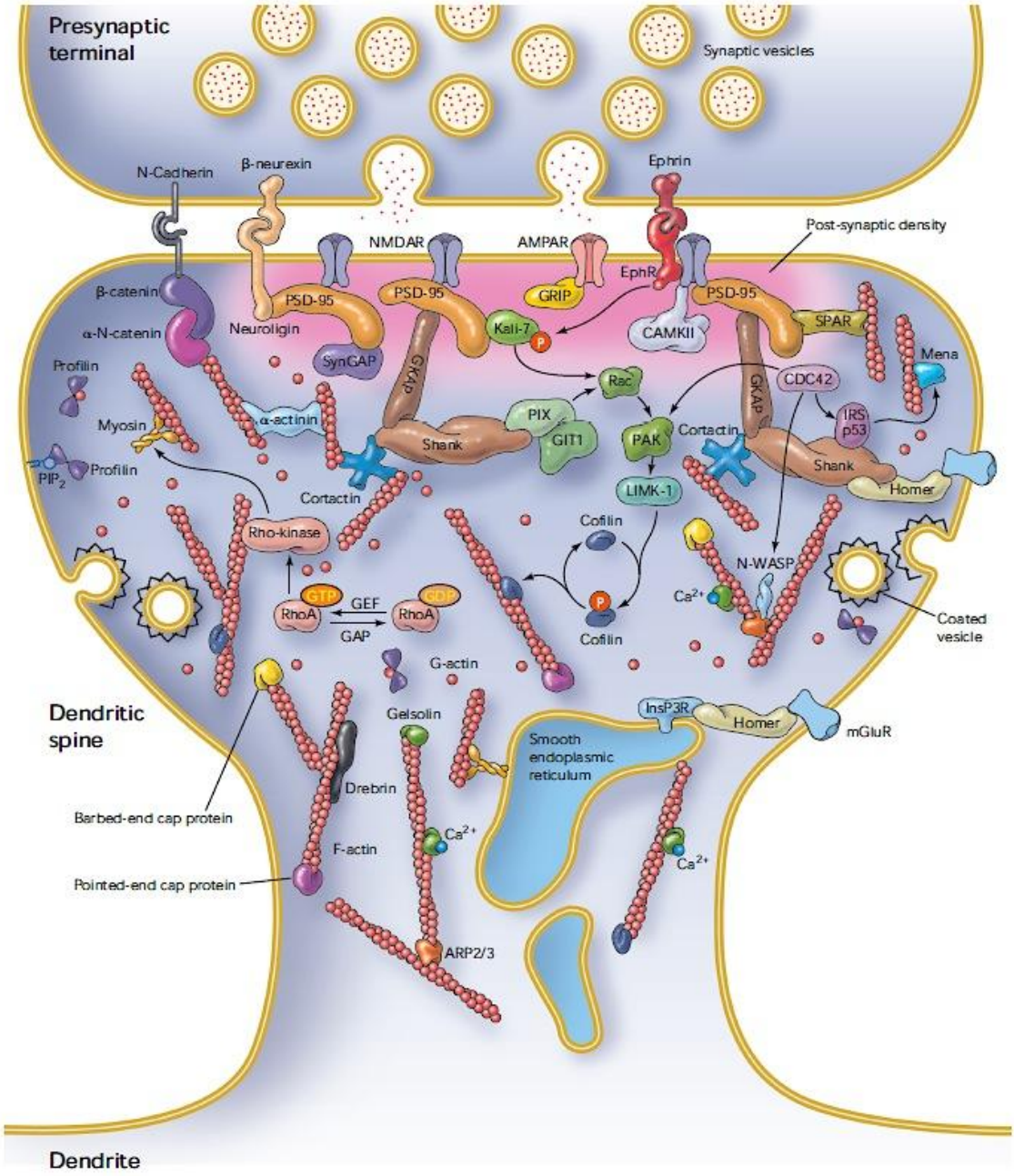

Figure 2. The molecular composition of spines. Schematic showing the numerous proteins that are present in dendritic spines. These include glutamate receptors (NMDAR, AMPAR, and mGluR), scaffolding proteins (PSD-95, Homer, Shank), cell-cell adhesion molecules (like neuroligin), and many proteins that regulate actin dynamics. Note that scaffold proteins localize NMDA and AMPA receptors at the postsynaptic density and link synaptic activity to changes in the actin cytoskeleton. Figure reprinted from (Calabrese et al. 2006).

ions (Mg^{2+}) (Mayer et al. 1984; Nowak et al. 1984). These properties confer specific functions for the NMDA and AMPA receptors in cellular models of memory formation (discussed in the next section) (Luscher and Malenka 2012).

Spines / synapses as substrates for memory – The hypothesis that dendritic spines are involved in learning and memory was initially formulated by Santiago Ramón y Cajal, who utilized novel staining techniques to visualize dendritic spines and carefully characterize their morphology and interaction with other neurons (Ramon y Cajal 1888; Ramon y Cajal 1891; Ramon y Cajal 1893). Several decades later, Donald Hebb introduced his theory linking changes in synaptic activity to memory formation (Hebb 1949). According to Hebb, learning alters neuronal connections such that enhanced activity of one neuron leads to a stronger postsynaptic response due to increased synaptic signaling; memories, therefore, are stored as long-lasting increases in synaptic transmission (Hebb 1949). Attempts to model this theory in neurons led to the discovery of the phenomenon known as long-term potentiation (LTP) (Bliss and Gardner-Medwin 1973; Bliss and Lomo 1973). When a rabbit hippocampal presynaptic neuron was stimulated with high-frequency action potentials, a long-lasting increase in synaptic transmission was observed in the postsynaptic neuron, suggesting that changes in neuronal activity enhance synaptic strength (Bliss and Gardner-Medwin 1973; Bliss and Lomo 1973). This effect was proven to be NMDA receptor-dependent (Bliss and Collingridge 1993). Under basal conditions, the NMDA receptor channel is blocked by a Mg^{2+} ion; high frequency stimulation, however, rapidly depolarizes the postsynaptic neuron, forcing the expulsion of the Mg^{2+} and allowing Ca^{2+} influx through the channel (Mayer et al. 1984; Nowak et al. 1984; MacDermott et al. 1986). Therefore, the NMDA receptor is not active unless two conditions are met: binding of glutamate (released from the presynaptic neuron) and depolarization of the membrane (resulting from the cumulative activity of other synaptic inputs) (Mayford et al. 2012). The resulting Ca^{2+} influx activates signaling molecules – particularly CaMKII – to regulate

synaptic plasticity (Colbran and Brown 2004). For example, CaMKII-mediated phosphorylation of AMPA receptors leads to their insertion into the synaptic membrane (Tan et al. 1994; Luscher et al. 1999), thereby strengthening synaptic transmission. The recruitment of AMPA receptors to the PSD is responsible for the enduring nature of LTP (Kauer et al. 1988), which is also dependent on the synthesis of new proteins (Stanton and Sarvey 1984; Deadwyler et al. 1987). In all, LTP presents an attractive model for memory storage because it pairs the simultaneous activity of pre- and postsynaptic neurons with a long-lasting enhancement of synaptic transmission.

In order to modulate the strength of synaptic transmission, there must be a process that counterbalances the increase in synaptic strength modeled by LTP. This mechanism, known as long-term depression (LTD), is elicited experimentally by low frequency neuronal stimulation, which causes a sustained decrease in synaptic transmission (Dudek and Bear 1992; Mulkey and Malenka 1992). Inhibition of NMDA receptor activity via the selective inhibitor DL-2-amino-5-phosphonovaleric acid (AP5), as well as reduction in intracellular Ca^{2+} via the chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), have been shown to prevent LTD induction (Dudek and Bear 1992; Mulkey and Malenka 1992). Therefore, both LTP and LTD require the influx of Ca^{2+} through NDMA receptor channels. The activity level of NMDA receptors determines whether LTP or LTD dominates. Strong NMDA receptor activity causes a large Ca^{2+} influx (leading to LTP expression), while low NMDA receptor activity only permits a small Ca^{2+} influx (leading to LTD expression) (Luscher and Malenka 2012). Furthermore, LTD depends on the removal of AMPA receptors from the synaptic membrane, which mechanistically agrees with the membrane insertion of these receptors during LTP (Luscher et al. 1999; Luthi et al. 1999). Finally, the processes of LTP and LTD mirror each other in that LTP promotes the phosphorylation of signaling proteins (Bliss and Collingridge 1993), while LTD is associated with the dephosphorylation of proteins via phosphatases such as protein phosphatase 1 (PP1) (Lisman 1989). Collectively, LTP and LTD represent cellular mechanisms of synaptic signaling

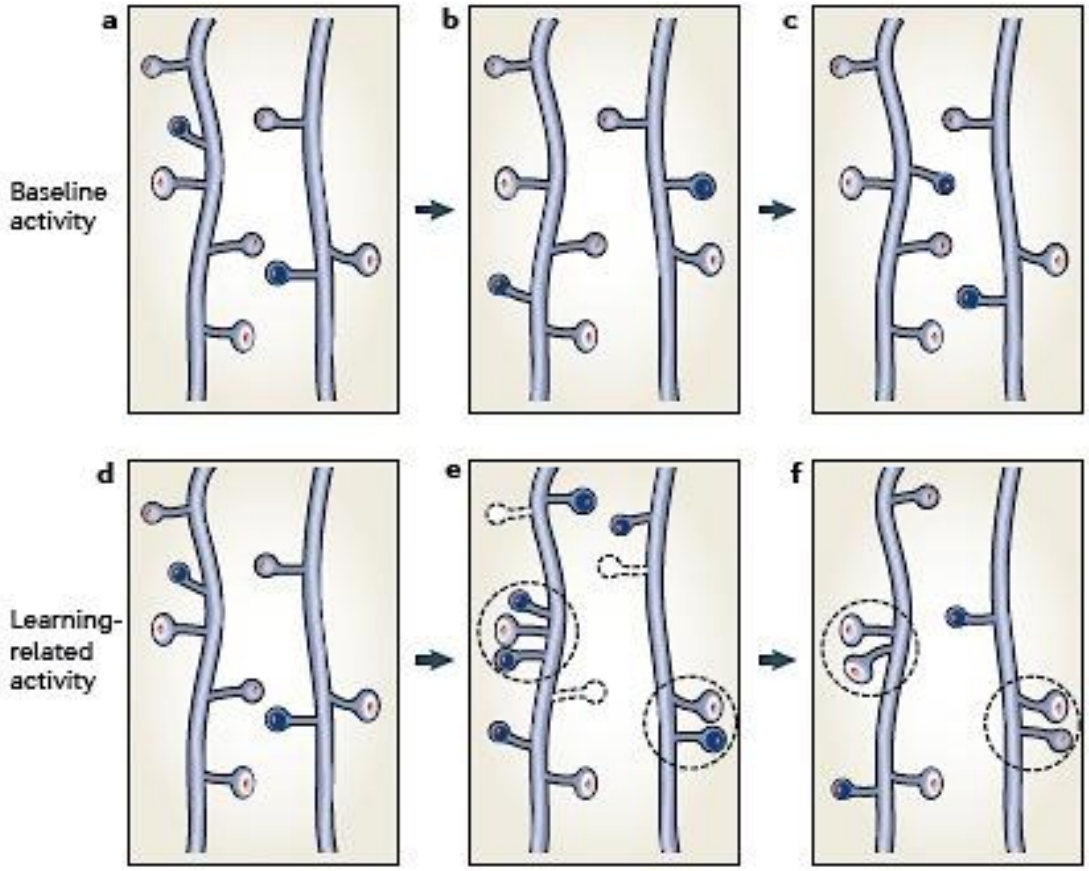

Figure 3. Structural plasticity of dendritic spines due to learning. *a-c*, Under baseline conditions, a subset of dendritic spines (dark spines) are formed or lost. The majority of spines (light spines) are larger and more stable. *d-f*, Learning increases the number of spines that are formed and lost. Also, learning can cause more stable spines to be pruned (dashed outlines). Learning-mediated spine growth can occur in clusters (dashed circles) that are more stable than non-clustered spines. The changes in spine density allow the connections between neurons to be altered, and the persistence of these alterations is the structural basis for memory storage. Figure reprinted from (Caroni et al. 2012).

modulation that model the alterations in synaptic strength that are thought to occur when memories are formed.

The structural modification of dendritic spines also mediates synaptic plasticity (Lamprecht and LeDoux 2004; Caroni et al. 2012). This was suggested by LTP induction experiments that resulted in the appearance of new spines (Desmond and Levy 1983; Chang and Greenough 1984; Engert and Bonhoeffer 1999; Nagerl et al. 2004), which could be blocked by treatment with the NMDA receptor antagonist AP5 (Engert and Bonhoeffer 1999). Conversely, LTD causes a loss of dendritic spines (Nagerl et al. 2004). The increase or decrease in spine density could regulate transmission by altering the number of synaptic contacts between neurons (Lamprecht and LeDoux 2004). Morphological plasticity is also apparent in single spines subjected to LTP or LTD induction. For example, activation of individual spines via glutamate uncaging (Matsuzaki et al. 2001) caused an increase in spine head size that was NMDA receptor-dependent (Matsuzaki et al. 2004). Induction of LTD, on the other hand, via low-frequency stimulation caused spine shrinkage (Nagerl et al. 2004; Oh et al. 2013). The spine head volume is proportional to the area of the PSD (Harris and Stevens 1989; Arellano et al. 2007). An LTP-mediated increase in spine head volume, therefore, enhances the PSD area; this is functionally relevant because more neurotransmitter receptors can be inserted into the PSD membrane, leading to stronger synaptic transmission (Takumi et al. 1999; Matsuzaki et al. 2001). While experimental manipulation of synaptic strength has been useful for the *in vitro* analysis of dendritic spine plasticity, studies of living animals have emphasized the importance of learning-associated spine formation and pruning (Caroni et al. 2012). For example, mice placed in an enriched environment (i.e. an environment that promotes social interaction and exploration and enhances learning (van Praag et al. 2000)) exhibit increased spine formation, as well as loss of pre-existing spines (Bednarek and Caroni 2011; Jung and Herms 2014). Intriguingly, a portion of spines that form due to learning cluster together and have increased stability (Figure 3), which supports the hypothesis that learned information is

encoded by specific patterns of synaptic connection (Fu et al. 2012). In all, these observations emphasize the connection between spine alteration, synaptic plasticity, and memory formation. Further solidifying this relationship is the well-documented correlation between abnormal spine density or morphology – and the resulting perturbation of synaptic signaling – with numerous neurological disorders, such as Alzheimer's disease, Fragile X syndrome, epilepsy, schizophrenia, and intellectual disability (Fiala et al. 2002; Penzes et al. 2011). Therefore, investigating the molecular mechanisms that facilitate spine formation is essential for determining how defects in spine morphology lead to cognitive impairment.

The Actin Cytoskeleton

The dynamic structural changes that occur as synaptic connections are modified during memory formation point to the cellular cytoskeleton as an essential mediator of neuronal communication. The observation that actin is enriched in dendritic spines (Fifkova and Delay 1982; Matus et al. 1982) has led to an extensive characterization of the signaling pathways that regulate actin dynamics in spines (Hotulainen and Hoogenraad 2010). The assembly and disassembly of actin structures allows the spine head to rapidly change shape (Fischer et al. 1998); also, disrupting the regulation of actin dynamics impedes the development of spines (Wegner et al. 2008). Therefore, the actin cytoskeleton must be tightly regulated to ensure that synaptic contacts are established and that these contacts can be remodeled in response to synaptic signaling (Cingolani and Goda 2008). While the role of actin in spines has been well-established, much work is still necessary to elucidate the molecular factors that promote or impede actin dynamics. Nevertheless, the integral function of actin in dendritic spine formation and morphology underscores the diversity of actin-dependent processes within the cell (Schoenenberger et al. 2011).

Actin structure and dynamics – In cells, actin exists in two forms: a monomeric form, called globular (G) actin, and a polymeric form, called filamentous (F) actin, which is composed of G-actin monomers (Straub 1942). Each monomer possesses a central cleft that binds the nucleotides adenosine diphosphate (ADP) or adenosine triphosphate (ATP), which are each associated with a Ca^{2+} or a Mg^{2+} ion that regulates actin filament flexibility (Kabsch et al. 1990; Orlova and Egelman 1993). The conformation of the G-actin protein results in a helical actin filament that is inherently polarized (Hanson 1963); each filament has a "barbed" (+) end and a "pointed" (–) end, based on EM studies using filaments coated with myosin subunits that bind to actin in an arrowhead-like pattern (Huxley 1963). Filament assembly is not instantaneous, since G-actin monomers must form dimers or trimers that act as seeds for filament growth (Wegner and Engel 1975; Cooper et al. 1983; Reutzel et al. 2004). Kinetic studies of monomers binding to filaments revealed that ATP-bound and ADP-bound G-actin have different rate constants for association and dissociation at the barbed and the pointed end (Pollard 1986). ATP-bound G-actin preferentially binds to the barbed end of the filament because the critical concentration there (i.e. the minimum concentration of monomers that will promote filament assembly) is lower than at the pointed end (Pollard 1986). Thus, net assembly occurs at the barbed end, and net disassembly occurs at the pointed end; this concomitant gain and loss of actin monomers on a filament is known as actin treadmilling (Wegner 1976). Binding of ATP-G-actin at the barbed end promotes the irreversible hydrolysis of ATP (Carlier et al. 1988), resulting in filaments composed of ADP-P_i-actin. The terminal phosphate group later dissociates so that the pointed end of the filament is composed of ADP-G-actin. The ADP-bound G-actin is then depolymerized; this provides free monomers to continue actin polymerization at the barbed end (Pantaloni et al. 2001).

The cycle of actin filament assembly and disassembly, as well as the formation of more complex cytoskeletal structures, such as branches or bundles, is mediated by a host of actin-binding proteins (Dominguez and Holmes 2011). For example, once the ADP-bound monomers

reach the end of the filament, they are depolymerized (via binding and severing of the filament by the protein ADF/cofilin (Carlier et al. 1997)), converted to ATP-G-actin (via the exchange of ADP for ATP by the protein profilin (Mockrin and Korn 1980)), and sequestered in the cytoplasm (by the protein thymosin β4 (Carlier et al. 1993)) for use in a new round of polymerization. Furthermore, the actin-related protein 2/3 (Arp2/3) complex promotes the formation of branched actin networks (Pollard 2007). Upon activation by nucleation promoting factors (NPFs), such as Wiskott-Adrich syndrome protein (WASP) (Machesky and Insall 1998), the Arp2/3 complex associates with a pre-existing actin filament and stimulates the formation of a new filament – at a characteristic 70° angle (Mullins et al. 1998) – by structurally mimicking a filament barbed end (Kelleher et al. 1995; Rouiller et al. 2008). Another class of actin nucleating proteins, called formins, mediate a different actin structure. By associating with actin dimers, formins sequentially introduce additional monomers into a growing, unbranched actin filament by harnessing the energy produced by ATP hydrolysis (Evangelista et al. 2002; Romero et al. 2004; Otomo et al. 2005). Capping proteins, such as gelsolin (Kurth et al. 1983) and capping protein (CP) (Isenberg et al. 1980), bind to the barbed ends of actin filaments to suppress polymerization (Zigmond 2004); this is hypothesized to provide a pool of G-actin (derived from the pointed ends of barbed-end-capped filaments) to be used for the selective polymerization of uncapped barbed ends (Akin and Mullins 2008). Finally, cross-linking proteins, such as α-actinin (Meyer and Aebi 1990), can link actin filaments into bundles that strengthen the overall cytoskeleton (Tseng et al. 2005). Collectively, the wide range of actin-binding proteins tightly regulates the structure and dynamics of actin filaments.

Actin-dependent cellular processes – The actin cytoskeleton takes part in multiple processes throughout the cell (Schoenenberger et al. 2011). For example, actin was first discovered because of its ability to interact with myosin fibers to generate the force needed to

contract muscle cells (Koubassova and Tsaturyan 2011). Many of the functions of the actin cytoskeleton revolve around the establishment of cell polarity. Actin is especially important for membrane trafficking because it assists both the endocytosis (Yarar et al. 2005) and exocytosis (Muallem et al. 1995) of secretory vesicles. Also, actin filaments act as tracks for myosin-dependent transport of molecular cargoes to specific regions of the cell (Schuh 2011). Actin plays several roles in cell division, as well (Heng and Koh 2010). For example, polymerization of actin promotes the separation of centrosomes and the assembly of mitotic spindles (Uzbekov et al. 2002), and actin filaments cooperate with myosin motor proteins to form a contractile ring around a dividing cell during cytokinesis (Mavrakis et al. 2014). These examples are only a subset of actin's structural functions in cells.

One of the most important roles of the actin cytoskeleton is to facilitate the migration of cells (Blanchoin et al. 2014). This process underlies many aspects of organismal development, wound healing, and immune response; aberrant cell migration, on the other hand, is implicated in disease states such as atherosclerosis and cancer (Horwitz and Webb 2003). Actin filaments form distinct cytoskeletal structures during cell migration. At the front, or leading edge, of the cell, actin forms a highly branched network called the lamellipodium (Small et al. 2002). Polymerization of actin at the lamellipodium plasma membrane exerts a force that pushes the plasma membrane outward, generating forward cell movement (Pollard and Borisy 2003). Extending from the lamellipodium are thin structures called filopodia, which are composed of thin, bundled actin filaments that actively search the environment and promote directional cell migration (Mattila and Lappalainen 2008). Finally, actin associates with myosin in long bundles called stress fibers; these structures generate contractile forces to facilitate cell movement (Burridge and Wittchen 2013). This force is created by binding of the stress fibers to complex structures called focal adhesions, which link the actin cytoskeleton to the extracellular matrix (ECM) through membrane-bound proteins called integrins (Gardel et al. 2010). The coordinated turnover of actin filaments and adhesions drives cell migration, and this process can be

characterized into distinct steps: protrusion of the actin-rich lamellipodia, formation of adhesions at the leading edge, translocation of the cell body, and detachment of adhesions at the rear of the cell (Lauffenburger and Horwitz 1996). Because actin dynamics are central to cell migration, the signaling proteins that mediate polymerization and depolymerization of actin filaments are tightly controlled. Interestingly, many of the molecular pathways that regulate actin dynamics in migrating cells are also important for the development of dendritic spines (Hotulainen and Hoogenraad 2010). Therefore, both cell migration and dendritic spine formation provide tractable models for studying the regulation of the actin cytoskeleton.

Actin in dendritic spines – Actin filaments were first visualized in dendritic spines via EM (Fifkova and Delay 1982; Matus et al. 1982; Caceres et al. 1983; Landis and Reese 1983). A recent investigation of the spine cytoskeleton, using platinum replica EM to better view the intact cytoskeleton (Svitkina 2009), showed that the spine head is enriched in short, branched actin filaments, the spine neck contains a mixture of branched and unbranched filaments, and the spine base contains longer, unbranched filaments (Korobova and Svitkina 2010) (Figure 4A). Expression of green fluorescent protein (GFP)-tagged actin in neurons has shown that spines are dynamic structures (Fischer et al. 1998; Star et al. 2002). Rapid changes in spine head shape are mediated by actin dynamics (Figure 4B), since treatment of neurons with the drug cytochalasin D, which inhibits actin polymerization (Cooper 1987), slows the turnover of actin filaments (visualized by photobleaching spines and measuring the recovery of GFP-actin) (Star et al. 2002) and inhibits spine head motility (Fischer et al. 1998). Advances in microscopy have revealed the complexity of actin filament dynamics within individual spines (Honkura et al. 2008; Tatavarty et al. 2009; Frost et al. 2010; Izeddin et al. 2011; Tatavarty et al. 2012). For example, photoactivation of GFP-actin demonstrated that spines contain separate pools of actin with distinct kinetics: a large pool at the tip of the spine that exhibits rapid turnover, a smaller spine pool at the base of the spine that turns over more slowly, and a pool that localizes

throughout the spine and mediates spine head expansion during induction of LTP (Honkura et al. 2008). Differences in actin polymerization rates were confirmed by tracking the dynamics of individual actin molecules, which revealed distinct nodes of rapid actin turnover within the spine (Frost et al. 2010). In general, actin is polymerized near the tips of spines and flows toward the spine base (Honkura et al. 2008; Tatavarty et al. 2009; Frost et al. 2010). The rapid polymerization and depolymerization of actin filaments creates a diverse continuum of spine morphologies, including mushroom-shaped spines, thin and elongated spines, and short spines without necks (Arellano et al. 2007). Furthermore, the shift from filopodia-like spine precursors to mature spines involves a reduction in actin flow velocity within the spine neck region (Tatavarty et al. 2012); this transformation is thought to represent a shift from formin-mediated to Arp2/3-mediated actin polymerization (Hotulainen et al. 2009).

The actin cytoskeleton and synaptic signaling are bidirectionally regulated; that is, the level of synaptic signaling modulates actin dynamics, and the structural alterations mediated by the actin skeleton influence changes in synaptic strength (Cingolani and Goda 2008). Induction of LTP, for example, causes an increase in actin polymerization throughout the hippocampus (including spines) that can persist for several weeks (Fukazawa et al. 2003; Okamoto et al. 2004; Lin et al. 2005). This is corroborated by a fluorescence resonance energy transfer (FRET) experiment assessing whether actin monomers fused with different fluorescent proteins can form filaments (and thus produce a FRET signal based on the proximity of individual monomers) (Okamoto et al. 2004). LTP induction caused an increase in the FRET signal (i.e. an increase in F-actin) while LTD reduced the FRET signal (Okamoto et al. 2004). Furthermore, application of the agonists NMDA or AMPA caused a stabilization of actin filaments, suggesting that enhanced synaptic transmission eventually leads to persistent spine head volumes (Fischer et al. 2000; Star et al. 2002). This is also supported by pharmacological inhibition of actin polymerization by the drug latrunculin (Spector et al. 1983), which has been shown to disrupt the maintenance of

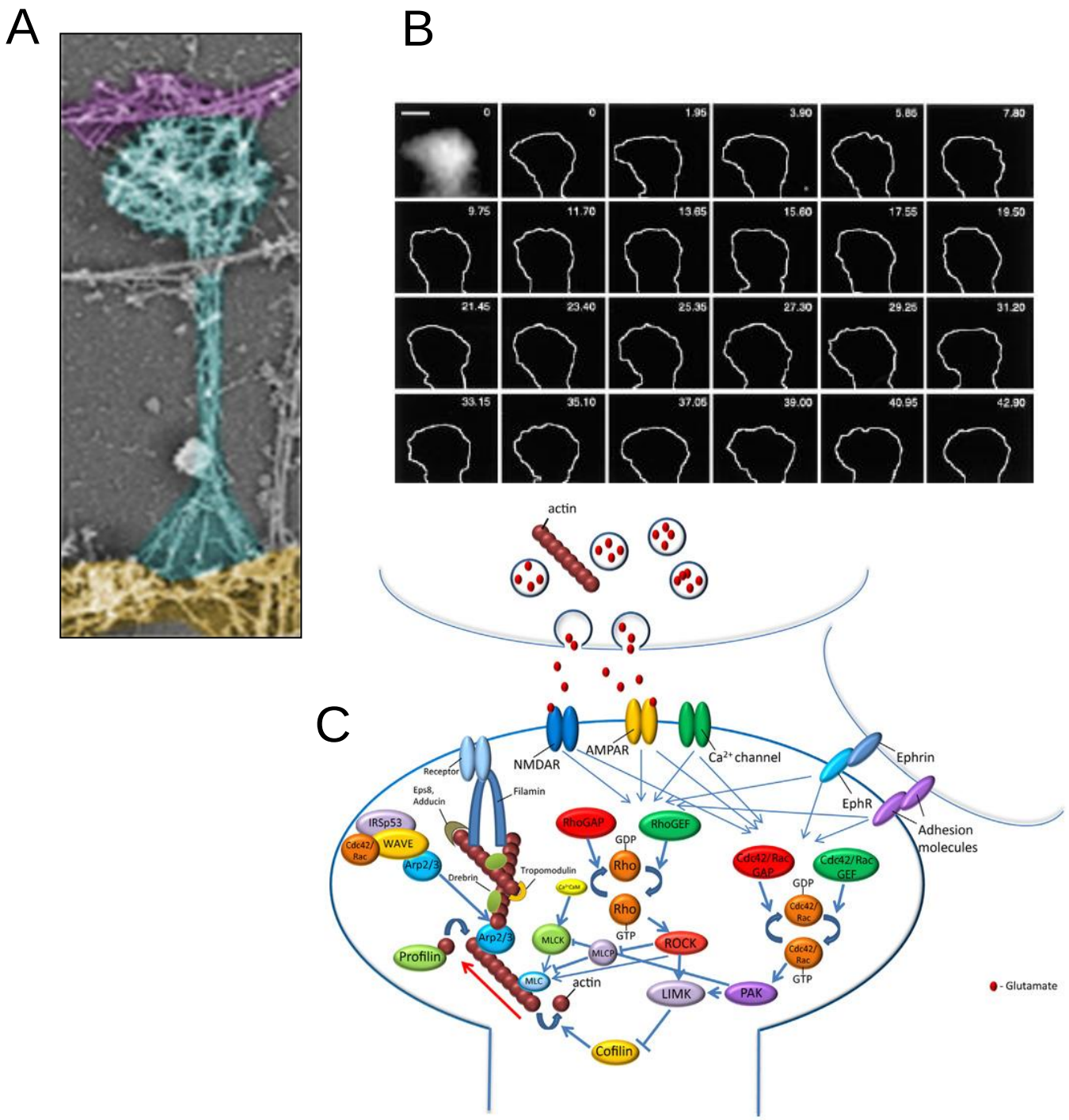

Figure 4. The actin cytoskeleton in dendritic spines. *A*, Pseudocolored EM image of a dendritic spine's cytoskeleton. The spine is colored cyan, the dendrite is colored yellow, and the presynaptic axon is colored purple. Figure reprinted from (Korobova and Svitkina 2010). *B*, Live imaging shows that spines are dynamic structures; the rapid changes in spine head shape are due to the tightly regulated polymerization and depolymerization of actin filaments. Figure reprinted from (Fischer et al. 1998). *C*, Schematic showing the regulation of the actin cytoskeleton in dendritic spines, which will be discussed in later sections. Figure reprinted from (Lamprecht 2014).

LTP (Kim and Lisman 1999; Krucker et al. 2000; Fukazawa et al. 2003; Ramachandran and Frey 2009). Latrunculin treatment also reduces the number of NMDA and AMPA receptors in spines (Allison et al. 1998). In all, these results emphasize the interplay between actin dynamics and synaptic signaling (Figure 4C), which is crucial for effectively modulating synaptic connections during memory storage.

The Rho Family of Small GTPases

The Rho family of small GTPases is an important group of regulatory proteins that, like the actin cytoskeleton, are central to numerous cellular functions such as cell polarity, progression through the cell cycle, cell-cell adhesion, and cell migration (Jaffe and Hall 2005). The Rho GTPases cycle between an active, guanosine triphosphate (GTP)-bound form and an inactive, guanosine diphosphate (GDP)-bound form (Allende 1988). This allows the GTPases to act as molecular switches that can activate downstream effector proteins in a tightly controlled manner (Jaffe and Hall 2005). In mammals, there are approximately 25 Rho family GTPases; however, the majority of these proteins have not been well characterized (Wennerberg and Der 2004). Three Rho GTPases – Rac, Cdc42, and Rho – have served as canonical members of this family because their effects on the actin cytoskeleton and on other cellular processes have been extensively investigated.

Rho GTPases and regulation of actin dynamics – One major function of the Rho GTPases in cells is to direct the organization of actin filaments into higher order structures that mediate cell migration (Murali and Rajalingam 2014). In a series of influential papers, Allen Hall's group correlated the expression of Rho, Rac, and Cdc42 to the aforementioned actin-rich cellular structures (Ridley and Hall 1992; Ridley et al. 1992; Nobes and Hall 1995). Exogenous expression of Rho in Swiss 3T3 fibroblasts, for example, promoted the formation of multiple actin stress fibers within the cell that were associated with adhesions (Ridley and Hall 1992).

Rac expression facilitated the creation of a branched network of actin filaments in the lamellipodium (Ridley et al. 1992), and Cdc42 expression caused an increase in filopodia along the cell periphery (Nobes and Hall 1995). These discoveries led to an intensive study of the signaling pathways that link these Rho GTPases to the formation of distinct actin-based structures (Murali and Rajalingam 2014). Rac-dependent lamellipodia formation, as well as Cdc42-dependent filopodia development, involves activation of the downstream effector protein p21-activated kinase (PAK) (Zhang et al. 1995), which phosphorylates several important substrates such as Lin11, Isl-1, and Mec-3 (LIM) kinase (Edwards et al. 1999). LIM kinase, in turn, inhibits the activity of the actin severing protein cofilin, leading to stabilized actin structures (Yang et al. 1998; Edwards et al. 1999). Rac and Cdc42 also utilize an Arp2/3-mediated pathway to establish branched actin networks (Schafer et al. 1998); Rac signals to the Arp2/3 complex via the WASP-family verprolin homologous protein (WAVE) complex, while Cdc42 activates WASP and the kinase insulin-receptor substrate p53 (IRSp53) (Aspenstrom et al. 1996; Symons et al. 1996; Miki et al. 1998; Krugmann et al. 2001). Rho activates a different signaling cascade to mediate stress fiber formation: Rho-associated kinase (ROCK) activation by Rho leads to inactivation of MLC phosphatase and activation of myosin light chain (MLC) (Kimura et al. 1996). These events activate myosin II, which then associates with actin to form stress fibers (Giuliano and Taylor 1990). The GTPases are spatially and temporally regulated, such that active Rac and Cdc42 are localized to the leading edge of the migrating cell, while active Rho is present at both the front and the rear of the cell (Pertz and Hahn 2004; Pertz et al. 2006; Machacek et al. 2009). Using FRET biosensors, Machacek et al. showed that, at the leading edge of mouse embryonic fibroblasts (MEFs), Rho activation precedes Rac and Cdc42 activation and is strictly associated with the extent of leading edge protrusion, while Rac and Cdc42 are still activated as the protrusion retracts (Machacek et al. 2009). This study underscores the precise regulation of Rho GTPases that is necessary for migration. Also, the

Rho GTPases are regulated by extensive crosstalk, so that the diverse actions of the GTPases can be coordinated to facilitate cell migration (Guilluy et al. 2011).

Because the Rho GTPases (specifically Rac, Cdc42, and Rho) are crucial for the regulation of actin dynamics, they have also been extensively studied for their effects on actin regulation within dendritic spines (Govek et al. 2005). In transgenic mice that expressed constitutively active Rac in their Purkinje neurons, an increase in spine density was observed (Luo 1996). This was corroborated by transfection of hippocampal neurons with a dominant negative version of Rac, which inhibited spine formation (Nakayama and Luo 2000). Expression of constitutively active Rho, however, blocked spine formation; this suggests an opposing role in spines compared to Rac (Nakayama and Luo 2000; Tashiro et al. 2000). Initial studies of Cdc42 suggested that it did not affect spine density or morphology (Tashiro et al. 2000). Later work showed that expression of a dominant negative Cdc42 mutant blocks the maturation of spines and increases the density of long, immature protrusions (Irie and Yamaguchi 2002). The effect of Cdc42 on spine density was confirmed using a loss-of-function Cdc42 mutant (Scott et al. 2003) and an RNA interference (RNAi) knockdown approach (Wegner et al. 2008). In all, these experiments demonstrate that both Rac and Cdc42 promote spine formation, while Rho inhibits it. Studies using advanced microscopy techniques have provided intriguing insight into Rho GTPase localization and dynamics. For example, expression of a Rac FRET biosensor (Itoh et al. 2002) that allows for the visualization of active Rac showed a distinct localization to dendritic spine heads and to the base of filopodial protrusions (Zhang et al. 2005). Another FRET-based study revealed distinct dynamics for activated Cdc42 and Rho (Murakoshi et al. 2011). Upon glutamate uncaging (to enhance synaptic transmission of an individual spine), active Rho exits the spine, while active Cdc42 remains within the spine; these effects were inhibited by the NMDA receptor antagonist AP5, suggesting that both Cdc42 and Rho are downstream effectors of NMDA receptor signaling (Murakoshi et al. 2011). This study also demonstrates that the Rho GTPases have distinct roles in modulating spine structural dynamics, since the extended

presence of Cdc42 in glutamate-stimulated spines correlated with a persistent increase in spine head size (Murakoshi et al. 2011). Therefore, the rapid alterations in spine density or morphology are ultimately dependent on Rho GTPase regulation of actin dynamics.

Rho GTPase regulatory proteins – The activation state of the GTPases (i.e. whether they are bound to GTP or GDP) is carefully controlled so that downstream effector proteins are not aberrantly activated (Figure 5). The GTPases bind to both GDP and GTP with a high affinity (Goody et al. 1991); therefore, the exchange of nucleotides must be enzymatically mediated to ensure rapid activation and inactivation of the GTPase. One family of proteins that regulates GTPase activation includes the guanine nucleotide exchange factors (GEFs) (Rossman et al. 2005). In humans, there are approximately 70 known proteins belonging to the diffuse B cell lymphoma (Dbl) subfamily of Rho GEFs, so named because they share homology with the first identified Rho GEF Dbl (Srivastava et al. 1986; Cherfils and Zeghouf 2013). The overabundance of GEFs compared to GTPases adds an additional regulatory component to GTPase activity because many GEFs contain multiple protein-protein interaction domains that are hypothesized to target the GTPases to specific cellular regions, where they can be activated by distinct upstream signals (Rossman et al. 2005). Elucidation of crystal structures for GEFs in complex with Rho GTPases has provided much insight into the mechanism of nucleotide exchange (Boriack-Sjodin et al. 1998; Goldberg 1998; Worthylake et al. 2000; Renault et al. 2001; Itzen et al. 2006). GTPases bind to nucleotides via specialized loop structures, called switch 1, switch 2, and the phosphate-binding (P)-loop; nucleotide binding is also stabilized by a Mg^{2+} ion (Vetter and Wittinghofer 2001). GEF interactions in this region cause either conformational or electrostatic changes that block Mg^{2+} binding, thus decreasing the affinity for GDP (Bos et al. 2007; Cherfils and Zeghouf 2013). The GDP is spontaneously replaced by GTP because the cellular concentration of GTP is much higher than that of GDP (Bos et al. 2007). GEFs also stabilize the GTPase once the GDP nucleotide has been displaced; this allows the

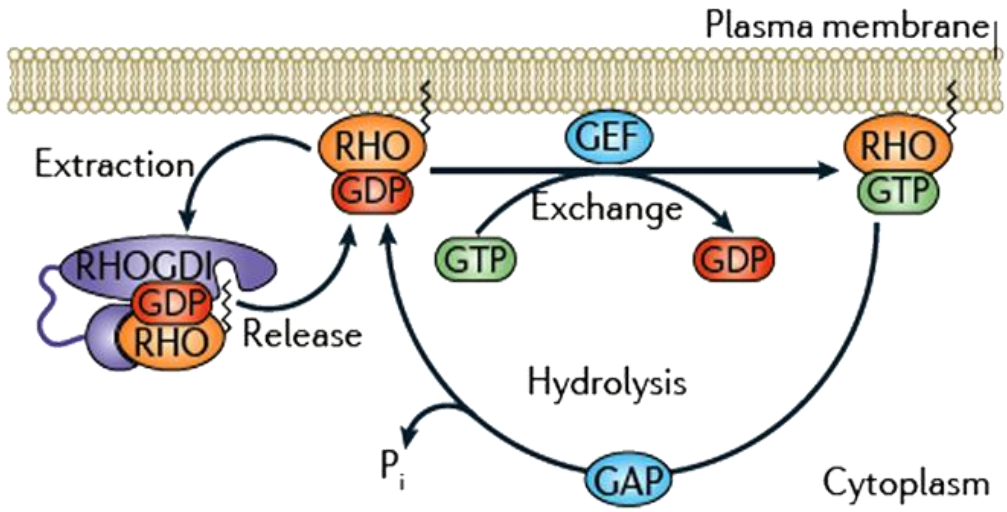

Figure 5. The Rho GTPase cycle. Membrane-bound Rho GTPases are activated by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP. Once activated, the GTPase can activate a host of downstream effector proteins. Inactivation of the GTPase is mediated by GTPase activating proteins (GAPs), which hydrolyze the GTP to produce GDP bound to the GTPase. A third class of regulatory molecules, the guanine nucleotide dissociation inhibitors (GDIs), sequesters inactive GTPases in the cytoplasm by preventing the GTPase from inserting into the membrane. Figure reprinted from (Garcia-Mata et al. 2011).

GEF to form a high-affinity interaction with the GTPase prior to binding by GTP (Klebe et al. 1995). In Dbl family Rho GEFs, nucleotide exchange is mediated by a characteristic domain architecture consisting of a Dbl homology (DH) domain and a pleckstrin homology (PH) domain (Rossman et al. 2005). The DH domain interacts with the switch regions of the GTPase to mediate nucleotide exchange (Soisson et al. 1998), while the PH domain localizes the GEF to the plasma membrane (Harlan et al. 1994). In some cases, the PH domain can also regulate GEF activity by interacting with the GTPase, which is thought to spatiotemporally control GTPase activity by linking GTPase nucleotide exchange to association with the membrane (Rossman et al. 2002). In all, GEFs are integral for GTPase signaling because they not only mediate nucleotide exchange but also direct the localized activation of GTPases in response to diverse upstream signals (Rossman et al. 2005).

While GEFs are essential for promoting the exchange of GDP to GTP, the reverse reaction is also necessary to prevent the GTPase from being constitutively activated. Therefore, a second class of GTPase regulatory proteins – the GTPase activating proteins (GAPs) – facilitates the dissociation of GTP (Scheffzek and Ahmadian 2005). As with Rho GEFs, there are almost three times as many GAPs as there are Rho GTPases, which reiterates the strict control of GTPase activation that occurs in cells (Cherfils and Zeghouf 2013). Instead of catalyzing the exchange of GTP for GDP, GAPs hydrolyze the terminal phosphate group from GTP to produce GDP (Bos et al. 2007). The GTPases themselves can perform this hydrolysis step, but it occurs very slowly (Neal et al. 1988). Therefore, GAPs are necessary because they make the hydrolysis step progress much faster (Scheffzek and Ahmadian 2005). Determination of crystal structures for GAP-GTPase complexes provided insight into the mechanism of GTP hydrolysis (Rittinger et al. 1997; Scheffzek et al. 1997). Interaction of a GAP with a GTPase stabilizes a specific glutamine residue in the nucleotide binding cleft of the GTPase; this glutamine, in turn, positions a water molecule so that a nucleophilic attack of the terminal phosphate group of GTP is more favorable (Bos et al. 2007). Furthermore, an arginine residue

from the GAP (the “arginine finger”) is inserted into the nucleotide binding cleft to stabilize the GDP-GTP intermediate state (Scheffzek and Ahmadian 2005). This intricate positioning of amino acids, which is mirrored by the specific conformation of GEFs to disrupt Mg^{2+} binding during GDP exchange, emphasizes the specificity of these GTPase regulatory proteins for catalyzing nucleotide exchange and hydrolysis. This specificity is also demonstrated by the domain architecture of the GAPs; they all possess a conserved GAP domain that, like the DH and PH domains of Dbl family Rho GEFs, is responsible for the protein’s function (Scheffzek and Ahmadian 2005). The GAPs also possess a diverse array of protein-protein interaction domains to enhance the control of GAP activity (Scheffzek and Ahmadian 2005). Collectively, the Rho family GEFs and GAPs perform opposing functions, but their multi-functional domain structures work in concert to control GTPase activation.

The third and final group of Rho GTPase regulatory molecules is composed of the guanine nucleotide dissociation inhibitors (GDIs) (Garcia-Mata et al. 2011). While the Rho family GEFs and GAPs greatly outnumber the Rho GTPases, there are only three known Rho GDIs – Rho GDI1-3 (Garcia-Mata et al. 2011). Early characterization of GDIs revealed that they can block the dissociation of GDP bound to GTPases (Ueda et al. 1990). These GTPases contain a specific post-translational modification – the addition of a geranylgeranyl group to the C-terminus (Maltese 1990) – that is responsible for attaching the GTPases to the plasma membrane prior to activation (Schaber et al. 1990). Rho GDIs prevent nucleotide exchange by sequestering inactive (GDP-bound) GTPases in the cytoplasm, which is accomplished by shielding the GTPase’s C-terminal geranylgeranyl group so that it cannot be inserted into the plasma membrane (Hori et al. 1991). Structural analysis has revealed that GDIs each contain an N-terminal GTPase switch loop binding region and a C-terminal geranylgeranyl binding region (Grizot et al. 2001). The C-terminal geranylgeranyl-associated region contains numerous hydrophobic residues to provide a favorable interaction with the lipid moiety (Hoffman et al. 2000; Grizot et al. 2001). Also, this region is more stable than the N-terminus (Keep et al. 1997).

The flexibility of the N-terminus is thought to assist in another function of GDIs: extraction of GTPases from the membrane (Nomanbhoy et al. 1999). This extraction mechanism could be accomplished by the stepwise binding of the GDI to the GTPase (via N-terminal interactions), followed by binding to the geranylgeranyl group (via C-terminal interactions) (Cherfils and Zeghouf 2013). Intriguingly, Rho GDIs can bind to both GDP- and GTP-bound GTPases, suggesting a protein chaperone role for GDIs (Hancock and Hall 1993). While the sequestering function of GDIs has been established, the mechanism by which the GTPase is released from the GDI is not fully known (Cherfils and Zeghouf 2013). One hypothesis is that the pattern of GDI phosphorylation can differentially mediate GTPase dissociation, as evidenced by the dissociation of Rac – but not Rho – by PAK-dependent phosphorylation of GDI (DerMardirossian et al. 2004). Once again, these results highlight the fine-tuned regulation of GTPase activity in the cells. Determining how the numerous GEFs, GAPs, and GDIs coordinate their activities is crucial for developing a more complete understanding of complex GTPase-centered cellular functions.

Regulation of Rho GTPases in dendritic spines – Because of the large number of GTPase regulatory proteins, the control of Rho GTPase activity by GEFs and GAPs during the process of dendritic spine formation represents a fruitful area of research. Recent studies have begun to elucidate the complex regulation of GTPases in spines (Tolias et al. 2011). GEFs that activate Rac or Cdc42 promote the formation of dendritic spines (Irie and Yamaguchi 2002; Ma et al. 2003; Zhang et al. 2003; Tolias et al. 2005), while GEFs that activate Rho promote spine retraction (Ryan et al. 2005; Margolis et al. 2010). For example, a reduction in expression of the Rac-specific GEF T lymphoma invasion and metastasis 1 (Tiam1) caused a decrease in dendritic spine and synapse density and blocked NMDA receptor-dependent spine growth (Tolias et al. 2005). Conversely, exogenous expression of the Rho-specific GEF Lbc (lymphoid blast crisis)'s first cousin (Lfc) caused an increase in longer, more immature protrusions (Ryan

et al. 2005), and reduction in Lfc expression inhibited the loss of spines caused by treatment with the AMPA receptor inhibitor 2,3-dihydroxy-6-nitro-7-sulfamoyl-beno(F)quinoxaline (NBQX) (Kang et al. 2009). These results mirror the aforementioned roles of Rac and Rho in spine formation and retraction, respectively (Luo 1996; Tashiro et al. 2000). Also, their opposing functions underscore the link between strict control of actin dynamics and synaptic signaling. Compared to the study of Rho family GEFs, not as much is known about the role of GAPs or GDIs in regulating spine and synapse development. In fact, there are currently no published reports of GDI-mediated spine formation. As would be expected, the reported functions of GAPs are counter to that of the corresponding GEFs. GAPs that inactivate Rac or Cdc42, for example, reduce dendritic spine density (Van de Ven et al. 2005; Buttery et al. 2006). Exogenous expression of the Rac-specific GAP α1-chimaerin causes a loss of spines (Van de Ven et al. 2005; Buttery et al. 2006). GAPs that inactivate Rho, however, promote spine formation and growth (Zhang and Macara 2008; Nadif Kasri et al. 2009). This is demonstrated by the GAP oligophrenin-1, which regulates dendritic spine length and modulates synaptic signaling by stabilizing AMPA receptors at the synapse (Govek et al. 2004; Nadif Kasri et al. 2009). In all, these studies reveal the complexity of balancing active GTPase levels. Future work is necessary to elucidate how these GEFs and GAPs are differentially regulated within spines; perhaps they have overlapping functions, or they could be activated by distinct upstream signaling proteins that are regulated in a spatiotemporal fashion (Tolias et al. 2011).

Since Rho GTPase regulatory proteins are integral for the formation and maintenance of dendritic spines, they are also implicated in the signaling mechanisms that underlie learning and memory (Tolias et al. 2011). For example, the Rac GEF kalirin-7 interacts with the AMPA receptor subunit GluR1, and knockdown of kalirin-7 reduces AMPA receptor-dependent synaptic transmission by reducing the number of AMPA receptors at the synapse (Xie et al. 2007). Kalirin-7 also interacts with the NMDA receptor subunit NR2B (Kiraly et al. 2011). In kalirin-7 knockout mice, NMDA receptor-dependent LTP and LTD are perturbed, and defects in

memory formation have been observed (Ma et al. 2008; Lemtiri-Chlieh et al. 2011). Similarly, the Rac GEF Tiam1 interacts with NMDA receptors (Tolias et al. 2005); Tiam1-mediated Rac activation has been implicated in both LTP, via interaction with tropomyosin-related kinase B (TrkB) (Lai et al. 2012), and LTD, via interaction with microtubule-associated protein 1B (MAP1B) (Benoist et al. 2013). Furthermore, the Rac GAPs breakpoint cluster region (Bcr) and active Bcr-related (Abr) are important for synaptic plasticity, since loss of these proteins inhibits the maintenance of LTP and perturbs memory-associated tasks (Oh et al. 2010). Just as it is necessary to elucidate the effects of GEF and GAP signaling on structural plasticity, it is also crucial to investigate how these regulatory proteins affect the strength of long-term synaptic communication, which underlies learning and memory.

The Asef Family of GEFs

One important group of GEFs includes the members of the Asef (APC-stimulated guanine nucleotide exchange factor) family, which belongs to the larger Dbl family of Rho GEFs. There are three known GEFs in this family: Asef1, Asef2, and collybistin (Figure 6). As their names suggest, Asef1 and Asef2 were initially identified because of their interaction with the tumor suppressor adenomatous polyposis coli (APC) (Kins et al. 2000; Hamann et al. 2007; Kawasaki et al. 2007); collybistin, which does not bind to APC (Reid et al. 1999; Kins et al. 2000), was grouped into the Asef family based on homology (Harvey et al. 2004). These GEFs have been implicated in numerous cellular processes, including cell migration (Kawasaki et al. 2003; Hamann et al. 2007; Kawasaki et al. 2007), cell-cell contact (Kawasaki et al. 2003; Muroya et al. 2007), tumor growth (Mitin et al. 2007; Kawasaki et al. 2009a; Kawasaki et al. 2010; Lyons et al. 2010), angiogenesis (Kawasaki et al. 2010), translation initiation (Sertie et al. 2010), scaffold protein and neurotransmitter receptor clustering (Kins et al. 2000; Harvey et al. 2004; Papadopoulos et al. 2007), and synaptic transmission (Papadopoulos et al. 2007;

Jedlicka et al. 2009; Chiou et al. 2011). This diversity of roles is due to the modulation of GTPase signaling through diverse upstream signals that converge on these GEFs.

Asef1 – Asef1 (*ARHGEF4*) was identified in a yeast two-hybrid screen for proteins that interact with the armadillo repeat domain of human APC (APC^{ARM}) (Kawasaki et al. 2000), as well as in a human neuronal teratocarcinoma screen (Thiesen et al. 2000). This protein contains an APC binding region (ABR), a Src homology 3 (SH3) domain, a Dbl homology (DH) domain, and a pleckstrin homology (PH) domain (Kawasaki et al. 2000; Thiesen et al. 2000). Crystal structure analysis has shown that Asef1 exists in an autoinhibited conformation, in which the SH3 and DH domains form extensive contacts that block GTPase binding and sterically hinder binding of APC to Asef1 (Mitin et al. 2007; Murayama et al. 2007; Zhang et al. 2012). It is hypothesized that intrinsic fluctuations in the autoinhibitory state of Asef1 allow APC^{ARM} to bind to the ABR when Asef1 is transiently in the "open" conformation (Zhang et al. 2012). Since APC^{ARM} binds very tightly to Asef1, this would promote a shift in equilibrium so that Asef1 is predominantly in the "open" conformation, leading to DH-domain-mediated GTPase activation (Zhang et al. 2012). The specificity of APC^{ARM}-mediated GTPase activation by Asef1 is currently unclear. Several groups have shown that Asef1 only activates Cdc42 (Gotthardt and Ahmadian 2007; Mitin et al. 2007), while others have reported that Asef1 can also activate Rac (Kawasaki et al. 2000). This discrepancy could be due to cell-type-specific GTPase selectivity by Asef1 (Kawasaki et al. 2007).

Asef1's function has been well-studied, particularly concerning its roles in cell migration, cell-cell adhesion, and tumor formation. Early work using short hairpin RNAs (shRNAs) targeting Asef1 showed that knockdown of endogenous Asef1 reduces cell migration in an APC-dependent manner (Kawasaki et al. 2003). Upstream signaling by various growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF), induces the localization of both Asef1 and APC at the leading edge of

migrating cells, where APC can then stimulate Asef1's GEF activity and promote Rac/Cdc42 activation, actin polymerization, and cell migration (Itoh et al. 2008; Kawasaki et al. 2009b). Both the localization of Asef1 and its role in migration are mediated by Asef1's PH domain, which preferentially binds to phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the plasma membrane (Muroya et al. 2007; Kawasaki et al. 2009b). Asef1's presence at the membrane could also be important for the formation of cell-cell contacts. Expression of constitutively active Asef1 (i.e. lacking the ABR and SH3 domains) causes a decrease in the localization of the cell-cell junction protein E-cadherin at the plasma membrane of Madin Darby canine kidney (MDCK) cells (Kawasaki et al. 2003); however, conflicting results from the same group later showed that constitutively active Asef1 causes an increase in membrane-associated E-cadherin in MDCK II cells (Muroya et al. 2007). This discrepancy has been attributed to differences in signaling when the cultured cells are sparser (less membrane-associated E-cadherin) or confluent (more E-cadherin at cell-cell junctions) (Kawasaki et al. 2013). Finally, recent work has implicated Asef1 in the formation and growth of tumor cells. Asef1 is highly expressed in colorectal tumors (Kawasaki et al. 2009a) and in TEL-AML1 leukemias (Lyons et al. 2010), and mutations in Asef1 have been found in patients with breast cancer (Sjoblom et al. 2006). Genetic deletion of Asef1 in mice resulted in the reduction of adenoma number and size in an $APC^{Min/+}$ background (Kawasaki et al. 2009a), which is a common model of colorectal cancer (Su et al. 1992).

Furthermore, deletion of Asef1 inhibited the growth of implanted melanoma cells and reduced the number of tumor blood vessels (Kawasaki et al. 2010); this latter result points to Asef1 as a regulator of angiogenesis, which is a crucial component of tumor growth (Ferrara and Kerbel 2005). Recently, a pathway linking Asef1, the receptor NOTCH3, and the microRNA (miRNA)miR-1 to the formation of colorectal tumors has been elucidated (Furukawa et al. 2013). Asef1 expression is upregulated in colorectal tumors by NOTCH3 signaling, which is repressed by miR-1 (Furukawa et al. 2013). This pathway represents an enticing avenue for colorectal

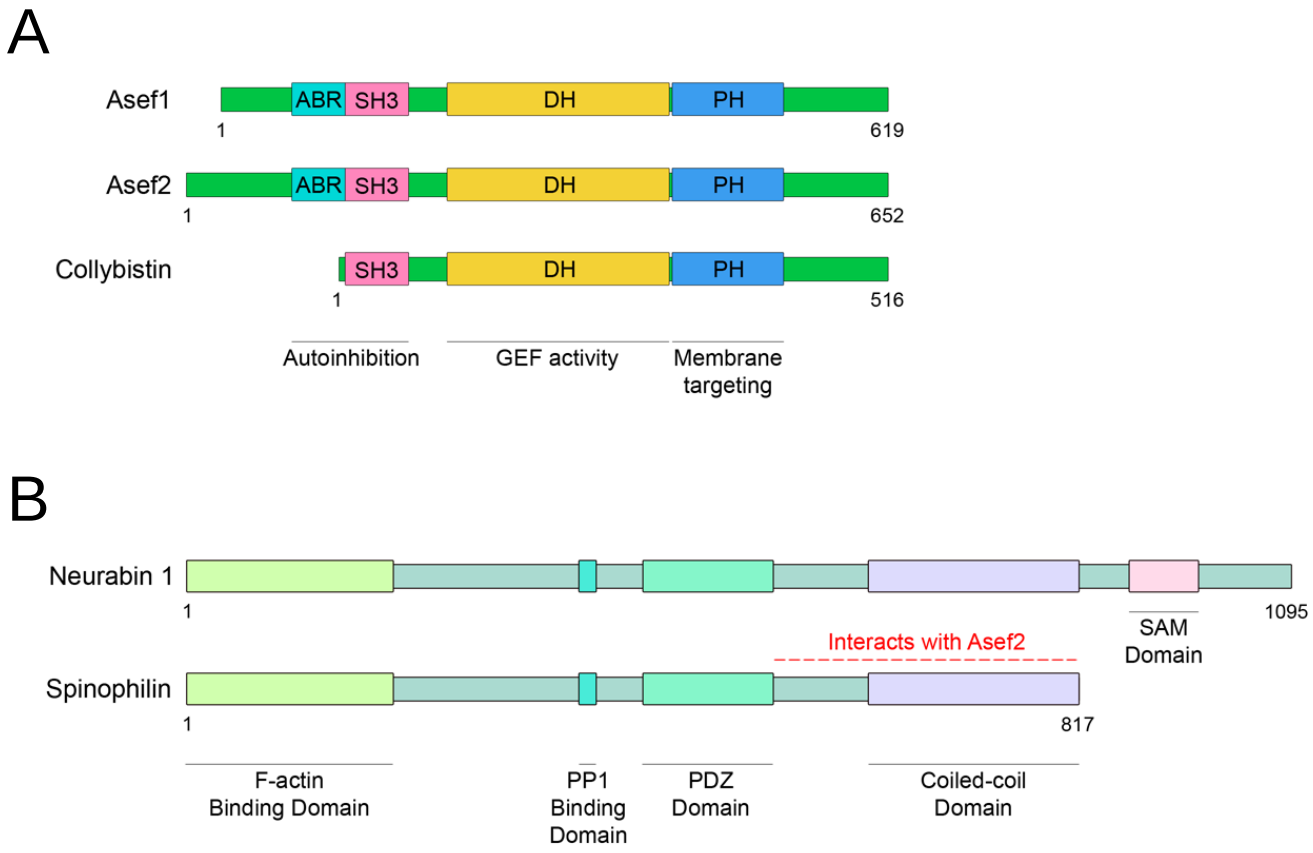

Figure 6. The Asef and neurabin protein families. *A*, Domain structures for Asef1, Asef2, and collybistin. The domain numbering is based on (Kawasaki et al. 2007). *B*, Domain structures for neurabin 1 and spinophilin. The domain numbering is based on (Kelker et al. 2007).

cancer treatments. In all, proper regulation of Asef1 activity is crucial for preventing the formation of cancer.

Collybistin – Collybistin (hPEM-2, *ARHGEF9*) was identified in a screen for potential Rho family GEFs (Reid et al. 1999) and in a yeast two-hybrid screen for proteins that interact with the inhibitory synapse scaffold protein gephyrin (Kins et al. 2000). Like Asef1, collybistin contains tandem DH-PH domains that are characteristic of most Dbl family GEFs (Rossman et al. 2005) (Figure 6). The SH3 domain of collybistin, however, is not always present, since isoforms lacking the SH3 domain have been identified (Kins et al. 2000; Harvey et al. 2004). Even though collybistin does not have an ABR, the SH3 domain is still thought to act in an autoinhibitory manner because the absence of this domain enhances collybistin function, including GTPase activation (Kins et al. 2000; Xiang et al. 2006; Reddy-Alla et al. 2010). The lack of an ABR also necessitates a collybistin-activating protein other than APC; in fact, both neuroligin 2, a cell-cell adhesion protein, and the α2 subunit of the GABA type A neurotransmitter receptor ($GABA_AR$) have been shown to promote the activation of collybistin (Poulopoulos et al. 2009; Saiepour et al. 2010). An alternative mechanism for collybistin activation involves the Cdc42-like GEF TC10, which binds to the PH domain – not the SH3 domain – of collybistin and stimulates GABAergic neurotransmission (Mayer et al. 2013). While Asef1 can activate both Rac and Cdc42 (Kawasaki et al. 2000; Gotthardt and Ahmadian 2007; Mitin et al. 2007), collybistin only activates Cdc42 (Reid et al. 1999; Xiang et al. 2006). Another key difference between Asef1 and collybistin is that, while Asef1 has a broader expression profile, including brain, colon, and skeletal muscle (Kawasaki et al. 2000; Hamann et al. 2007), collybistin is almost exclusively expressed in the brain (Reid et al. 1999; Kneussel et al. 2001; Hamann et al. 2007).

Collybistin's most well-characterized role in neurons is to promote the clustering of the scaffold protein gephyrin at sites of inhibitory synapses (Kins et al. 2000; Harvey et al. 2004).

This function is enhanced when the SH3 domain is absent (Harvey et al. 2004), emphasizing the importance of this domain in regulating collybistin activity. Furthermore, collybistin-dependent gephyrin clustering is mediated by the GEF's PH domain, which selectively binds to phosphatidyl 3-phosphate (PI3P) at the plasma membrane (Kalscheuer et al. 2009). Deletion of the PH domain or mutations that disrupt PIP binding both inhibit gephyrin clustering (Reddy-Alla et al. 2010; Tyagarajan et al. 2011); intriguingly, gephyrin clustering is not regulated by collybistin's GEF activity (Reddy-Alla et al. 2010). Targeting of gephyrin to inhibitory synapses by collybistin also promotes the clustering of GABAergic neurotransmitter components, including $GABA_AR$ subunits (Harvey et al. 2004; Papadopoulos et al. 2007). The importance of collybistin-mediated gephyrin clustering is emphasized in collybistin knockout mice, which exhibit reduced inhibitory neurotransmission (Papadopoulos et al. 2007; Jedlicka et al. 2009). Furthermore, mutations in collybistin have been associated with intellectual disability, epilepsy, and hyperekplexia, a condition characterized by an enhanced startle response (Harvey et al. 2004; Marco et al. 2008; Kalscheuer et al. 2009). In one patient with hyperekplexia, for example, a glycine to alanine mutation at residue 55 (G55A) in the SH3 domain of collybistin causes a reduction in gephyrin and $GABA_AR$ clustering (Harvey et al. 2004; Tyagarajan et al. 2011). Therefore, collybistin's ability to localize gephyrin and GABAergic signaling molecules is crucial for efficient inhibitory neurotransmission.

Asef2 – The final member of the Asef family of GEFs is Asef2 (SPATA13), which bears many similarities to Asef1. For example, Asef2 shares the same domain architecture as Asef1 (Figure 6), including the presence of an ABR (Hamann et al. 2007; Kawasaki et al. 2007). A crystal structure for Asef2 has not been determined; therefore, the mechanism of Asef2 activation is thought to mirror that of Asef1 (i.e. ABR-SH3-mediated release from autoinhibition by binding of the APC^{ARM}) (Hamann et al. 2007; Kawasaki et al. 2007). This is substantiated by deletion of Asef2's ABR-SH3 region, which enhances GTPase activation (Hamann et al. 2007;

Kawasaki et al. 2007). The C-terminus is also hypothesized to regulate Asef2's autoinhibitory state via interactions with the N-terminus that impede GTPase activation (Hamann et al. 2007) Another similarity to Asef1 is that the GTPase specificity of Asef2 is cell-type specific (Kawasaki et al. 2007); Asef2 has been shown to activate both Cdc42 (Hamann et al. 2007; Bristow et al. 2009) and Rac (Kawasaki et al. 2007; Bristow et al. 2009), as well as indirectly cause a reduction in active Rho (Bristow et al. 2009). As a result, expression of either wild-type or constitutively active Asef2 promotes cell migration (Kawasaki et al. 2007; Bristow et al. 2009) and the assembly and disassembly of cellular adhesions (Bristow et al. 2009). In randomly migrating cells plated on fibronectin, this increase in cell migration is dependent on Rac, but not Cdc42, activation and on a pathway involving phosphoinositol 3-kinase (PI3K) and Akt (Bristow et al. 2009). Surprisingly, when Asef2-expressing cells are plated on type 1 collagen, instead of fibronectin, cell migration is inhibited (Jean et al. 2013); this effect is mediated by activation of Rac, which promotes the activation of myosin II, thereby enhancing cell contractility, and slows the turnover of cell adhesions (Jean et al. 2013). In HeLa cells expressing Asef2-targeting small interfering RNAs (siRNAs), HGF-promoted cell migration is abrogated (Sagara et al. 2009); therefore, Asef2 – like Asef1 (Kawasaki et al. 2009b) – is downstream of HGF signaling. Furthermore, Asef2 is highly expressed in colorectal tumors, and deletion of Asef2 is mice resulted in reduction in adenoma growth and in the number of angiogenic blood vessels (Kawasaki et al. 2009a). In fact, deletion of both Asef1 and Asef2 showed an even greater reduction in tumor growth (Kawasaki et al. 2009a). Therefore, Asef1 and Asef2 have overlapping roles in the regulation of cancer cell formation.

While the neuronal function of collybistin has been extensively studied, not much is known about the roles of Asef1 and Asef2 in the brain. Asef1 is expressed in the brain (Kawasaki et al. 2000; Hamann et al. 2007), and deletion or duplication of a region of chromosome 2, which contains Asef1, has been linked to attention-deficit hyperactivity disorder (ADHD), epilepsy, and intellectual disability (Dharmadhikari et al. 2012). This suggests that

Asef1 could regulate processes that are crucial for normal cognitive function, such as dendritic spine and synapse formation, but there is currently no evidence supporting this. Asef2, which is expressed in numerous regions of the brain (Yoshizawa et al. 2003; Becker et al. 2008; Walther and Mann 2011; Cajigas et al. 2012), has also been associated with neurological disorders. For example, deletion of a single nucleotide in Asef2 created a frameshift mutation in a child diagnosed with autism (Iossifov et al. 2012). Also, Asef2 was identified in an analysis of genes that are mutated more frequently in patients exhibiting both depression and alcohol dependence (Edwards et al. 2012). Another role for Asef2 in the brain is to mediate the migration of neuronal precursor cells (Toriyama et al. 2012). Phosphorylation of the microtubule-binding protein doublecortin (DCX) causes it to disassociate from microtubules. DCX then interacts with Asef2 and stimulates Asef2's GEF activity for Rac (Toriyama et al. 2012). Finally, Asef2 has been shown to interact with a scaffold protein, called spinophilin, that is integral for neuronal function (Sagara et al. 2009). This interaction was studied in the context of cell migration; spinophilin was shown to co-localize with both Asef2 and APC upon HGF stimulation, and knockdown of spinophilin inhibited cell migration and blocked Asef2-dependent filopodia formation (Sagara et al. 2009). The neuronal functions of spinophilin, including its role in dendritic spine formation, will be discussed later. Because Asef2 has been linked to neurological disorders, and because it interacts with an important neuronal signaling protein, Asef2 could be integral for processes underlying cognitive functions such as learning and memory. Chapter II describes a novel role for Asef2 in the formation of dendritic spines and synapses.

Regulation by phosphorylation – Investigations into the activation mechanism of the Asef family GEFs have mainly involved the interaction of proteins that induce conformational changes in the GEF and release it from autoinhibition (Xiang et al. 2006; Hamann et al. 2007; Mitin et al. 2007; Murayama et al. 2007). Other possible mechanisms of regulation, such as post-translational modification of the GEFs, could exist. The addition of moieties, such as

phosphate groups, to a protein is a common mechanism for tightly regulating the protein's activity (Walsh et al. 2005). Not much is known about the post-translational modification of Asef family GEFs; for example, the phosphorylation state of collybistin has not been elucidated. Conversely, phosphorylation of Asef1 on tyrosine 94 – in the ABR – by Src family kinases was shown to promote Rac and Cdc42 activation upon stimulation with EGF (Itoh et al. 2008). Although this tyrosine is conserved in Asef2, Asef2 is not phosphorylated at this site (Itoh et al. 2008). This result suggests that Asef1 and Asef2 could be differentially regulated via distinct phosphorylation sites. Therefore, it is important to identify putative phosphorylation sites in these GEFs. Chapter III details the mapping of phosphorylation sites on Asef2, as well as the effect of phosphorylation on Asef2-dependent Rac activation, cell migration, and adhesion turnover.

The Neurabin Family of Actin-Binding Proteins

In cells, multiple signaling networks can be integrated by scaffold proteins, which utilize diverse protein-protein interaction domains to simultaneously bind regulatory proteins and coordinate their activity. One such group of proteins, the neurabin (neural tissue-specific F-actin binding protein (Nakanishi et al. 1997)) family, is a well-studied example of the multiple roles that scaffolds can perform. This family comprises two structurally related proteins, neurabin 1 and spinophilin (also known as neurabin 2), that are integral for a wide range of processes, including organization of the actin cytoskeleton (Nakanishi et al. 1997; Satoh et al. 1998; Schuler and Peti 2008), modulation of neurotransmitter receptor activity and synaptic plasticity (Yan et al. 1999; Feng et al. 2000; Terry-Lorenzo et al. 2002; Terry-Lorenzo et al. 2005; Allen et al. 2006; Hu et al. 2006; Hu et al. 2007), tumor progression (Ferrer et al. 2011a; Ferrer et al. 2011b; Molina-Pinelo et al. 2011; Aigelsreiter et al. 2013; Estevez-Garcia et al. 2013; Aigelsreiter et al. 2014), neurite outgrowth (Nakanishi et al. 1997; Orioli et al. 2006; Causeret et al. 2007), platelet activation (Ma et al. 2012), and cell migration (Causeret et al. 2007; Sagara et

al. 2009). Therefore, investigating the proteins that are regulated by the neurabin family is vital for our understanding of these diverse cellular activities.

Domain organization and actin binding – Neurabin 1 and spinophilin possess a similar domain structure (Figure 6). Given their primary roles as scaffold proteins, it is not surprising that they both contain several domains associated with protein-protein interactions. These include a postsynaptic density 95 (PSD95) / Discs large (DLG) / zona occludens 1 (ZO-1) (PDZ) domain and a C-terminal coiled-coil domain (Allen et al. 1997; Nakanishi et al. 1997; Satoh et al. 1998). The coiled-coil domain also allows the neurabins to form homo- and heterodimers. Neurabin 1 also has a sterile α motif (SAM) domain at its C-terminus that is hypothesized to interact with other proteins but is not a dimerization domain (Ju et al. 2007). In addition, neurabin 1 and spinophilin contain a small protein phosphatase 1 (PP1)-binding domain (Allen et al. 1997; MacMillan et al. 1999), whose function will be described shortly. Finally, the neurabins possess an N-terminal F-actin binding (FAB) domain that is responsible for binding, bundling, and cross-linking actin filaments (Nakanishi et al. 1997; Satoh et al. 1998; Terry-Lorenzo et al. 2005; Schuler and Peti 2008). An actin capping role for this domain has also been postulated (Barnes et al. 2004; Schuler and Peti 2008). Phosphorylation of neurabin 1 and spinophilin by kinases such as PKA (McAvoy et al. 1999; Hsieh-Wilson et al. 2003) and CAMKII (Grossman et al. 2004) inhibits actin binding; this provides a mechanism for regulating the localization of spinophilin-bound proteins (Hsieh-Wilson et al. 2003). The FAB domain is responsible for localizing the neurabins to distinct subcellular regions (Grossman et al. 2002; Oliver et al. 2002; Barnes et al. 2004). The domain organization of the neurabins makes them perfectly suitable as scaffolds because they can utilize their diverse protein-protein interaction domains to link signaling molecules to the cell's cytoskeleton.

Roles in synaptic signaling and spine formation – While spinophilin is expressed in multiple tissues, including the brain (Allen et al. 1997; Satoh et al. 1998), neurabin 1 is expressed almost exclusively in the brain (Nakanishi et al. 1997; Burnett et al. 1998). The neurabins are particularly enriched in dendritic spines (Allen et al. 1997; Nakanishi et al. 1997; Satoh et al. 1998; Muly et al. 2004a; Muly et al. 2004b), which makes sense because spines are themselves enriched in F-actin (Wulf et al. 1979; Drenckhahn et al. 1984; Allison et al. 1998). The neurabins' localization to spines, as well as their likely scaffolding function, made them attractive candidates for regulators of synaptic activity. In fact, their most well-characterized role is to direct the localization of PP1 (Allen et al. 1997; MacMillan et al. 1999; McAvoy et al. 1999; Terry-Lorenzo et al. 2002; Carmody et al. 2004), a phosphatase that regulates the activity of multiple neurotransmitter receptors (Munton et al. 2004). Both neurabins have been shown to preferentially interact with the γ1 isoform of PP1 (Terry-Lorenzo et al. 2002; Carmody et al. 2004; Carmody et al. 2008), which localizes to spines (Ouimet et al. 1995; Strack et al. 1999; Bordelon et al. 2005). PP1 dephosphorylates glutamatergic neurotransmitters, thus inactivating them (Wang et al. 1991; Wang et al. 1994). Mutation of the PP1-binding region of either neurabin 1 or spinophilin disrupts PP1-mediated dephosphorylation of the AMPA receptor subunit GluR1 (Terry-Lorenzo et al. 2002), suggesting that spinophilin can regulate receptor activity by modulating the localization of PP1 (Yan et al. 1999). Furthermore, genetic deletion of spinophilin blocked the negative regulation by PP1 of both AMPA receptor and NMDA receptor currents (Feng et al. 2000). The neurabins can also interact directly with AMPA and NMDA receptor subunits (Kelker et al. 2007), thereby emphasizing their role in regulating receptor function.

The neurabin-mediated targeting of PP1 to glutamatergic receptors also has a physiological purpose – modulating synaptic plasticity. Expression of neurabin 1, but not a PP1-binding-deficient mutant, promoted LTD and and inhibited LTP in hippocampal slices (Hu et al. 2006); similarly, knockout of spinophilin was shown to inhibit LTD (Feng et al. 2000). These

alterations in synaptic transmission are also correlated with morphological changes. Expression of neurabin 1 promoted the formation of dendritic spines and immature filopodial protrusions (Oliver et al. 2002; Zito et al. 2004; Terry-Lorenzo et al. 2005). Intriguingly, knockout of spinophilin caused an increase in spine density in young, but not adult, mice (Feng et al. 2000). These opposing results could represent differential regulation of neurabin 1 versus spinophilin. Nevertheless, the neurabins are integral for proper synaptic signaling, as deficits in neurabin 1 or spinophilin have been linked to impaired contextual fear memory (Wu et al. 2008), enhanced depression-related responses (Kim et al. 2011), and neurological disorders such as schizophrenia (Law et al. 2004).

Neurabins as scaffold proteins for GEFs – PP1 is just one of many binding partners for the neurabin family. Rho family GEFs represent another important group of proteins whose localization is mediated by the neurabins. For example, spinophilin has been shown to promote the targeting of GEFs such as Tiam1 and Lfc to the plasma membrane in non-neuronal cells (Buchsbaum et al. 2003; Ryan et al. 2005; Rajagopal et al. 2010). Spinophilin also can regulate GEF activity, since knockdown of spinophilin was shown to block Tiam1-dependent Rac activation (Rajagopal et al. 2010). As stated earlier, spinophilin interacts with the GEF Asef2 and mediates cell migration (Sagara et al. 2009). Neurabin 1, however, does not associate with Asef2 (Sagara et al. 2009), suggesting that neurabin 1 and spinophilin can differentially regulate the function of GEFs. Not as much is known about neurabin 1's association with GEFs, except that it interacts with Lfc and with the neuron-specific GEF kalirin-7 (Penzes et al. 2001; Ryan et al. 2005). It is interesting to note that all of these GEFs are important for dendritic spine formation (Penzes et al. 2001; Ryan et al. 2005; Tolias et al. 2005; Zhang and Macara 2006; Tolias et al. 2007). Little is currently known about the functional role of neurabin 1- or spinophilin-dependent GEF localization in the process of spine development. Chapter II presents a novel mechanism for spinophilin-mediated spine formation. Also, these GEFs

interact with overlapping regions of the C-terminus of either neurabin 1 or spinophilin (Penzes et al. 2001; Buchsbaum et al. 2003; Ryan et al. 2005; Sagara et al. 2009); therefore, the neurabins represent an important hub for GEF signaling pathways. Future work is necessary to fully understand the complex coordination of Rho GEF function by the neurabin family.

Hypotheses

Rho family GTPase-mediated regulation of spine and synapse density must be tightly controlled to ensure efficient neuronal communication. Previous work has demonstrated that Rho family GEFs are integral for this process; however, many of the known GEFs are not well-characterized in spines. Asef2 is a GEF that has been predominantly studied in the context of cell migration, but its role in the dendritic spine formation is unknown. Its association with the spine-associated protein spinophilin, though, suggests that Asef2 could mediate spine and synapse development. Given that Asef2 activates Rac in migrating cells, we hypothesized that Asef2 could also promote Rac activity in neurons, leading to an increase in spine and synapse density. Spinophilin has been shown to direct Rho family GEFs to the plasma membrane; therefore, we also hypothesized that Asef2-mediated signaling could depend on targeting by spinophilin.

A second hypothesis involves the regulatory mechanism of Asef2 activity. While Asef2 autoinhibition has been studied, it is also possible that Asef2 function could be modulated by post-translational modifications such as phosphorylation. We hypothesized that Asef2 could be phosphorylated at distinct residues, and that the GEF activity of Asef2 could be dependent on Asef2's phosphorylation state. Therefore, mutation of putative phosphorylation sites to inhibit phosphorylation would block Asef2-mediated Rac activation. This would, in turn, disrupt Asef2's function in actin-dependent processes such as cell migration and adhesion turnover.

CHAPTER II

THE GUANINE NUCLEOTIDE EXCHANGE FACTOR (GEF) ASEF2 PROMOTES DENDRITIC SPINE FORMATION VIA RAC ACTIVATION AND SPINOPHILIN-DEPENDENT TARGETING

J. Corey Evans[1], Mingjian Shi[1], and Donna J. Webb[1,2]

[1]Department of Biological Sciences and the Kennedy Center for Research on Human Development, [2]Department of Cancer Biology, Vanderbilt University, Nashville, TN 37235

This article is in review at *The Journal of Biological Chemistry*.

Abstract

Dendritic spines are actin-rich protrusions that establish excitatory synaptic contacts with surrounding neurons. Reorganization of the actin cytoskeleton is critical for the development and plasticity of dendritic spines, which is the basis for learning and memory. Rho family GTPases are emerging as important modulators of spine and synapse function, predominantly through their ability to regulate actin dynamics. Much less is known, however, about the function of GEFs, which activate these GTPases, in spine and synapse development. In this study, we show that the Rho family GEF Asef2 is found at synaptic sites, where it promotes dendritic spine and synapse formation. Knockdown of endogenous Asef2 with shRNAs impairs spine and synapse formation, while exogenous expression of Asef2 causes an increase in spine and synapse density. This effect of Asef2 on spines and synapses is abrogated by expression of GEF-activity-deficient Asef2 mutants or by knockdown of Rac, suggesting that Asef2-Rac signaling mediates spine development. Because Asef2 interacts with the F-actin-binding protein spinophilin, which localizes to spines, we investigated the role of spinophilin in Asef2-promoted spine formation. Spinophilin recruits Asef2 to spines, and knockdown of spinophilin hinders spine and synapse formation in Asef2-expressing neurons. These results collectively point to spinophilin-Asef2-Rac signaling as a novel mechanism for the development of dendritic spines and synapses.

Introduction

Neurons form cell-cell junctions called synapses that comprise pre- and postsynaptic terminals that propagate signals from one neuron to another. Most excitatory synapses form on dendritic spines, which are postsynaptic protrusive structures enriched in actin (Gray 1959; Matus et al. 1982; Nimchinsky et al. 2002). Spines display a wide range of morphologies, from immature, filopodia-like protrusions to more mature protrusions composed of a mushroom-shaped spine head and a thin neck (Papa et al. 1995; Dailey and Smith 1996; Ziv and Smith 1996). Spine development and plasticity are essential for normal cognitive function and underlie processes such as learning and memory. Abnormalities in spine formation and morphology are associated with numerous neurological and intellectual disorders, including autism, schizophrenia, epilepsy, Fragile X syndrome, and Alzheimer's disease (Fiala et al. 2002; Penzes et al. 2011), underscoring the importance of these structures in cognition.

The development and morphological plasticity of dendritic spines is associated with the assembly and disassembly of actin filaments (Fischer et al. 1998; Matus 2000; Korobova and Svitkina 2010). Actin reorganization, in turn, is tightly regulated by the Rho family of small GTPases, including Rac, Cdc42, and Rho (Jaffe and Hall 2005). Like other small GTPases, the Rho GTPases, cycle between an active (GTP-bound) form and an inactive (GDP-bound) form. This cycling is regulated by two families of proteins: GEFs, which catalyze the exchange of GDP for GTP, and GTPase activating proteins (GAPs), which promote GTP hydrolysis (Trahey and McCormick 1987; West et al. 1990; Cherfils and Zeghouf 2013). While the roles of Rac, Cdc42, and Rho in modulating spine and synapse formation have been characterized (Luo 1996; Tashiro et al. 2000; Irie and Yamaguchi 2002; Scott et al. 2003; Wegner et al. 2008), much less is known about the GEFs and GAPs that regulate them. Recent work, however, suggests that these proteins play a critical role in spine development (Tolias et al. 2011). For example, the Rac GEF Tiam1 has been shown to mediate spine morphogenesis through its association with N-methyl-D-aspartate (NMDA)-type glutamate receptors, Eph receptors, and the polarity protein

PAR-3 (Tolias et al. 2005; Zhang and Macara 2006; Tolias et al. 2007). In addition, the Rho GAP oligophrenin-1 regulates the plasticity and maturation of spines and synapses, and loss of function of this protein is associated with some intellectual disorders (Billuart et al. 1998; Govek et al. 2004; Nadif Kasri et al. 2009). Therefore, these studies highlight the importance of GEFs and GAPs in regulating spine and synapse function and point to a need to investigate the contribution of other GEFs and GAPs to these processes.

Asef2 (SPATA13, FLJ31208) is a 652-amino acid GEF that activates Rac and Cdc42 (Hamann et al. 2007; Kawasaki et al. 2007). Asef2 is composed of several conserved domains that include: an adenomatous polyposis coli (APC) binding region (ABR), a Src homology 3 (SH3) domain, a Dbl homology (DH) domain, and a pleckstrin homology (PH) domain (Hamann et al. 2007) (Figure 6). The DH domain promotes GTP exchange, whereas the PH domain contributes to membrane localization (Hamann et al. 2007; Kawasaki et al. 2007; Muroya et al. 2007; Bristow et al. 2009). The ABR and SH3 domains work together to modulate the activation state of Asef2 (Hamann et al. 2007). The autoinhibited form of Asef2 prohibits nucleotide exchange via the DH domain; however, binding of the tumor suppressor APC to the ABR-SH3 tandem region induces a conformational change in Asef2, thus stimulating the GEF activity of this protein (Hamann et al. 2007; Murayama et al. 2007). In the mammalian brain, Asef2 is expressed in various regions, including the cerebral cortex, the amygdala, the olfactory bulb, and the hippocampus (Yoshizawa et al. 2003; Becker et al. 2008; Walther and Mann 2011; Cajigas et al. 2012), but its function in the central nervous system is currently not known.

Although most of the Asef2 binding partners remain to be identified, Asef2 has been shown to interact with the actin binding protein spinophilin (neurabin II) (Sagara et al. 2009). Spinophilin is highly expressed in the brain, and it localizes to dendritic spines in hippocampal neurons, through an N-terminal F-actin binding domain (Allen et al. 1997; Satoh et al. 1998; Grossman et al. 2002; Barnes et al. 2004). Spinophilin has been shown to regulate the formation and morphology of dendritic spines and to modulate glutamatergic synaptic

transmission (Feng et al. 2000). Moreover, spinophilin knockout mice display defects in associative learning (Stafstrom-Davis et al. 2001), further emphasizing the importance of this protein in regulating synaptic function. Spinophilin could mediate these effects on spines and synapses, at least in part, through its interaction with proteins such as Asef2. This led us to investigate the role of Asef2 in the development of dendritic spines and synapses.

In this study, we show that spinophilin recruits Asef2 to synaptic sites. Asef2, in turn, promotes the formation of dendritic spines and synapses in hippocampal neurons by a Rac-dependent signaling mechanism. These results indicate that spinophilin-Asef2-Rac signaling is important in spine and synapse development.

Experimental Procedures

Reagents – Asef2 polyclonal antibody was made by 21st Century Biochemicals (Marlboro, MA), as previously described (Bristow et al. 2009). Spinophilin polyclonal antibody was obtained from Novus Biologicals (Littleton, CO). SV2 monoclonal antibody was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). PSD95 monoclonal antibody (clone 7E3-1B8) was purchased from EMD Millipore (Billerica, MA). FLAG monoclonal antibody (clone M2) was obtained from Sigma (St. Louis, MO). Alexa Fluor® 488 anti-rabbit, Alexa Fluor® 555, 647, and 680 anti-mouse, Alexa Fluor® 546 phalloidin, and ProLong® Gold antifade reagent were purchased from Life Technologies (Grand Island, NY). Aqua-Poly/Mount was from Polysciences, Inc. (Warrington, PA).

Plasmids – Full length human Asef2 cDNA (Bristow et al. 2009) was tagged with enhanced green fluorescent protein (EGFP, from Clontech, Mountain View, CA) by insertion into a neuronal expression vector (pTαS2) that contains the neuron-specific α1-tubulin promoter (Gloster et al. 1999). This vector was kindly provided by Freda Miller (University of Toronto, Toronto, Ontario). Asef2 GEF-activity deficient mutants were generated by site-directed muta-

genesis using the following primers: Asef2-K382A, forward (5'-CTCACACCAGTGC-AGGCGATCTGCAAATAC-3') and reverse (5'-GTATTTGCAGATCGCCTGCACTGGTGTGAG-3'); Asef2-K385A, forward (5'-CAGAAGATCTGCGCATACCCGCTGCAG-3') and reverse (5'CTGCAGCGGGTATGCGCAGATCTTCTG-3'). Asef2 short hairpin RNA (shRNA) constructs were created by inserting 64-mer sense and antisense oligonucleotides into the pSUPER vector, as described previously (Zhang and Macara 2008). The oligonucleotides contained the following target sequences: Asef2 shRNA #1 (5'-CCAGCAGATGATCGATATA-3'), and Asef2 shRNA #2 (5'-GCGACTACAACAATATAAA-3'). A non-targeting shRNA was constructed by inserting the sequence 5'-CAGTCGCGTTTGCGACTGG-3' into the pSUPER vector. Similarly, Rac shRNAs were generated using the following target sequences: Rac shRNA #1 (5'-GTGGTATCCTGAAGTACGA-3'), and Rac shRNA #2 (5'-GCAAACAGACGTGTTCTTA-3'). shRNAs targeting spinophilin were made using the following target sequences: spinophilin shRNA #1 (5'-GGACTATGACCGACGCAAT-3'), and spinophilin shRNA #2 (5'-AGGAGAATAAGGAGCGGAT-3'). Rac1 cDNA and glutathione-S-transferase (GST)-tagged p21-activated kinase (PAK) binding domain (PBD) cDNA were generously provided by Alan Hall (Memorial Sloan-Kettering Cancer Center, New York, NY). mCherry cDNA was a kind gift from Roger Tsien (University of California, San Diego, CA). Spinophilin cDNA, kindly provided by Roger Colbran (Vanderbilt University, Nashville, TN), was inserted into pTαS2 vector containing mCherry. mCerulean cDNA, which was a generous gift from David Piston (Vanderbilt University, Nashville, TN), was also inserted into pTαS2 vector.

Cell culture and transfection – Hippocampal neurons were isolated from day 19 rat embryos and were cultured at low density using an established protocol (Goslin 1998). At day *in vitro* (DIV) 5, the neurons were transfected using a modified calcium phosphate protocol (Wegner et al. 2008). Rat 2 fibroblasts (R2Fs) and HT1080 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Grand Island, NY) that was supplemented

with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA) and penicillin/streptomycin (Life Technologies, Grand Island, NY). HT1080 cells were transfected using Lipofectamine® 2000 (Life Technologies, Grand Island, NY), and R2Fs were transfected using an Amaxa Nucleofector™ kit (Lonza, Cologne, Germany), according to the manufacturer's instructions.

Immunocytochemistry – For SV2 and phalloidin staining, neurons were fixed at DIV11 with 4% paraformaldehyde (PFA)/4% sucrose in phosphate-buffered saline (PBS) for 15 min at room temperature. For PSD95 staining, DIV11 neurons were fixed with PFA/sucrose for 3 min at room temperature, followed by ice cold methanol for 10 min. Separate sets of neurons were used to stain for SV2 and PSD95. For endogenous protein staining, neurons were fixed at DIV14 with either PFA/sucrose for 3 min and then cold 10% formalin for 10 min, or PFA/sucrose for 15 min. After three washes in PBS, coverslips were permeabilized with 0.2% Triton X-100 in PBS for 10 min and washed three times again. The coverslips were blocked with 20% goat serum in PBS for approximately 1 h. Primary antibodies were diluted in 5% goat serum in PBS, and coverslips were incubated with the antibodies overnight at 4°C. After at least 1 h of washes in PBS, the coverslips were incubated with secondary antibodies, which were diluted in 5% goat serum in PBS, for 45 min at room temperature. The coverslips were washed again for 1 h and were then mounted with either ProLong® Gold antifade reagent or Aqua Poly/Mount for visualization.

Microscopy and image analysis – Fixed neurons were visualized with a Retiga EXi CCD camera (QImaging, Surrey, British Columbia) linked to an Olympus IX71 inverted microscope (Melville, NY) with a PlanApo 60X OTIRFM objective (NA 1.45). MetaMorph software (Molecular Devices, Sunnyvale, CA), which was integrated with a Lambda 10-2 automated controller (Sutter Instruments, Novato, CA), was used to acquire and analyze

images. For Alexa Fluor® 488 and enhanced GFP images, an Endow GFP Bandpass filter cube (excitation HQ470/40, emission HQ525/50, Q495LP dichroic mirror) (Chroma, Brattleboro, VT) was utilized. A TRITC/Cy3 cube (excitation HQ545/30, emission HQ610/75, Q570LP dichroic mirror) was used to visualize mCherry, as well as Alexa Fluor® 546 and 555. For Alexa Fluor® 647 imaging, a Cy5^{TM} cube (excitation HQ620/60, emission HQ- 700/75, Q660LP dichroic mirror) was utilized.

A Quorum WaveFX-X1 spinning disk confocal system containing a Yokogawa CSU-X1 spinning disk (Yokogawa Electric Corporation, Newnan, GA) with Borealis upgrade/modifications (Guelph, Canada) was utilized for live-cell imaging. Images were obtained via an EM-CCD camera (Hamamatsu, Hamamatsu City, Japan) on a Nikon Eclipse Ti microscope (Melville, NY) with MetaMorph software and an Apo TIRF 60X objective (NA 1.49). mCerulean, GFP, and mCherry images were acquired by exciting laser lines at 441nm, 491 nm, and 561 nm, respectively (Semrock, Rochester, NY); the emission filters for these fluorophores were 470/24, 525/50, and 593/40, respectively (Semrock, Rochester, NY). Neurons were maintained in 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 30 mM glucose, pH 7.4 at 37°C using a temperature-controlled chamber (Live Cell Instrument, Seoul, Korea).

Dendritic spine and synapse densities were quantified along primary and secondary dendrites. Spines were defined as protrusions that contacted presynaptic terminals (identified by immunostaining for SV2). For analysis of knockdown efficiency in neurons, DIV11 cells were immunostained for the endogenous protein, and the average fluorescence intensity of staining in the soma was obtained. To measure Asef2 localization to spines, neurons were analyzed as previously described (Lin et al. 2010). SPSS Statistics, version 22 (Armonk, NY), was used for statistical analyses. Student's t-tests were used to compare two means. One-way ANOVA was performed to compare multiple means, followed by post hoc tests (Games-Howell pairwise comparison tests) to determine the level of significance ($p < 0.05$). Data are shown as means ± s.e.m.

Rac activity assay – This assay was performed as described previously (Knaus et al. 2007; Jean et al. 2013). Briefly, HT1080 cells were transiently co-transfected with FLAG-tagged Rac cDNA and either GFP, GFP-Asef2, GFP-Asef2-K382A, or GFP-Asef2-K385A cDNAs. After 24 h, the cells were lysed and incubated with GST-tagged PBD, which was bound to glutathione sepharose beads (GE Healthcare Life Sciences, Pittsburgh, PA) for 1 h at 4°C with end-over-end mixing. The amount of active Rac pulled down by the GST-PBD beads was assayed by Western blot. For quantification, the amounts of active Rac were normalized to the total Rac amounts.

Results

Endogenous Asef2 is in dendritic spines and synapses – Previous work has shown that Asef2 is expressed in various regions of the mammalian brain (Yoshizawa et al. 2003; Becker et al. 2008; Walther and Mann 2011; Cajigas et al. 2012), including the hippocampus (Walther and Mann 2011; Cajigas et al. 2012). Since the hippocampus is functionally linked to learning and memory (Scoville and Milner 1957; Bird and Burgess 2008), which are processes that are dependent on proper dendritic spine formation (Engert and Bonhoeffer 1999; Maletic-Savatic et al. 1999; Leuner et al. 2003; Nagerl et al. 2004; De Roo et al. 2008), we hypothesized that Asef2 plays a role in the development of spines. To test this hypothesis, we first assessed the localization of endogenous Asef2 in hippocampal neurons. In DIV14 neurons, Asef2 was seen throughout the dendrites, including in small puncta along the dendrites that appeared to be spines (Figure 7). To show that these dendritic puncta were spines, we co-stained for two synaptic markers, synaptic vesicle protein 2 (SV2) and postsynaptic density protein 95 (PSD95). Indeed, Asef2 puncta were observed with the synaptic markers (Figure 7), suggesting that Asef2 is present at synaptic sites (i.e. dendritic spines).

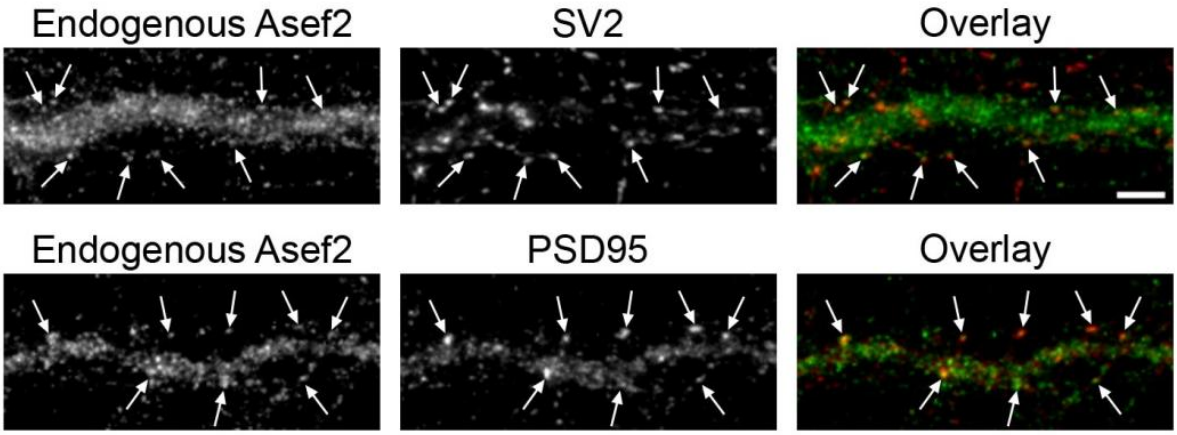

Figure 7. Asef2 is present at synaptic sites. Hippocampal neurons were fixed at day *in vitro* (DIV) 14 and immunostained for endogenous Asef2 and SV2 (upper panels) or PSD95 (lower panels). Overlays of the images show that Asef2 puncta is found with the synaptic markers (right panels, arrows). Bar, 5 μm.

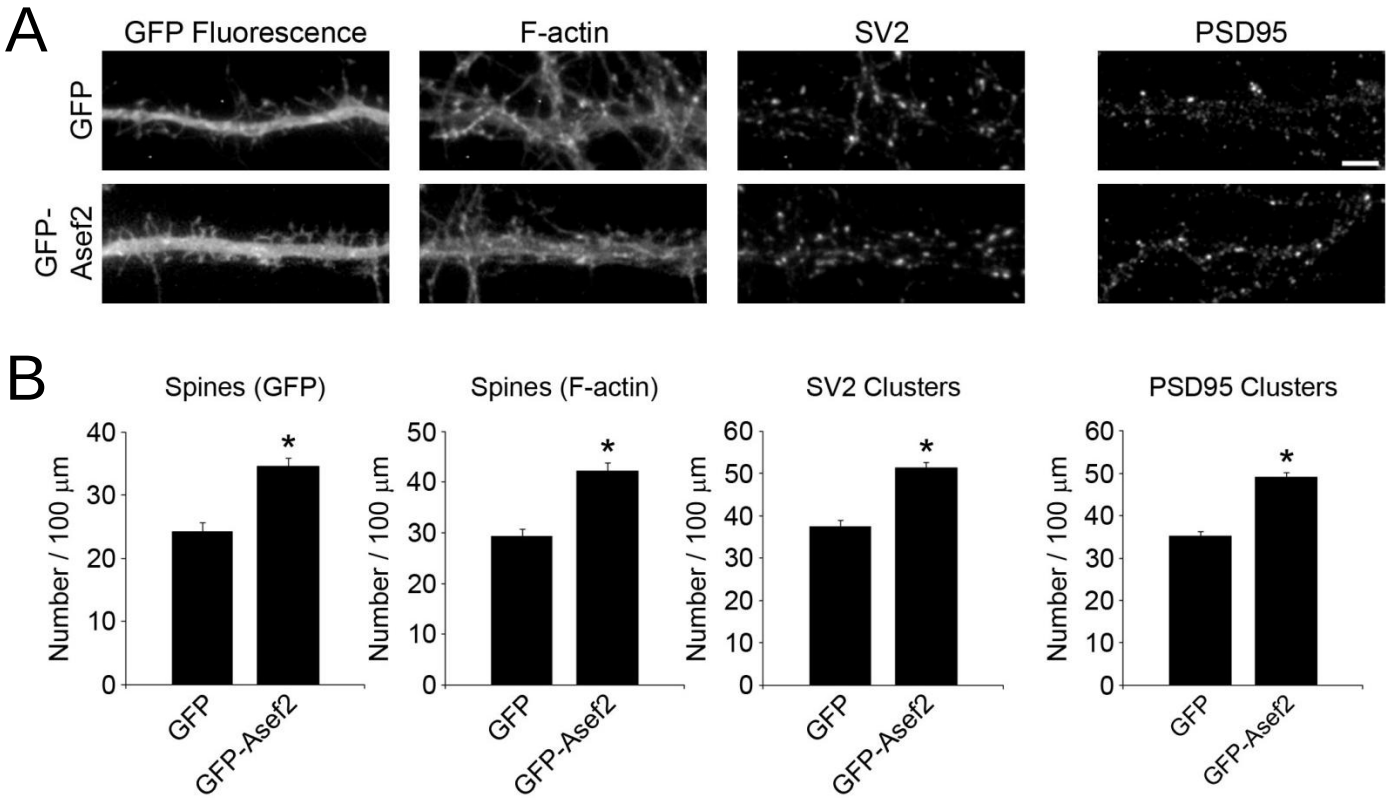

Figure 8. Asef2 promotes dendritic spine and synapse formation. *A*, DIV5 neurons were transfected with GFP or GFP-Asef2, then fixed and stained for F-actin (phalloidin), SV2, or PSD95 at DIV11. Bar, 5 μm. *B,* Quantification of spine density, using either GFP fluorescence or F-actin (phalloidin) staining, as well as synapse density (SV2 and PSD95 clusters) in GFP- and GFP-Asef2-expressing neurons. Error bars represent s.e.m. for 40-72 dendrites from five independent experiments (*, $p < 0.001$).

Expression of Asef2 promotes dendritic spine and synapse formation – To continue investigating the role of Asef2 in spine development, we generated a GFP-tagged Asef2 construct and expressed it in hippocampal neurons. We transfected neurons at DIV5, before spines and synapses have formed, and assessed spine and synapse density at DIV11, when these structures are prevalent. Expression of GFP-Asef2 caused an increase in the density of dendritic spines compared to expression of GFP alone, as determined using GFP fluorescence (Figure 8). The neurons were also stained with fluorescently-labeled phalloidin, which binds to F-actin and is a commonly used marker for dendritic spines (Wulf et al. 1979; Drenckhahn et al. 1984; Allison et al. 1998). Similar to the spine density quantified using GFP fluorescence, the density of spines quantified using phalloidin was increased in GFP-Asef2-expressing neurons (Figure 8). Furthermore, GFP-Asef2 expression caused an increase in the number of synapses, as determined by SV2 and PSD95 staining, compared to GFP expression alone (Figure 8). These results suggest that Asef2 promotes the formation of dendritic spines and synapses in hippocampal neurons.

Knockdown of endogenous Asef2 impairs spine and synapse development – We next used a short hairpin RNA (shRNA) approach to knock down endogenous Asef2 in neurons and examined the effect on spines and synapses. We generated two shRNAs, which targeted the rat sequence of Asef2, and assessed their ability to reduce endogenous Asef2 expression. Neurons were stained for endogenous Asef2 at DIV11, and the intensity of Asef2 staining in the soma was used to quantify the percentage of Asef2 knockdown (Figure 9). Both shRNAs reduced endogenous Asef2 expression by approximately 30%, compared to endogenous Asef2 expression in non-transfected neurons (Figure 9). Expression of either Asef2 shRNA caused an approximately 50% decrease in dendritic spine density as compared to expression of empty pSUPER vector or a non-targeting shRNA (NT shRNA) (Figure 10). Similar decreases in synaptic density were also observed in Asef2 shRNA-transfected neurons (Figure 10).

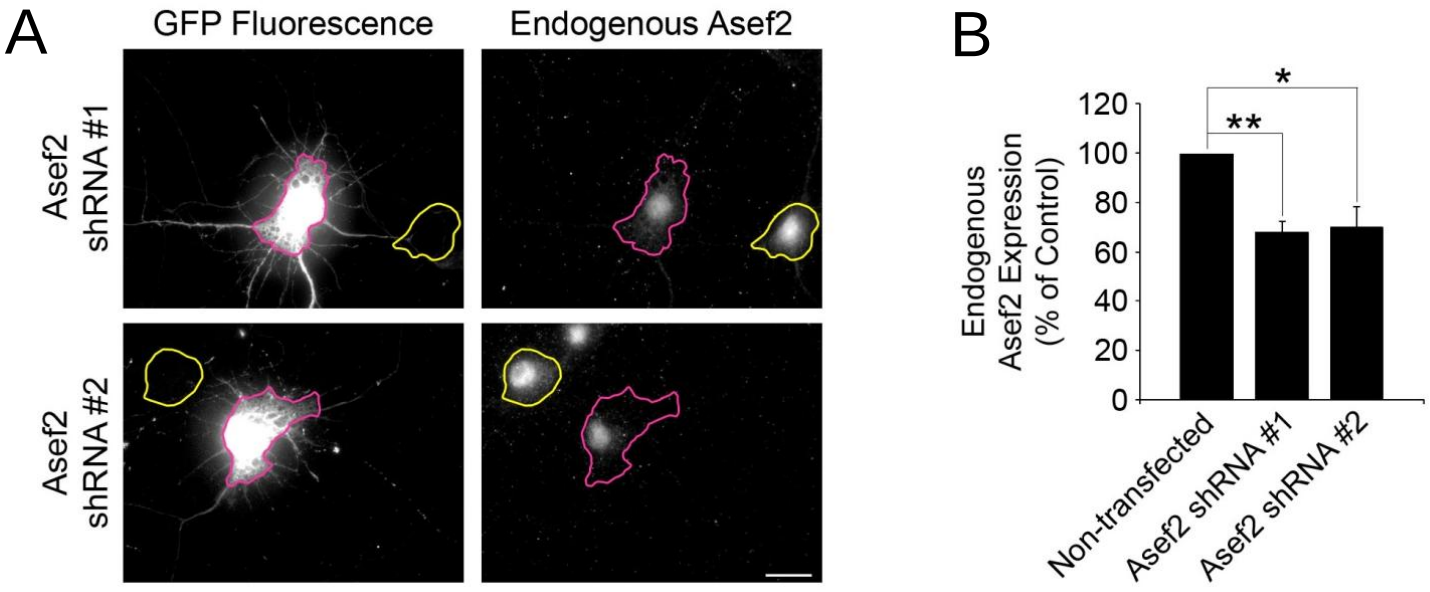

Figure 9. Expression of endogenous Asef2 is knocked down by shRNAs. *A,* Neurons were co-transfected at DIV5 with GFP and shRNAs targeting Asef2, then fixed at DIV11 and immunostained for endogenous Asef2. Transfected cell somas are outlined in magenta, and non-transfected cell somas are outlined in yellow. Bar, 20 μm. *B,* The amount of endogenous Asef2 was quantified by measuring the fluorescence intensity of Asef2 in the somas of non-transfected and shRNA-expressing neurons. Error bars represent s.e.m. for 45 cells from three separate experiments (*, p = 0.02; **, p = 0.002).

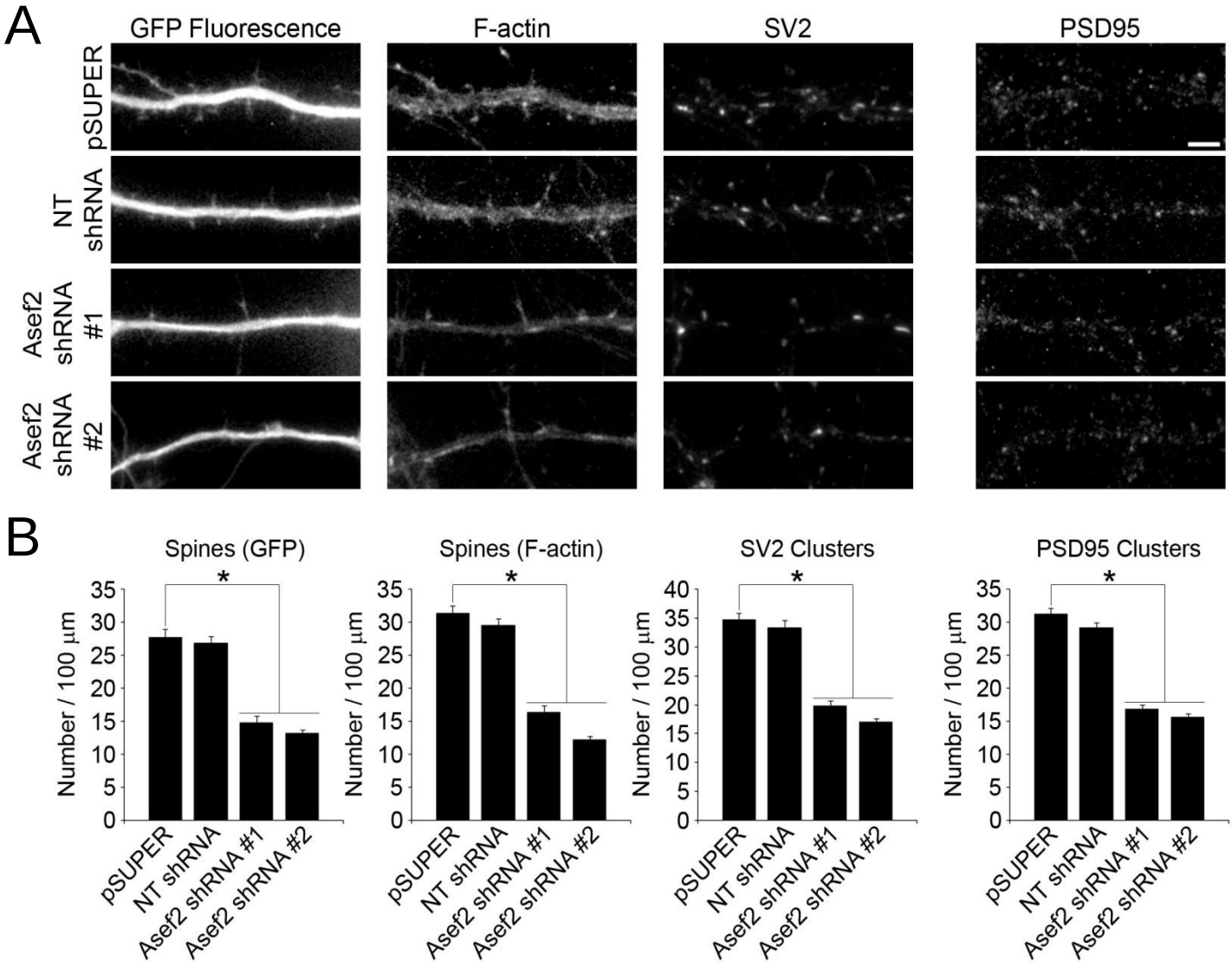

Figure 10. Asef2 knockdown inhibits spine and synapse development. *A,* Neurons were co-transfected with GFP and either empty pSUPER vector, non-targeting shRNA (NT shRNA), or Asef2 shRNAs at DIV5, then fixed and immunostained for F-actin (phalloidin), SV2, or PSD95 at DIV11. Bar, 5 µm. *B,* Quantification of spine and synapse density for control (empty pSUPER vector or NT shRNA) and Asef2 shRNA-expressing neurons. Error bars represent s.e.m. for 45 dendrites from three separate experiments (*, $p < 0.001$).

Therefore, these results suggest that endogenous Asef2 is an important regulator of dendritic spine and synapse formation in developing neurons.

The effect of Asef2 on spines and synapses is dependent on its GEF activity – Because GEF activity is important for Asef2 function (Kawasaki et al. 2007; Bristow et al. 2009; Kawasaki et al. 2009b; Jean et al. 2013), we hypothesized that Asef2 promotes spine and synapse formation through activation of GTPases. To initially test this hypothesis, we mutated two residues, lysine 382 or lysine 385, to alanine (K382A and K385A, respectively). These residues are highly conserved among members of the Dbl GEF family, which includes Asef2 (Figure 11A); previous studies showed that lysine to alanine mutation of these residues in Dbl family GEFs, including Tiam1 and collybistin, greatly diminished their GEF activity (Worthylake et al. 2000; Zhu et al. 2000; Reddy-Alla et al. 2010). We tested these mutants for their GEF activity towards Rac using a Rac activation assay. For this assay, the GST-tagged binding domain from the Rac effector PAK (GST-PBD) was used to detect the active form of Rac from cell lysates of GFP-, GFP-Asef2-, GFP-Asef2-K382A-, and GFP-Asef2-K385A-expressing cells. Expression of GFP-Asef2 resulted in an increase in active Rac, compared to expression of GFP (Figure 11B,C). In contrast, expression of either Asef2-K382A or Asef2-K385A abolished the Asef2-promoted effect on active Rac, (Figure 11B,C), indicating that these residues are crucial for the ability of Asef2 to activate Rac. We then transfected neurons with either GFP, GFP-Asef2, or the GEF-activity deficient mutants and quantified spine and synapse density. Expression of GFP-Asef2 caused a significant increase in spine and synapse density compared to GFP expression (Figure 12). In contrast, expression of GFP-Asef2-K382A or GFP-Asef2-K385A did not lead to an increase in the number of spines and synapses (Figure 12), suggesting that the GEF activity of Asef2 is critical for its ability to promote spine and synapse formation.

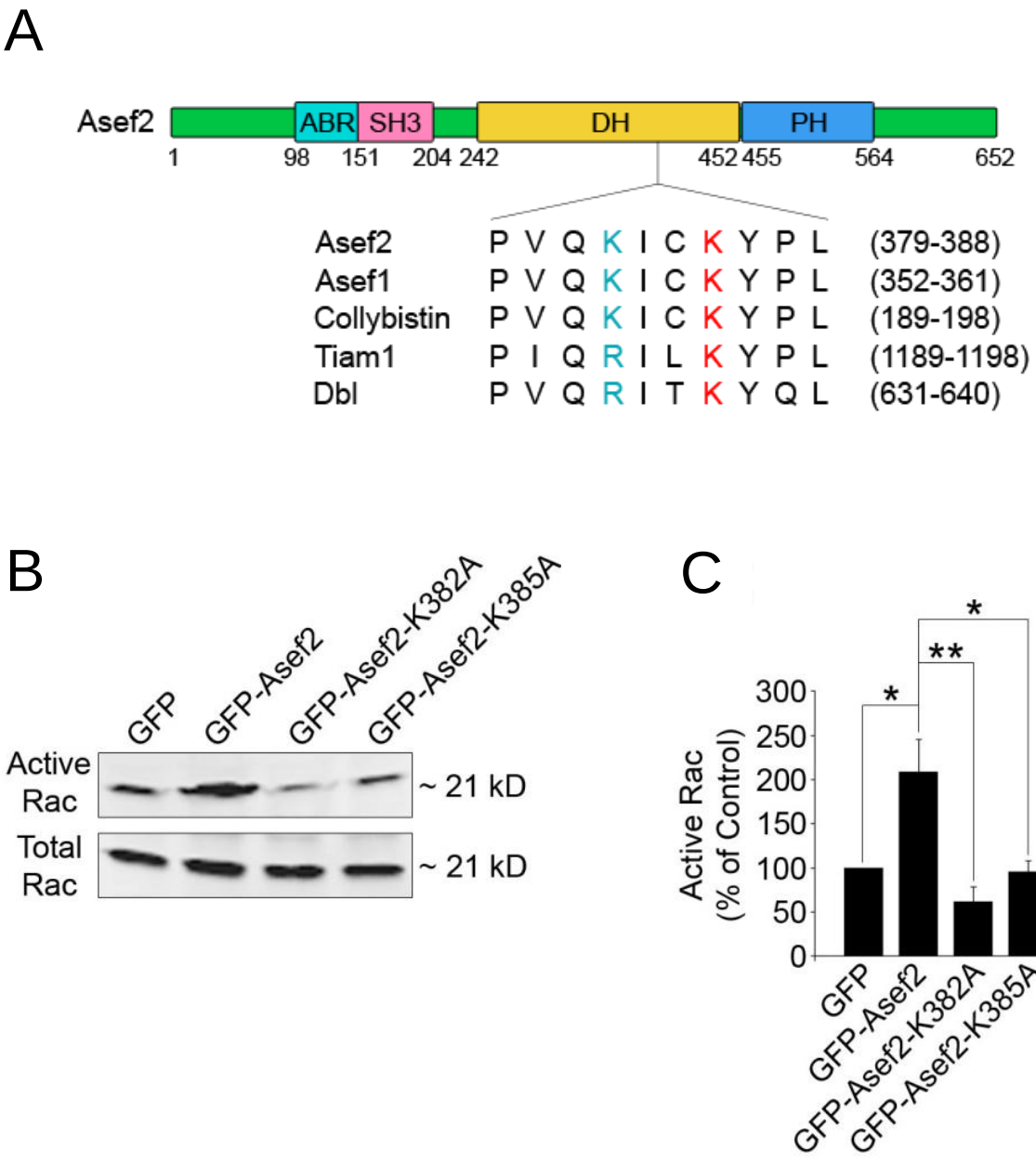

Figure 11. Mutations in the DH domain of Asef2 inhibit Rac activation. *A,* Schematic comparing a portion of the DH domain amino acid sequences of several Dbl family GEFs. Asef2-K382 (and corresponding residues in other GEFs) is shown in blue, and Asef2-K385 (and corresponding residues in other GEFs) is shown in red. *B,* HT1080 cells were co-transfected with FLAG-Rac and either GFP, GFP-Asef2, GFP-Asef2-K382A or GFP-Asef2-K385A, and three days later the active form of Rac was pulled down from lysates from these cells. The amounts of total Rac in the lysates is shown as a control. *C,* Quantification of the amount of active Rac from four independent experiments. Error bars represent s.e.m. (*, $p < 0.02$, **, $p = 0.001$).

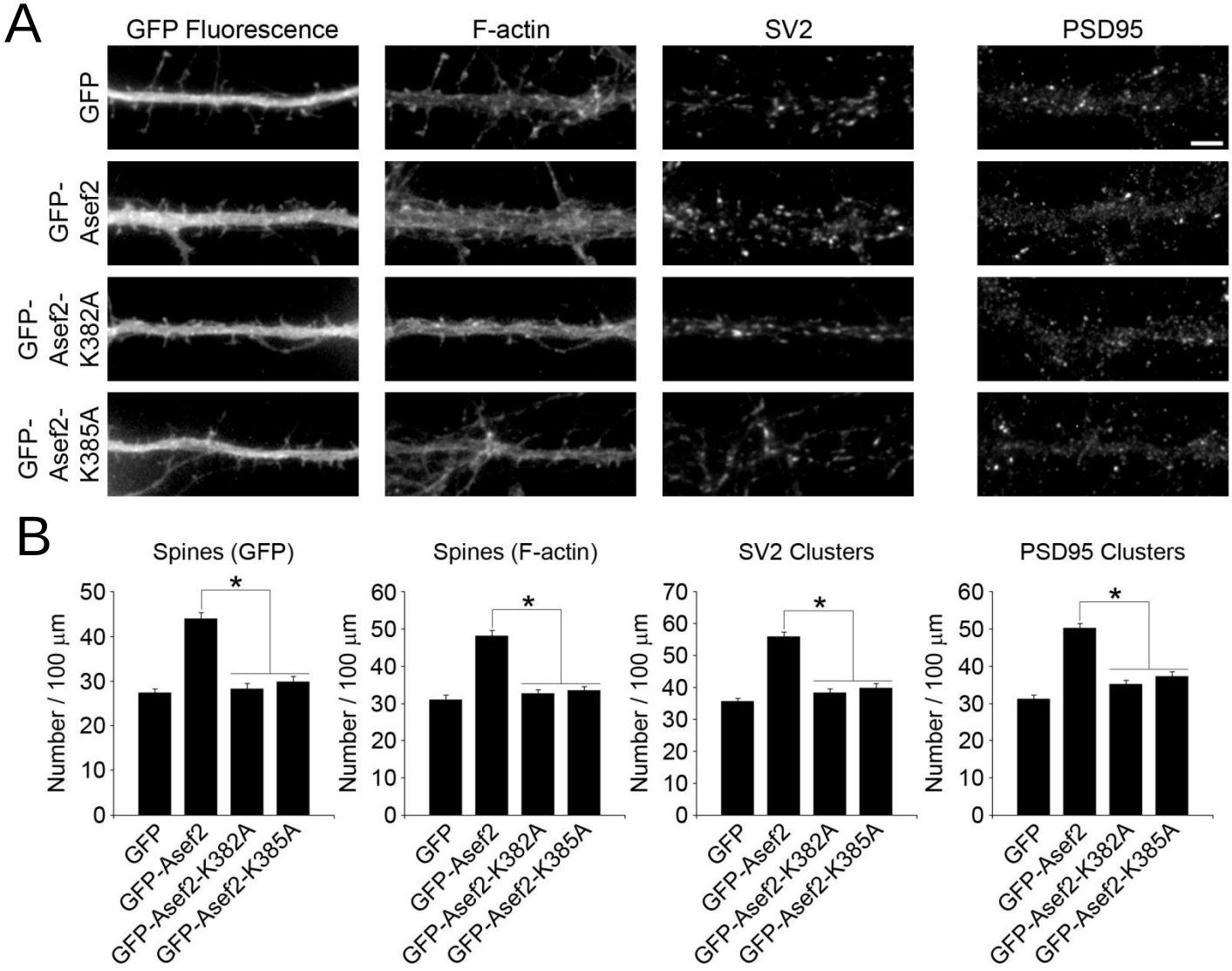

Figure 12. Abrogation of Asef2 GEF activity blocks spine and synapse formation. *A,* Neurons were transfected with the indicated constructs and stained for F-actin (phalloidin), SV2, or PSD95. Bar, 5 μm. *B,* Quantification of spine and synapse density in GFP-, GFP-Asef2-, GFP-Asef2-K382A-, and GFP-Asef2-K385A-expressing neurons. Error bars represent s.e.m. for 45-59 dendrites from three independent experiments (*, $p < 0.001$).

Knockdown of endogenous Rac impedes Asef2-dependent spine and synapse formation – Because the GEF activity of Asef2 is crucial for its effect on spines and synapses and previous work has shown that Rac regulates spine formation (Luo 1996; Nakayama and Luo 2000; Tashiro et al. 2000), we hypothesized that Asef2-mediated spine and synapse formation is Rac-dependent. To test this hypothesis, we knocked down endogenous Rac using shRNAs. Two shRNAs, which targeted the rat sequence of Rac, reduced the expression of the endogenous protein by approximately 80% compared to empty pSUPER vector or to NT shRNA (Figure 13). We then co-transfected these shRNAs with either GFP or GFP-Asef2 in neurons at DIV5 and assessed spine and synapse density at DIV11. Knockdown of Rac by transfection with Rac shRNAs in GFP-expressing neurons caused a decrease in the density of spines and synapses compared to neurons transfected with either empty pSUPER vector or NT shRNA (Figure 14), supporting the importance of Rac signaling for the formation of spines. Transfection of Rac shRNAs in Asef2-expressing neurons resulted in a significant decrease in spine and synapse density (Figure 14), which abolished the Asef2-promoted effect on these structures. These results suggest that Asef2 signaling through Rac mediates the formation of dendritic spines and synapses.

Spinophilin regulates Asef2-promoted spine and synapse formation – Spinophilin, which binds to F-actin, is a known Asef2-interacting protein that has previously been shown to regulate dendritic spine formation and synaptic function (Allen et al. 1997; Yan et al. 1999; Feng et al. 2000; Kelker et al. 2007; Sagara et al. 2009). Therefore, we next investigated the role of spinophilin in Asef2-mediated spine and synapse formation. Immunostaining of DIV14 neurons revealed that endogenous Asef2 and spinophilin co-localized at distinct sites along the dendrite (Figure 15). Quantification showed that Asef2 was present in 84 ± 1% of spinophilin puncta, indicating that Asef2 co-localized with spinophilin in neurons. To determine whether spinophilin affects the function of Asef2 in spines and synapses, we generated two shRNAs to knock down

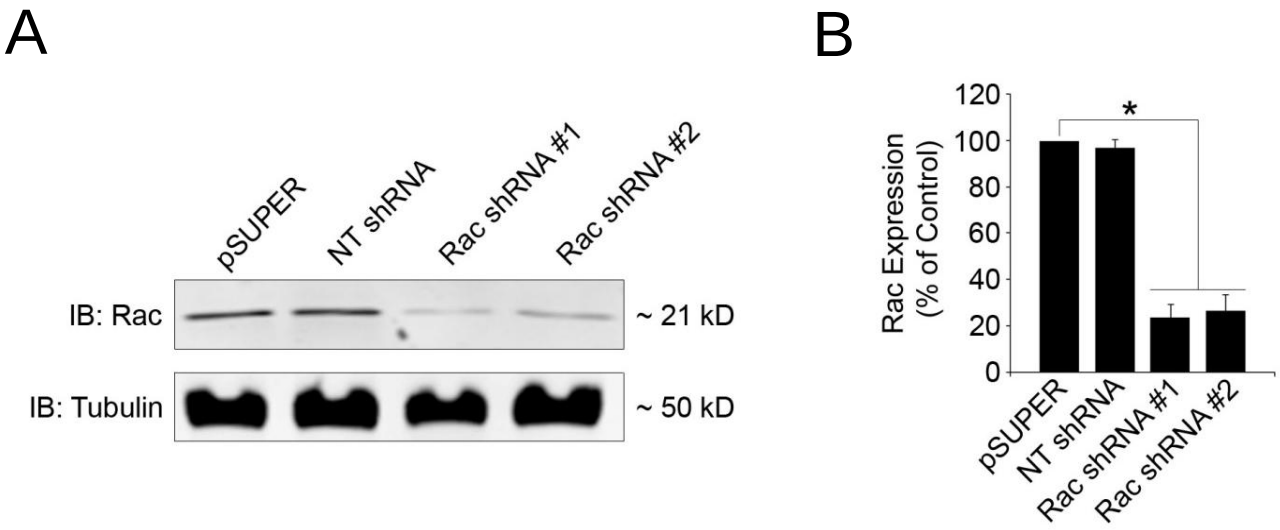

Figure 13. Knockdown of endogenous Rac. *A,* Cell lysates from R2F cells, which were transfected with either empty pSUPER vector, NT shRNA, or Rac shRNAs, were immunoblotted for Rac as well as tubulin for a loading control. *B,* Quantification of endogenous Rac from three independent experiments. Error bars represent s.e.m. (*, $p < 0.001$).

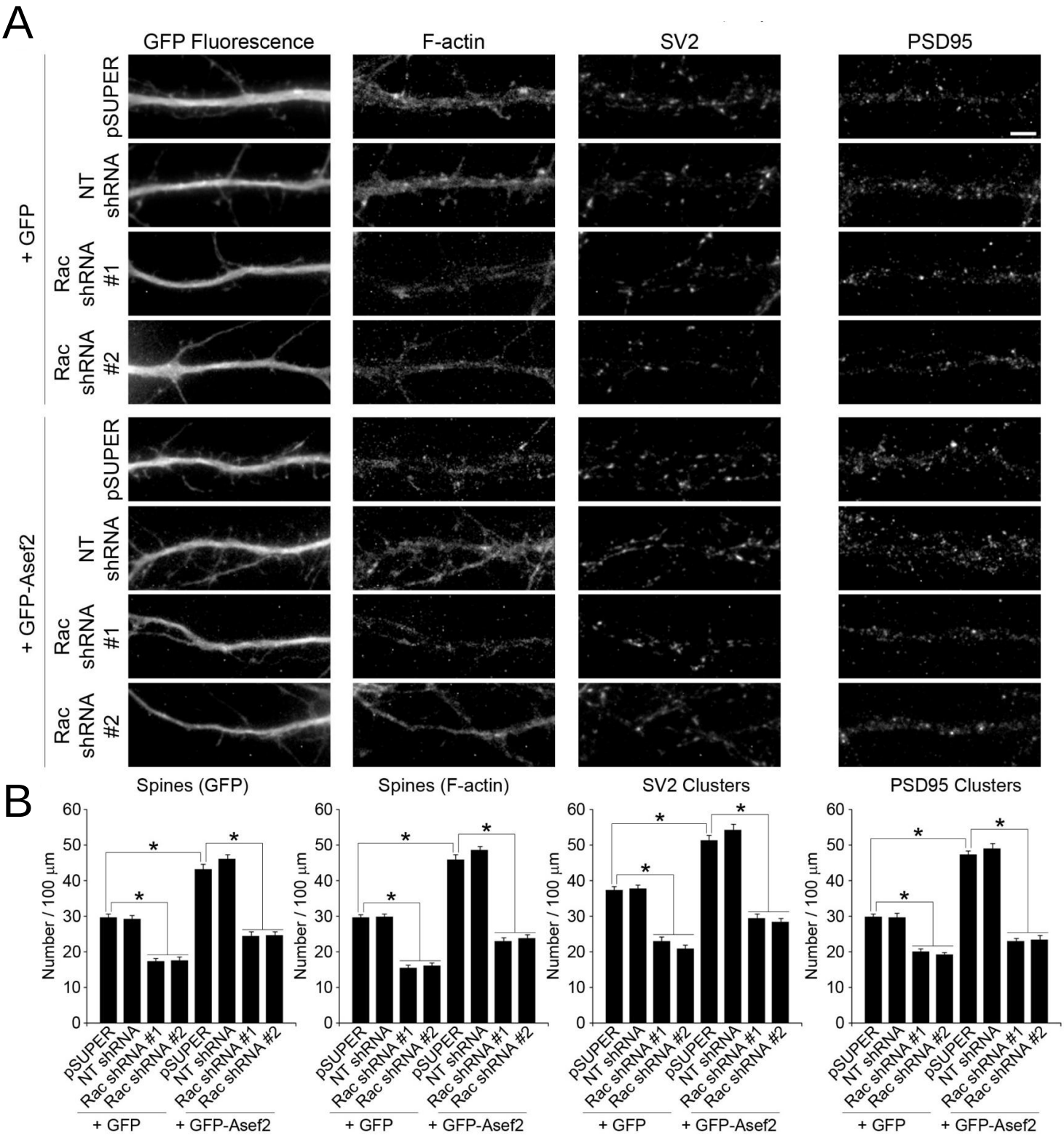

Figure 14. Knockdown of Rac inhibits Asef2-mediated spine and synapse formation. *A,* Neurons were co-transfected with GFP or GFP-Asef2 and either pSUPER vector, NT shRNA, or Rac shRNAs at DIV5. The cells were then fixed at DIV11 and stained for F-actin (phalloidin), SV2, or PSD95. Bar, 5µm. *B,* Quantification of spine and synapse density in GFP- and GFP-Asef2 neurons transfected with either controls (empty pSUPER vector or NT shRNA) or Rac shRNAs. Error bars represent s.e.m. for 45 dendrites from three separate experiments (*, $p < 0.001$).

endogenous expression of the protein. Co-transfection of the shRNAs with either GFP or GFP-Asef2 in neurons showed that the spinophilin shRNAs decreased endogenous levels of the protein by approximately 50% (Figure 16). The spinophilin shRNAs caused a significant decrease in spine and synapse density as compared to either empty pSUPER vector or NT shRNA in GFP-expressing neurons (Figure 17), indicating that endogenous spinophilin is a key regulator of spine and synapse formation. Moreover, the spinophilin shRNAs completely abrogated the Asef2-promoted effect on spines and synapses (Figure 17), suggesting that spinophilin is an important contributor to Asef2 signaling in the regulation of spine and synapse formation.

Spinophilin recruits Asef2 to dendritic spines – Since spinophilin localizes prominently to dendritic spines and is a binding partner for Asef2 (Allen et al. 1997; Satoh et al. 1998; Sagara et al. 2009), we hypothesized that spinophilin targets Asef2 to spines. Intriguingly, co-expression of mCherry-spinophilin and GFP-Asef2 caused a stark change in the localization of Asef2, as shown by live-cell confocal imaging (Figure 18A). Although GFP-Asef2 alone was seen throughout dendrites, GFP-Asef2, when co-expressed with mCherry-spinophilin, was observed to localize predominantly to dendritic spines with very little accumulation in the dendrites (Figure 18A). Indeed, the localization of Asef2 mirrored that of mCherry-spinophilin, with both proteins co-localizing in dendritic spines (Figure 18A). Quantification of the spine-to-shaft ratio showed an approximately 2.6-fold increase when mCherry-spinophilin was co-expressed with GFP-Asef2 (Figure 18B). A linescan analysis of the fluorescence intensity in representative spines further demonstrated that expression of mCherry-spinophilin altered GFP-Asef2 localization (Figure 18C). Whereas GFP was found in both spines and dendrites in linescans for mCherry and mCherry-spinophilin-expressing neurons, GFP-Asef2 exhibited an intensity peak almost exclusively in the spine when co-

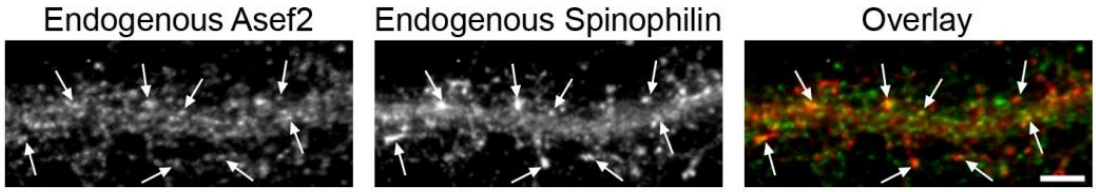

Figure 15. Localization of endogenous Asef2 and spinophilin in neurons. Neurons were fixed at DIV14 and immunostained for endogenous Asef2 and spinophilin. An overlay of the images shows co-localization of the proteins along the dendrite (arrows). Bar, 5 μm.

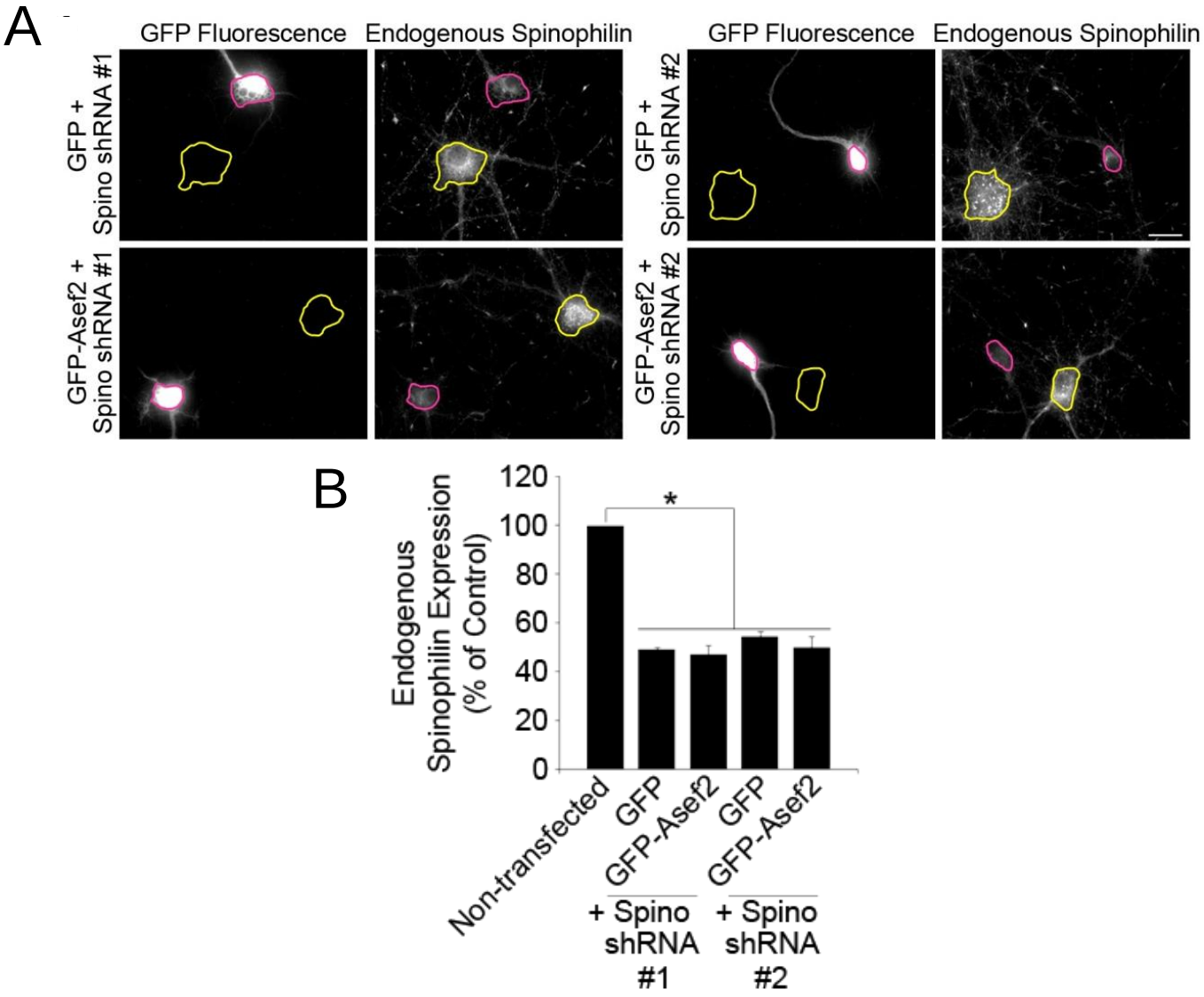

Figure 16. Knockdown of endogenous spinophilin by shRNAs. *A*, Neurons were co-transfected at DIV5 with GFP or GFP-Asef2 and with either empty pSUPER vector, NT shRNA, or spinophilin shRNAs (Spino shRNA). Cells were then fixed at DIV11 and immunostained for endogenous spinophilin. Transfected cell somas are outlined in magenta, and non-transfected cell somas are outlined in yellow. Bar, 20 μm. *B*, The amount of endogenous spinophilin was quantified by measuring the fluorescence intensity of spinophilin in the somas of non-transfected and shRNA-expressing neurons. Error bars represent s.e.m. for 45 somas from three separate experiments (* $p < 0.007$).

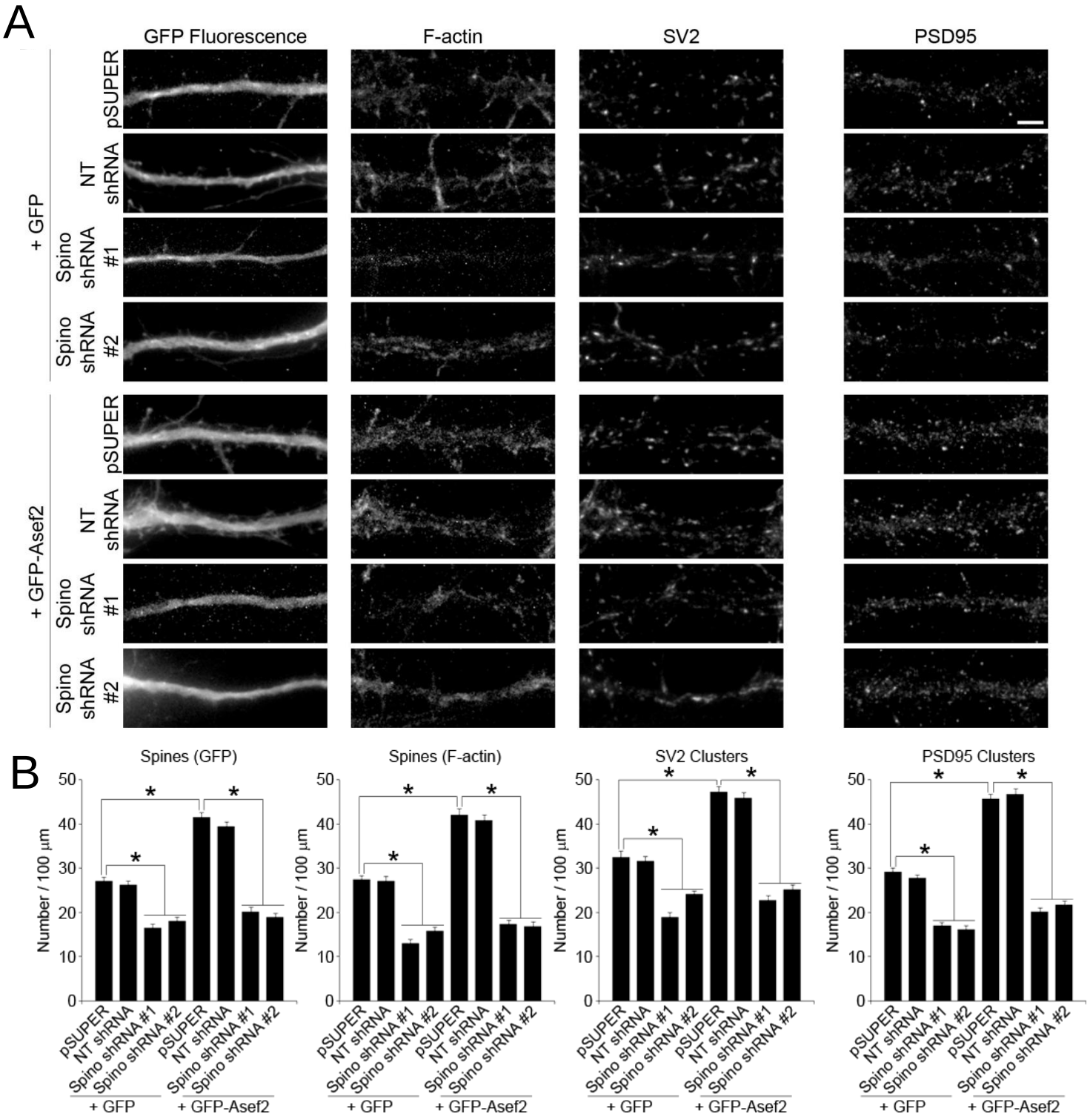

Figure 17. Spinophilin knockdown blocks Asef2-dependent spine and synapse development. *A,* Neurons were co-transfected with GFP or GFP-Asef2 and either empty pSUPER vector, NT shRNA, or spinophilin shRNAs at DIV5. Cell were fixed at DIV11 and stained for F-actin (phalloidin), SV2, or PSD95. Bar, 5µm. *B,* Quantification of spine and synapse density in GFP- and GFP-Asef2 neurons transfected with either controls (empty pSUPER vector or NT shRNA) or spinophilin shRNAs. Error bars represent s.e.m. for 45 dendrites from three separate experiments (*, $p < 0.001$).

expressed with mCherry-spinophilin (Figure 18C). These results suggest that spinophilin localizes Asef2 to dendritic spines, where it regulates the development of spines and synapses.

Discussion

The function of Rho family GEFs in spine development represents an increasingly exciting area of study, given that not much is currently known about the contribution of these proteins to spine and synapse formation. Our results indicate a previously unknown role for the Rho family GEF Asef2 in the formation of dendritic spines and synapses in hippocampal neurons. Proper development of spines is dependent on the endogenous expression of Asef2, because knockdown of Asef2 causes a decrease in spine and synapse density. Knockdown of other Rho family GEFs, including Tiam1 and kalirin-7, have also been shown to affect spine and synapse function (Ma et al. 2003; Tolias et al. 2005; Zhang and Macara 2006; Tolias et al. 2007; Xie et al. 2007). Asef2 signals through Rac to mediate spine formation, as demonstrated by GEF-activity deficient Asef2 mutants and by knockdown of Rac in Asef2-expressing neurons. Moreover, exogenous expression of Asef2 causes a significant increase in spine density, which also occurs when constitutively active Rac is expressed in neurons (Luo 1996; Nakayama and Luo 2000; Tashiro et al. 2000).

To better understand the mechanism of Asef2-dependent spine formation, we investigated the role of the scaffolding protein spinophilin. Our work shows that spinophilin localizes Asef2 to spines, which has not been previously shown. Furthermore, this observation highlights the increasing importance of spinophilin-GEF signaling as a key component of the development of spines. Spinophilin has been shown to interact with several Rho family GEFs in addition to Asef2: kalirin-7, Tiam1, and Lfc (Penzes et al. 2001; Buchsbaum et al. 2003; Ryan et al. 2005; Rajagopal et al. 2010). Interestingly, spinophilin expression alters the localization of endogenous Tiam1 in non-neuronal cells (Buchsbaum et al. 2003; Rajagopal et al. 2010), while spinophilin expression results in relocation of Lfc to the cell periphery in neuroblast N2a cells

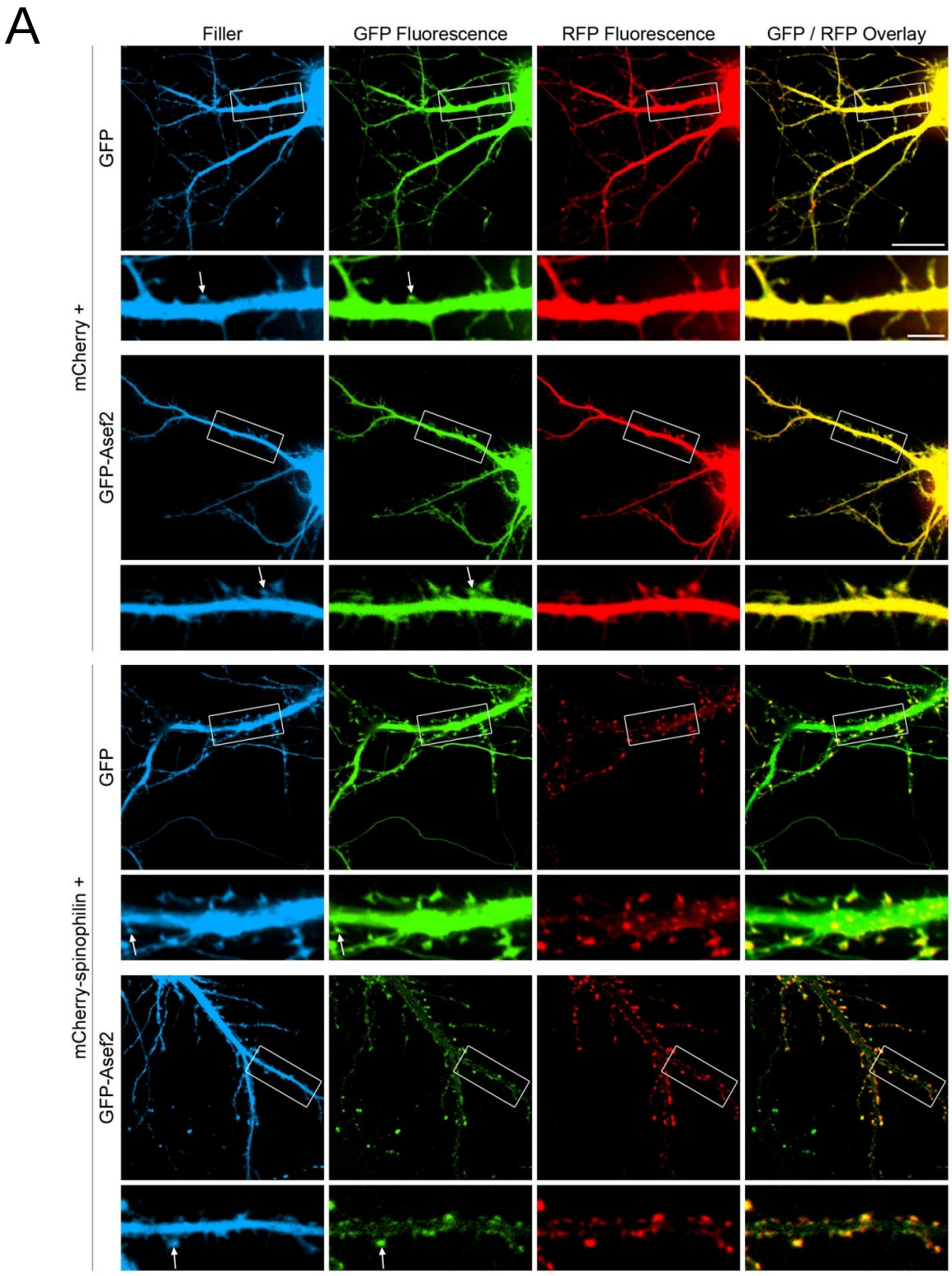

Figure 18. Spinophilin targets Asef2 to dendritic spines. *A,* Neurons were co-transfected with GFP or GFP-Asef2, mCerulean (Filler), and either mCherry or mCherry-spinophilin at DIV5. The neurons were visualized at DIV11 using live-cell confocal microscopy. Bar, 20 μm. Higher magnification images of the boxed regions are shown below. Arrows indicate spines that were used for linescan analyses in panel (C) (see next page). Bar, 5 μm.

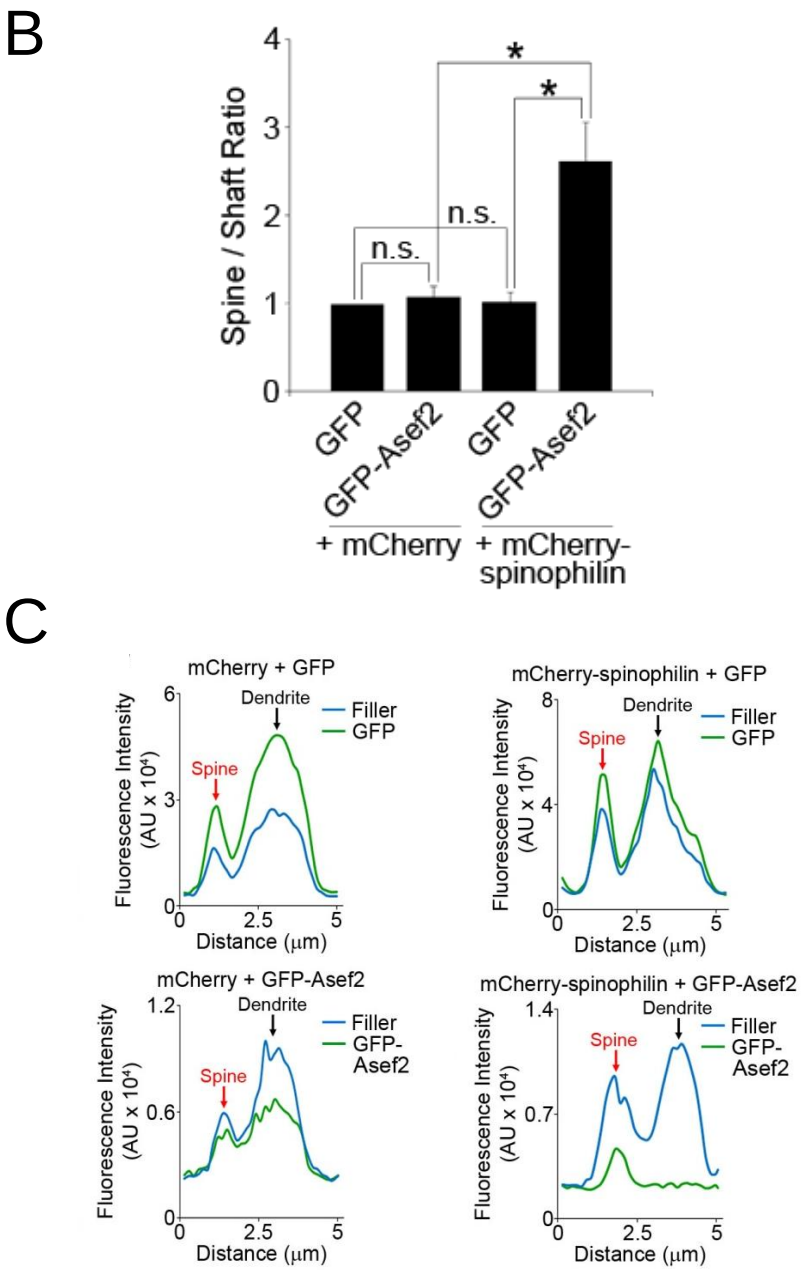

Figure 18. Spinophilin targets Asef2 to dendritic spines (continued from previous page). *B,* Fluorescence intensities of GFP and GFP-Asef2 were quantified in dendritic spines heads and were normalized to neighboring shafts. Error bars represent s.e.m. from five independent experiments (*, $p < 0.001$). "n.s." denotes no statistically significant difference. *C,* Linescan graphs showing the fluorescence intensities of mCerulean (Filler) and GFP through the dendrites and along dendritic spines. Red arrows represent the dendritic spine peaks, whereas black arrows denote the dendrite peaks.

(Ryan et al. 2005). Taken together, these data point to spinophilin as a key regulator of Rho family GEF localization.

Intriguingly, endogenous Asef2 distributed throughout dendrites, including the dendrite shaft and spines, under basal conditions. Other GEFs, such as Lfc, have similarly been found to distribute to dendritic shafts under basal conditions (Ryan et al. 2005). In the case of Lfc, neuronal stimulation caused it to translocate from dendrites to spines (Ryan et al. 2005). This change in localization is thought to occur via targeting by spinophilin (Ryan et al. 2005). Therefore, spinophilin-mediated targeting of Asef2 to spines could occur predominantly in response to synaptic signaling. Uncovering the upstream signals that regulate spinophilin-promoted Asef2 localization to spines is an interesting avenue for future study.

In our study, knockdown of spinophilin causes a decrease in dendritic spine density. This phenotype differs from that observed by Feng, et al., who reported that spinophilin knockout resulted in an increase in spine density in young mice (Feng et al. 2000). A possible explanation for this discrepancy is that the knockout spine density measurements were performed on neurons from the caudatoputamen. Spinophilin could have a different function in these neurons versus the hippocampal neurons used in our study. Another explanation could be that spinophilin mediates these effects on spines by associating with different binding partners. In the study by Feng, et al., the interaction of spinophilin with protein phosphatase-1 appears to be important in the modulation of spine dynamics (Feng et al. 2000). Whereas in our study the Asef2-spinophilin interaction is critical for the effects that we observe on spine development.

The C-terminus of spinophilin acts as a centralized scaffold for several GEFs, including Asef2 (Sagara et al. 2009). This region of spinophilin contains a PDZ domain and a coiled-coil domain, which both facilitate protein-protein interactions (Sarrouilhe et al. 2006). Based on yeast two-hybrid screening, the C-terminus of kalirin-7 appears to associate with the PDZ domain of spinophilin (Penzes et al. 2001). Lfc, on the other hand, interacts with the coiled-coil domain of spinophilin, whereas Tiam1 is thought to associate with a region spanning the PDZ

and coiled-coil domains (Buchsbaum et al. 2003; Ryan et al. 2005). The specific region within the spinophilin C-terminus that interacts with Asef2 is currently unknown. However, Asef2 most likely does not associate with spinophilin via coiled-coil interactions, because Asef2 does not have a coiled-coil domain (Sagara et al. 2009). Continued work is necessary to determine the specific regions that are essential for the Asef2-spinophilin interaction and whether this interaction affects the binding of spinophilin to other GEFs.

In summary, our results reveal a new function for Asef2 in promoting the formation of dendritic spines and synapses in hippocampal neurons. Asef2-mediated spine and synapse development occurs via the scaffold protein spinophilin, which targets Asef2 to spines. Asef2 most likely activates Rac locally to facilitate the formation of new spines. Spinophilin also maintains Asef2 in spines, which suggests that Asef2-mediated Rac signaling is involved in spine stability. The proper formation and maintenance of spines is crucial for efficient synaptic signaling. Interestingly, a nucleotide deletion in Asef2 has been linked to autism, and a single-nucleotide polymorphism in Asef2 has been linked to the co-occurrence of alcohol dependence and depression (Edwards et al. 2012; Iossifov et al. 2012); these disorders are associated with spine defects (Fiala et al. 2002; Gass and Olive 2012; Ota and Duman 2013). Collectively, these data point to Asef2 as a key signaling protein in regulating the development of dendritic spines and synapses, which is critical for maintaining normal cognitive and behavioral function.

CHAPTER III

PHOSPHORYLATION OF SERINE 106 IN ASEF2 REGULATES CELL MIGRATION AND ADHESION TURNOVER

J. Corey Evans[#,1], Kelly M. Hines[#,2,3,4], Jay G. Forsythe[#,2,3,4], Begum Erdogan[1], Mingjian Shi[1], Salisha Hill[5], Kristie L. Rose[5,6], John A. McLean[2,3,4], and Donna J. Webb[1,4,7]

[1]Department of Biological Sciences and the Vanderbilt Kennedy Center for Research on Human Development, [2]Department of Chemistry, [3]Vanderbilt Institute for Chemical Biology (VICB), [4]Vanderbilt Institute for Integrative Biosystems Research and Education (VIIBRE), [5]Mass Spectrometry Research Center, [6]Department of Biochemistry, Vanderbilt University, Nashville, TN 37235

[#]These authors have contributed to an equal extent.

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Abstract

Asef2, a 652-amino acid protein, is a guanine nucleotide exchange factor (GEF) that regulates cell migration and other processes via activation of Rho family GTPases, including Rac. Binding of the tumor suppressor adenomatous polyposis coli (APC) to Asef2 is known to induce its GEF activity; however, little is currently known about other modes of Asef2 regulation. Here, we investigated the role of phosphorylation in regulating Asef2 activity and function. Using high-resolution mass spectrometry (MS) and tandem mass spectrometry (MS/MS), we obtained complete coverage of all phosphorylatable residues and identified six phosphorylation sites. One of these, serine 106 (S106), was particularly intriguing as a potential regulator of Asef2 activity because of its location within the APC-binding domain. Interestingly, mutation of this serine to alanine (S106A), a non-phosphorylatable analogue, greatly diminished the ability of Asef2 to activate Rac, while a phosphomimetic mutation (serine to aspartic acid, S106D) enhanced Rac activation. Furthermore, expression of these mutants in HT1080 cells demonstrated that phosphorylation of S106 is critical for Asef2-promoted migration and for cell-matrix adhesion assembly and disassembly (adhesion turnover), which is a process that facilitates efficient migration. Collectively, our results show that phosphorylation of S106 modulates Asef2 GEF activity and Asef2-mediated cell migration and adhesion turnover.

Introduction

Cell migration is a complex, actin-dependent process that plays a central role in embryonic development and wound healing (Vicente-Manzanares and Horwitz 2011). The tightly controlled signaling pathways that mediate cell migration can be altered in pathological states, such as tumor metastasis and atherosclerosis (Yamaguchi et al. 2005; Hopkins 2013). Cell migration involves several canonical steps: the extension of actin-rich protrusions, the assembly of nascent adhesions at the leading edge, the translocation of the cell body, and the retraction of the rear of the cell (Lauffenburger and Horwitz 1996). The assembly of leading edge adhesions, which are sites of contact between cells and the extracellular matrix, stabilizes protrusions and provides traction to propel the forward movement of cells (Lauffenburger and Horwitz 1996; Beningo et al. 2001; Laukaitis et al. 2001). Once formed, nascent adhesions can disassemble, or they can continue to grow into larger, more stable adhesions (Webb et al. 2004; Choi et al. 2008). The constant assembly and disassembly of leading edge adhesions, termed adhesion turnover, is crucial for efficient cell migration (Webb et al. 2002; Webb et al. 2004) but not well understood on a molecular level.

Small GTPases that comprise the Rho family, including Rho, Rac, and Cdc42, are key modulators of cell migration through their ability to regulate processes underlying migration, such as adhesion assembly, disassembly, and maturation (Nobes and Hall 1995; Chrzanowska-Wodnicka and Burridge 1996; Rottner et al. 1999; Vicente-Manzanares and Horwitz 2011). Rho family GTPases, like other small GTPases, function by cycling between a GTP-bound active form and a GDP-bound inactive form (Jaffe and Hall 2005). This cycling is dependent on GEFs that catalyze the exchange of GDP for GTP and GTPase activating proteins (GAPs), which promote the hydrolysis of GTP (Trahey and McCormick 1987; Gibbs et al. 1988; West et al. 1990; Cherfils and Zeghouf 2013). Upon activation by GEFs, the Rho GTPases, in turn, activate a series of downstream effector proteins that regulate adhesion and actin dynamics (Ridley and Hall 1992; Ridley et al. 1992; Nobes and Hall 1995). While the role of the Rho GTPases in

regulating cell migration has been studied, less is known about the function of the various GEFs and GAPs in modulating migration and its underlying processes.

Asef2 is a recently discovered GEF that has been implicated in the regulation of cell migration (Hamann et al. 2007; Kawasaki et al. 2007; Bristow et al. 2009). This 652-amino acid protein is composed of several functional domains: an APC-binding region (ABR), a Src homology 3 (SH3) domain, a Dbl homology (DH) domain, and a pleckstrin homology (PH) domain (Hamann et al. 2007). The DH domain mediates GTP exchange for Rac and Cdc42, while the PH domain is most likely involved in membrane localization (Hamann et al. 2007; Kawasaki et al. 2007; Bristow et al. 2009). The ABR and SH3 domains work in concert to regulate Asef2 activity (Hamann et al. 2007). Asef2 exists in an auto-inhibited conformation that prevents nucleotide exchange by the DH domain; once the tumor suppressor APC binds to the tandem ABR and SH3 domains, Asef2 undergoes a conformational change that stimulates its GEF activity (Hamann et al. 2007; Murayama et al. 2007; Zhang et al. 2012). While the mechanism of APC binding to Asef2 and relieving autoinhibition has been studied (Hamann et al. 2007), little is currently known about other potential modes of Asef2 regulation. For example, post-translational modification of Asef2 is one possible avenue of modulating its activity and function. The addition of chemical moieties, such as acetyl, phosphate, or glycosyl groups, to a protein is a common mechanism for altering its conformation, localization, and activity (Walsh et al. 2005). Indeed, it has previously been shown that phosphorylation of GEFs is necessary for proper function (Kato et al. 2000; Servitja et al. 2003; Miyamoto et al. 2013). These data point to a possible role for phosphorylation in regulating Asef2 activity and function.

Here, we describe the identification of phosphorylation sites in Asef2 using a liquid chromatography-mass spectrometry (LC-MS) approach consisting of high-mass resolution Orbitrap MS, data-dependent tandem MS (MS/MS), multiple protease and denaturing strategies, and bioinformatics-based peptide and protein assignments (Gant-Branum et al. 2010). This methodology yielded a 94.5% sequence coverage and identified six sites of

phosphorylation. The portion of the sequence that was not covered does not contain serine, threonine, or tyrosine residues; therefore, 100% coverage of possible phosphorylatable sites was achieved. The majority of these sites are clustered in the N-terminus of Asef2; one site (S106) is located in the ABR domain (Hamann et al. 2007; Kawasaki et al. 2007), suggesting that it could regulate Asef2 activity. Indeed, we show that S106 phosphorylation is crucial for Asef2-promoted Rac activation, cell migration, and adhesion turnover, pointing to a new regulatory mechanism for Asef2 activity and function.

Experimental Procedures

Reagents and plasmids – Mouse IgG agarose, FLAG M2-agarose affinity gel, FLAG peptide (DYKDDDDK), FLAG monoclonal antibody (clone M2), and fibronectin were obtained from Sigma (St. Louis, MO). Sodium vanadate was purchased from Fischer Scientific (Fairlawn, NJ), and calyculin A was obtained from EMD Millipore (Billerica, MA). Peroxovanadate was prepared as previously described (Gant-Branum et al. 2010). Glutathione sepharose beads were purchased from GE Healthcare Life Sciences (Piscataway, NJ). Phosphoserine polyclonal antibody (catalog number 61-8100) was obtained from Life Technologies (Grand Island, NY). GFP-Asef2 was generated by cloning human Asef2 (accession number: NM_153023.2) into EGFP-C3 vector (Clontech, Mountain View, CA) at *EcoRI* sites as previously described (Bristow et al. 2009). FLAG-CFP was prepared as previously described (Gant-Branum et al. 2010), and FLAG-CFP-Asef2 was generated by inserting human Asef2 into the FLAG-CFP vector at *EcoRI* sites. Asef2 serine 106 mutants were created via site-directed mutagenesis using the following primers: serine 106 to alanine (Asef2-S106A), forward (5'-GGTACTGAGCCCGCTGCCTTAGT-GGAT-3') and reverse (5'-ATCCACTAAGGCAGCGGGCTCAGTACC-3'); serine 106 to aspartic acid (Asef2-S106D), forward (5'-GGTACTGAGCCCGATGCCTTAGTGGAT-3') and reverse (5'-ATCCACTAAGGCATCGGGCTCAGTACC-3'). mCherry-paxillin was a generous gift from Steve Hanks (Vanderbilt University, Nashville, TN).

Cell culture and transfection – HT1080 fibrosarcoma cells and human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Grand Island, NY), which was supplemented with 10% fetal bovine serum (FBS; Thermo Scientific, Waltham, MA) and penicillin/streptomycin (Life Technologies, Grand Island, NY). These cells were maintained in an incubator with 5% carbon dioxide (CO_2) at 37 °C. Cells were transiently transfected with appropriate cDNAs using Lipofectamine™ 2000 (Life Technologies, Grand Island, NY) according to the manufacturer's instructions.

Protein purification – HEK293 cells were cultured in eight 150 mm dishes (Corning, Tewksbury, MA) for 24 h and then transfected with FLAG-CFP-Asef2 cDNA (12 μg per dish). After approximately 40 h, cells were treated with 1 mM peroxovanadate and 50 nM calyculin A for 10 min and then extracted with 25 mM Tris, 137 mM NaCl, 1% NP-40, 10% glycerol, and 2 mM EDTA (pH 7.4) containing a phosphatase inhibitor cocktail (Sigma, St. Louis, MO; catalogue number P2714) for 30 min on ice. The lysate was precleared with mouse IgG agarose for 1 h at 4 °C with end-over-end mixing; the lysate was then precleared a second time by overnight incubation with IgG agarose. After preclearing, the lysate was incubated with FLAG-agarose for 2 h at 4 °C with end-over-end mixing, and the beads were washed three times (15 min each, 4 °C) with 25 mM Tris and 100 mM NaCl (pH 7.4). FLAG-CFP-Asef2 protein was eluted from the beads by incubation with 0.2 mg/mL FLAG peptide suspended in 25 mM Tris and 100 mM NaCl (pH 7.4) for 30 min at 4 °C; this elution was repeated, and the eluates were pooled. The eluate was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% slab, followed by staining with Coomassie® Brilliant Blue R-250 (EMD Millipore, Billerica, MA) to determine the protein purity.

To examine serine phosphorylation, HEK293 cells from a single 150 mm dish per condition were transfected with 8 μg of either FLAG-CFP or FLAG-CFP-Asef2 cDNAs and were immunoprecipitated using the aforementioned protocol. The eluates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with either phosphoserine polyclonal antibody or M2 FLAG monoclonal antibody, followed by incubation with AlexaFluor 680 anti-rabbit IgG (Life Technologies, Grand Island, NY) or IRDye 800 anti-mouse IgG (Rockland Immunochemicals Inc., Gilbertsville, PA). Membranes were scanned with a LI-COR® Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

Enzymatic proteolysis – Purified Asef2 was separated into three aliquots containing equal amounts of protein and was subjected to enzymatic digestion using trypsin, chymotrypsin, and Glu-C proteases (Promega, Madison, WI), as described previously (Gant-Branum et al. 2010). Briefly, approximately 5 μg of purified Asef2 was resuspended in 75 μL of 25 mM ammonium bicarbonate and was aliquoted into three 25 μL samples containing approximately 1.7 μg of protein. Reduction and alkylation of cysteine sulfhydryl groups were performed by the addition of 1.5 μL of 45 mM dithiothreitol (DTT) and incubation for 30 min at 55 °C, followed by the addition of 2.5 μL of 100 mM iodoacetamide (IAM) and incubation in darkness for 30 min at room temperature. Digestion was performed by adding 42 ng of trypsin, chymotrypsin, or Glu-C at a ratio of 1:40 protease/protein (w/w), followed by incubation at 37 °C for 16, 4, and 6 h, respectively. To quench proteolysis, 1 μL of 88% formic acid was added. The digested material was lyophilized and reconstituted in 25 μL of 0.1% formic acid for MS analysis.

Two additional trypsin digestions were performed at strongly denaturing conditions using heat and organic solvent. Both samples contained approximately 2 μg of purified and aliquoted protein. For denaturation by heat, the sample was reconstituted with 25 mM ammonium bicarbonate and denatured for 15 min at 90 °C. For denaturation by high organic solvent, the

respective sample was reconstituted with 20 μL of acetonitrile (HPLC Grade) and 5 μL of 25 mM ammonium bicarbonate to achieve a solution of 80% acetonitrile (Russell et al. 2001; Strader et al. 2006). Both samples were treated with DTT and IAM as described above to reduce and alkylate cysteine sulfhydryl groups. Digestion was performed by adding 52 ng (1:40 protease/protein, w/w) of trypsin (Promega, Madison, WI) to each sample. The high organic solvent digestion (referred to as $Trypsin^{Org}$) was stopped after 1 h of incubation at 37 °C, while the high temperature denatured digestion (referred to as $Trypsin^{Temp}$) was allowed to proceed for 16 h at 37 °C. The digestions were quenched, dried, and reconstituted as described above.

LC-MS/MS – Initial digestions of Asef2 were loaded onto a reverse-phase capillary trap column using a helium-pressurized cell (pressure bomb). The trap column (360 μm OD x 150 μm ID) was fitted with a filter end-fitting (IDEX Health & Science, Oak Harbor, WA) and packed with 4 cm of C18 reverse phase material (Jupiter, 5 μm beads, 300 Å; Phenomenex, Torrance, CA). After sample loading, an M-520 microfilter union (IDEX Health & Science) was used to connect the trap column to a capillary analytical column (360 μm OD x 100 μm ID) equipped with a laser-pulled emitter tip and was packed with 20 cm of C18 material (Jupiter, 3 μm beads, 300Å; Phenomenex, Torrance, CA). Peptides were gradient-eluted at a flow rate of 500 nL/min using an Eksigent NanoLC Ultra HPLC, and the mobile phase solvents consisted of 0.1% formic acid, 99.9% water (solvent A) and 0.1% formic acid, 99.9% acetonitrile (solvent B). The gradient consisted of the following: 2-45% B in 40 min, 45-90% B in 10 min, 90% B for 5 min, 90-2% B in 10 min. Subsequent trypsin digestions of Asef2 were loaded directly onto the capillary analytical column using the Eksigent NanoLC Ultra HPLC and autosampler, and the same reverse phase gradient was performed. Upon gradient-elution, peptides were mass analyzed on a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a nanoelectrospray ionization source. The instrument was operated using a data-dependent

method with dynamic exclusion enabled. Full scan (m/z 300-2000) spectra were acquired with the Orbitrap as the mass analyzer (resolution 60,000), and the top ten most abundant ions in each MS scan were selected for fragmentation in the LTQ. An isolation width of 2 m/*z*, activation time of 10 ms, and normalized collision energy of 35% were used to generate MS/MS spectra. The MS^n AGC target value was set to $1x10^4$, and the maximum injection time was 100 ms.

Bioinformatics – For peptide identification, tandem mass spectra were converted into DTA files using Scansifter and searched using a custom version of SEQUEST (Thermo Fisher Scientific, San Jose, CA) on the Vanderbilt ACCRE Linux cluster against a concatenated forward and reversed (decoy) database containing the *Homo sapiens* subset of the UniProtKB (www.uniprot.org) protein database, which was appended with the Asef2 sequence containing the PCR fragment "IRL" prior to the N-terminus methionine for improved coverage of the N-terminus of Asef2. The chymotrypsin digestion was searched with non-specific protease conditions. A maximum of 3 missed cleavages was allowed for trypsin digests, where cleavage was restricted to the C-terminal side of arginine (R) and lysine (K) residues, and 6 missed cleavages were allowed for Glu-C with cleavage restricted to the C-terminal side of glutamic acid (E) residues. Spectra were searched using a 2.5 Da mass tolerance for the precursor peptide mass, and parameters were set to search for monoisotopic masses of the product ions. Allowable variable modifications were limited to carbamidomethyl derivatization of cysteine, oxidation of methionine, and phosphorylation of serine, tyrosine, and threonine. Scaffold version 4.3.2 (Proteome Software Inc., Portland, OR) was used to visualize and validate peptide and protein identifications based on MS/MS data. A minimum probability threshold of 95% was required for peptide identifications; however, most peptides achieved probabilities of 99% or greater. For protein identification, the minimum requirements were four identified peptides per protein and a protein probability threshold of 99% (Keller et al. 2002; Nesvizhskii et al. 2003). For all samples, the decoy false discovery rates (FDR) were 0.0% at the protein level and

0.00% at the peptide level. Identifications made to Asef2 were based on the sequence associated with accession number A2VEA_HUMAN, and Asef2 was identified in all samples with 100% probability. All potential peptides and phosphopeptides achieving the minimum probability threshold were manually validated. Additionally, several peptides not found by the bioinformatics were manually identified and validated.

Migration analysis and microscopy – HT1080 cells were transfected with 1.5 μg of GFP, GFP-Asef2, GFP-Asef2-S106A, or GFP-Asef2-S106D cDNAs and were incubated for 24 h at 37 °C. Then, the cells were plated on tissue culture dishes that were coated with 5 μg/mL fibronectin (diluted in Dulbecco's Phosphate Buffered Saline (DPBS, Life Technologies, Grand Island, NY)) and allowed to adhere for 1 h at 37 °C. Prior to imaging, the culture medium was replaced with SFM4MAb^{TM} medium (Hyclone, Logan, UT) supplemented with 2% FBS. Images were obtained every 5 min for 6 h using an inverted Olympus IX71 microscope (Melville, NY) with a Retiga EXi CCD camera (QImaging, Surrey, BC), a 10X objective (NA 0.3), and MetaMorph software (Molecular Devices, Sunnyvale, CA) connected to a Lambda 10-2 automated controller (Sutter Instruments, Novato, CA). GFP-expressing cells were visualized with an Endow GFP Bandpass filter cube (excitation HQ470/40, emission HQ525/50, Q495LP dichroic mirror) (Chroma, Brattleboro, VT). MetaMorph software was used to track cell movement, and the migration speed was calculated by dividing the net distance traveled (μm) by the migration time (h). Wind-Rose plots were generated as previously described (Jean et al. 2013). SPSS Statistics, version 22 (Armonk, NY), was used for statistical analyses of migration, adhesion turnover, and Rac activity assays. One-way ANOVA was performed to compare multiple means, followed by post hoc tests (Games-Howell pairwise comparison tests) to determine the level of significance ($p < 0.05$).

Adhesion turnover assay – HT1080 cells were co-transfected with 1.5 μg mCherry-paxillin cDNA and 1.5 μg of either GFP, GFP-Asef2, GFP-Asef2-S106A, or GFP-Asef2-S106D cDNAs and were incubated for 24 h. Cells were then plated on glass-bottom dishes coated with fibronectin (5 μg/mL) and were allowed to adhere for 1 h at 37 °C. Fluorescent time-lapse images were acquired at 15 s intervals for 20 min using the Olympus IX71 microscope setup described above with a PlanApo 60X OTIRM objective (NA 1.45) and Metamorph software. mCherry was visualized with a TRITC/Cy3 cube (excitation HQ545/30, emission HQ610/75, Q570LP dichroic mirror). The $t_{1/2}$ values for adhesion assembly and disassembly were measured as previously described (Webb et al. 2004; Bristow et al. 2009).

Rac activity assay – The Rac binding domain (termed p21-binding domain, or PBD) from the effector p21-activated kinase (PAK) was tagged with glutathione-S-transferase (GST), expressed, and attached to glutathione sepharose beads as previously described (Knaus et al. 2007). HT1080 cells were cultured on 60 mm tissue culture dishes coated with 5 μg/mL fibronectin and co-transfected with 2 μg FLAG-Rac1 cDNA and 4 μg of either GFP, GFP-Asef2, GFP-Asef2-S106A, or GFP-Asef2-S106D cDNAs. After 24 h, cells were lysed and assayed for Rac activity as previously described (Knaus et al. 2007; Jean et al. 2013). Briefly, cells were lysed with 50 mM Tris, 1% NP-40, 10% glycerol, 100 mM NaCl, 2 mM $MgCl_2$, and a protease inhibitor cocktail, pH 7.5 (lysis buffer). A small fraction of each lysate was kept to determine the amount of total Rac. The remaining lysate was incubated with GST-PBD beads for 1 h at 4 °C with end-over-end mixing. The beads were washed three times with lysis buffer. Then, the bound protein was eluted from the beads with Laemmli sample buffer and analyzed via Western blot. The amount of active Rac pulled down was normalized to total Rac for each condition.

Results and Discussion

Identification of phosphorylation sites in human Asef2 – Because Asef2 phosphorylation, which could be an important regulatory mechanism for the activity and function of this protein, had not been previously investigated, we utilized an LC-MS/MS-based approach to uncover potential phosphorylation sites in Asef2 (Gant-Branum et al. 2010). To perform MS analyses, FLAG-CFP-Asef2 was expressed in HEK293 cells and then purified according to the immunoprecipitation protocol outlined in Figure 19A (Gant-Branum et al. 2010). A predominant band with a molecular mass corresponding to that of FLAG-CFP-Asef2 was observed when the immunoprecipitated protein sample was subjected to SDS-PAGE followed by Coomassie® Blue staining (Figure 19B). This band was confirmed to be FLAG-CFP-Asef2 via Western blot analysis (Figure 19C). We next examined the phosphorylation state of Asef2 by using a phosphoserine antibody; a distinct band was observed for the Asef2 sample compared to the control sample (FLAG-CFP), demonstrating that Asef2 is phosphorylated on serine residues (Figure 19C). Collectively, these results indicate that the immunoprecipitated protein sample is suitable for MS analysis to identify specific phosphorylated residues in Asef2.

Multiple proteases were used to obtain complete coverage of the potential sites of phosphorylation in Asef2. Initially, trypsin, chymotrypsin, and Glu-C digestions were used, providing partial (86%) sequence coverage, with 93% coverage of serine, threonine, and tyrosine residues. However, two significant stretches of the Asef2 protein sequence from R492-K518 and R561-Y596 (Figure 20A) were not covered in the trypsin, chymotrypsin, or Glu-C digestions. These regions have a high abundance of aspartic acid and glutamic acid residues, and therefore may not provide peptides of suitable length for MS analysis upon digestion with Glu-C. While these amino acid sequences have multiple lysine and arginine residues, regions R492-K518 and R561-Y596 were found to be inaccessible or resistant to trypsin and chymotrypsin under standard digestion conditions.

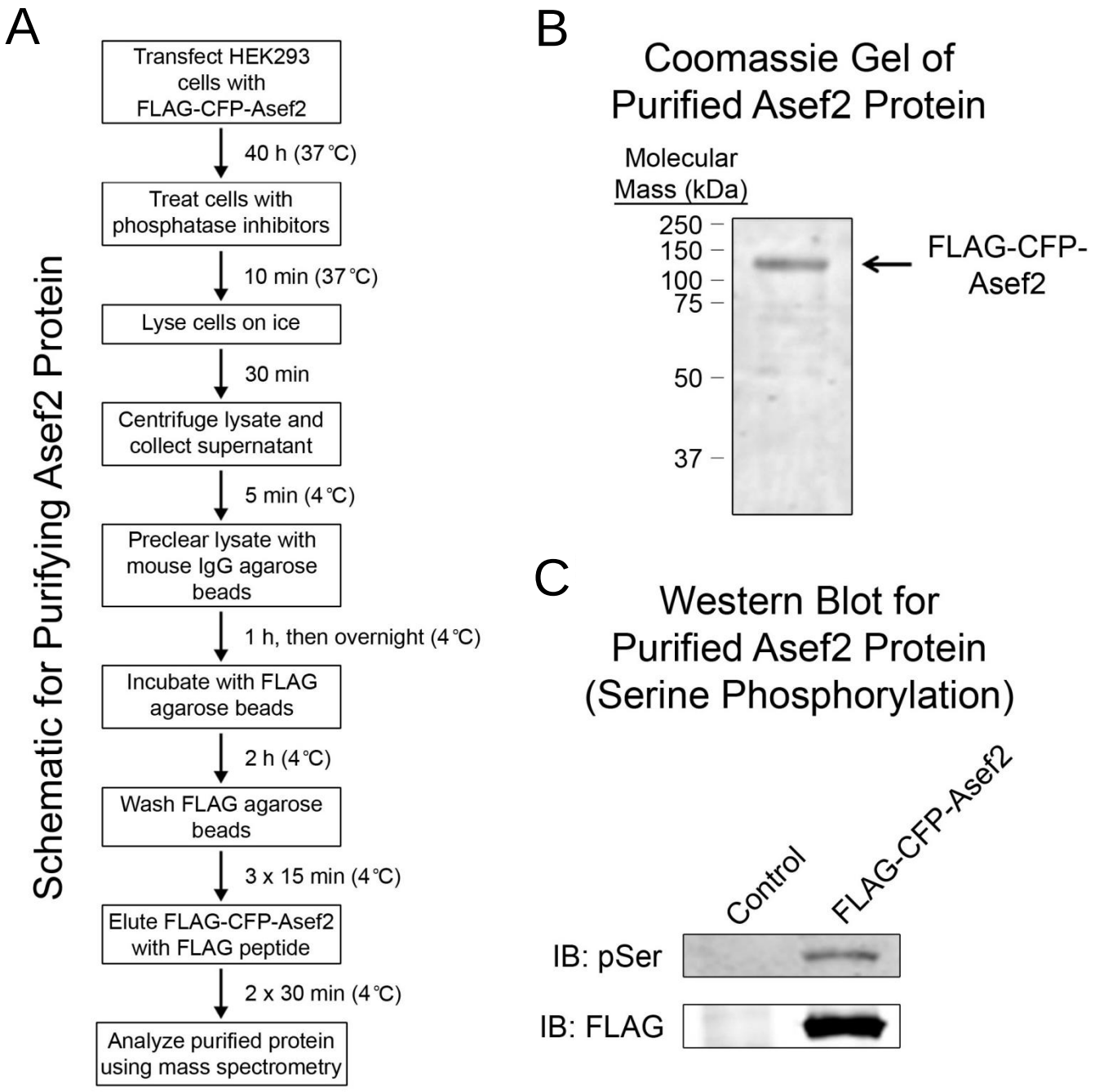


Figure 19. Purification of FLAG-CFP-Asef2. *A*, Schematic showing the protocol used to purify FLAG-CFP-Asef2 for MS analysis. *B*, SDS-PAGE gel of immunoprecipitated FLAG-CFP-Asef2 that was stained with Coomassie® Blue. The arrow points to the band representing purified FLAG-CFP-Asef2 in the eluted sample. *C*, Purified FLAG-CFP (Control) and FLAG-CFP-Asef2 were subjected to SDS-PAGE, followed by immunoblotting (IB) with phosphoserine (pSer, upper panel) and FLAG (lower panel) antibodies. These panels show that FLAG-CFP-Asef2 is phosphorylated on serine residues.

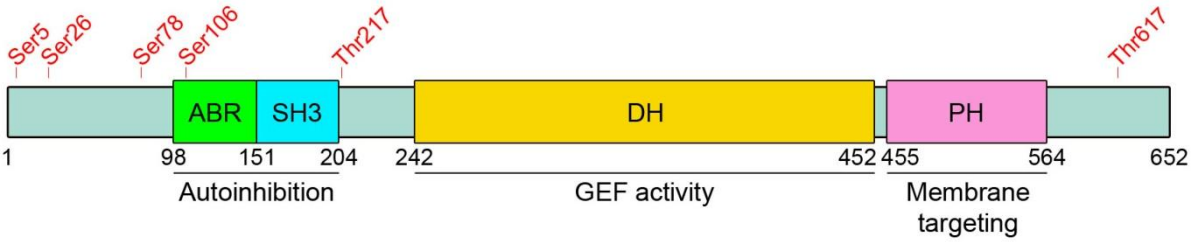
In order to obtain sequence coverage of regions R492-K518 and R561-Y596 of Asef2, additional trypsin digestions were performed using two strongly denaturing approaches: heat and high organic solvent concentration. Digestions done in mixed aqueous-organic solvent conditions have been demonstrated to increase peptide identifications from proteolysis-resistant proteins, while simultaneously allowing for shorter digestion times (Russell et al. 2001; Strader et al. 2006). Our results showed that an additional 44 residues were covered in regions R492-K518 and R561-Y596 with the strongly denaturing digestions, which were observed as the following peptides: ^{494}*DMLYYK*^{499} and ^{517}*DK*DCNLSVK^{525} in the Trypsin^{Heat} digestion, and ^{563}*VQEDKEMGMEISENQKKLAMLNAQK*^{587}, ^{588}*AGHGKSKGY*NRCPVAPPHQGLHPIHQR^{614}, and ^{552}WLQACADER*RR*^{562} in the Trypsin^{Org} digestion, where the italicized portions contributed to the 44 additional residues observed. Of these 44 amino acids, approximately 64% were observed in the high organic solvent digestion (Trypsin^{Org}). Combined, the two strongly denaturing digestions yielded nearly 82% sequence coverage and accessed additional regions of Asef2 compared to the more conventional, aqueous-based digestions. A sequence coverage of 94.5% was achieved with the five different digestions (Figure 20C), and complete coverage of the serine, threonine, and tyrosine residues were obtained in the identified peptides.

The identified phosphopeptides are shown in Table 1, along with the type of enzymatic digestion used and the associated mass error. Phosphopeptide identities were initially revealed by SEQUEST, but each was manually validated to confirm the location of phosphorylation. Four sites of phosphorylation were identified in the digestions using standard conditions: pS5, pS78, pS106 and pT217. Two additional sites, pS26 and pT617, were identified from the strongly denatured digestions. Five (pS5, pS26, pS78, pS106 and pT217) of the six total sites of phosphorylation were observed in multiple digests. An example of MS/MS data for the phosphorylated peptide, ^{94}ASNVSSDGGTEP**pS**ALVDDNGSEEDFSYEDLCQASPR^{129} is shown

A

1	MTSASPEDQN	APVGCPKGAR	RRRPISVIGG	VSLYGTNQTE	ELDNLLTQPA
51	SRPPMPAHQV	PPYKAVSARF	RPFTFSQSTP	IGLDRVGRRR	QMRASNVSSD
101	GGTEPSALVD	DNGSEEDFSY	EDLCQASPRY	LQPGGEQLAI	NELISDGNVV
151	CAEALWDHVT	MDDQELGFKA	GDVIQVLEAS	NKDWWWGRSE	DKEAWFPASF
201	VRLRVNQEEL	SENSSSTPSE	EQDEEASQSR	HRHCENKQQM	RTNVIREIMD
251	TERVYIKHLR	DICEGYIRQC	RKHTGMFTVA	QLATIFGNIE	DIYKFQRKFL
301	KDLEKQYNKE	EPHLSEIGSC	FLQNQEGFAI	YSEYCNNHPG	ACLELANLMK
351	QGKYRHFFEA	CRLLQQMIDI	AIDGFLLTPV	QKICKYPLQL	AELLKYTTQE
401	HGDYSNIKAA	YEAMKNVACL	INERKRKLES	IDKIARWQVS	IVGWEGLDIL
451	DRSSELIHSG	ELTKITKQGK	SQQRTFFLFD	HQLVSCKKDL	LRRDMLYYKG
501	RLDMDEMELV	DLGDGRDKDC	NLSVKNAFKL	VSRTTDEVYL	FCAKKQEDKA
551	RWLQACADER	RRVQEDKEMG	MEISENQKKL	AMLNAQKAGH	GKSKGYNRCP
601	VAPPHQGLHP	IHQRHITMPT	SVPQQQVFGL	AEPKRKSSLF	WHTFNRLTPF
651	RK				

B



C

Protease	# of Amino Acids	Sequence Coverage
Chymotrypsin	477 / 652	73.2%
Glu-C	181 / 652	27.8%
Trypsin	371 / 652	56.9%
Trypsin^{Temp}	426 / 652	65.3%
Trypsin^{Org}	390 / 652	59.8%
Total	**616 / 652**	**94.5%**

Figure 20. Phosphorylation sites identified in Asef2. *A*, The protein sequence of Asef2 is shown with the phosphorylation sites that were detected by MS in red. Serine, threonine, and tyrosine residues that did not have detectable phosphorylation are shown in blue. Colored shading represents the conserved domains in Asef2 as shown in panel B. Underlined residues were not detected in the MS analyses. *B*, Schematic of Asef2 showing conserved domains and the location of the six identified phosphorylation sites (red). The domain numbering is based on Kawasaki et al.(Kawasaki et al. 2007) *C*, Summary of Asef2 amino acid sequence coverage by MS analyses. Purified Asef2 samples were treated with multiple proteases (trypsin, chymotrypsin, or Glu-C) to achieve high sequence coverage. Additional trypsin digestions were performed with strongly denaturing conditions, which included high temperature (90 ^{o}C, Trypsin^{Temp}) or a high percentage of organic solvent (80% acetonitrile, Trypsin^{Org}), to obtain sequence coverage of regions that were resistant to trypsin under standard digestion conditions.

Table 1. Phosphorylation Sites Identified in Asef2 by MS Analysis

Peptide[a]	Sequence Position	Protease[b]	*m/z*[c] (Charge)	Mass error (ppm)
-LMTSA**pS**PEDQNAPVGC*PK[17]	S5	Trypsin[Org]	991.42 (+2)	0.91
[2]TSA**pS**PEDQNAPVGC*PK[17]	S5	Chymo	869.36 (+2)	-1.27
[22]RRPI**pS**VIGGVSLYGTNQTEELDNLLTQPASRPPMPAHQVPPYK[64]	S26	Trypsin[Org]	962.30 (+5)	1.77
[23]RPI**pS**VIGGVSLYGTNQTEELDNLLTQPASRPPMPAHQVPPYK[64]	S26	Trypsin[Temp]	1163.59 (+4)	0.52
[23]RPI**pS**VIGGVSLYGTNQTEELDNLLTQPASRPPMPAHQVPPYK[64]	S26	Trypsin[Org]	1163.59 (+4)	2.49
[70]FRPFTFSQ**pS**TPIGLDR[85]	S78	Trypsin	650.32 (+3)	0.92
[70]FRPFTFSQ**pS**TPIGLDR[85]	S78	Trypsin[Temp]	650.32 (+3)	0.77
[70]FRPFTFSQ**pS**TPIGLDR[85]	S78	Trypsin[Org]	650.32 (+3)	2.00
[70]FRPFTFSQ**pS**TPIGLDRVGR[88]	S78	Trypsin[Org]	566.04 (+4)	0.53
[70]FRPFTFSQ**pS**TPIGLDRVGRR[89]	S78	Trypsin[Org]	605.06 (+4)	0.66
[94]ASNVSSDGGTEP**pS**ALVDDNGSEEDFSYEDLC*QASPR[129]	S106	Trypsin	1295.86 (+3)	-0.23
[94]ASNVSSDGGTEP**pS**ALVDDNGSEEDFSYEDLC*QASPR[129]	S106	Trypsin[Temp]	1295.86 (+3)	-0.39
[94]ASNVSSDGGTEP**pS**ALVDDNGSEEDFSYEDLC*QASPR[129]	S106	Trypsin[Org]	1295.86 (+3)	1.00
[205]VNQEELSENSSS**pT**PSEEQDEEASQSR[230]	T217	Trypsin	992.73 (+3)	-0.40
[205]VNQEELSENSSS**pT**PSEEQDEEASQSR[230]	T217	Trypsin[Temp]	992.73 (+3)	0.71
[203]LRVNQEELSENSSS**pT**PSEEQDEEASQSR[230]	T217	Trypsin[Org]	1082.46 (+3)	0.00
[210]LSENSSS**pT**PSEEQDEEASQSRHRHC*E[235]	T217	Glu-C	774.81 (+4)	-0.26
[615]HI**pT**MPTSVPQQQVFGLAEPK[634]	T617	Trypsin[Org]	763.38 (+3)	1.18

[a] The hyphen "-" indicates the peptide occurs at the N-terminus of Asef2, and includes a residual PCR fragment leucine (L) prior to the start of Asef2; the "p" denotes a site of phosphorylation; an asterisk "*" denotes carboxyamidomethylation of cysteine.
[b] "Chymo" refers to digestion using chymotrypsin. The superscript "Org" denotes sample denatured by a high percentage of organic solvent; the superscript "Temp" denotes sample denatured by heat.
[c] The *m/z* values shown here are truncated to two decimal places; however, mass errors were calculated with *m/z* values extended to four decimal places.

in Figure 21. This peptide was conserved in all three tryptic digests, and the observation of y_{23}^{+3} enabled the exact site of phosphorylation (pS106) to be discerned.

Serine phosphorylation stimulates Asef2 GEF activity – A majority of the confirmed phosphorylation sites in Asef2 are concentrated at the N-terminus (Figure 20B). One residue (S106) is in the ABR domain, while four others (S5, S26, S78, and T217) bracket the adjacent ABR-SH3 domains. The sixth phosphorylation site (T617), conversely, resides in the C-terminus of Asef2 (Figure 20B). Of these six phosphorylation sites, S106 was particularly intriguing because of its location in the ABR domain, which is a critical region for Asef2 activation (Hamann et al. 2007; Kawasaki et al. 2007). This led us to hypothesize that phosphorylation of S106 is an important regulatory mechanism for Asef2 GEF activity. To investigate the effect of S106 phosphorylation on Asef2 activity, we mutated this residue to either alanine (S106A) or aspartic acid (S106D) using site-directed mutagenesis; these substitutions represent non-phosphorylatable and phosphomimetic analogues, respectively (Langan et al. 1971; Thorsness and Koshland 1987; Tarrant and Cole 2009). Then, we assessed the effect of S106 mutation on the activation of the small GTPase Rac using a GTPase activity assay (Bristow et al. 2009; Jean et al. 2013). In this assay, the GST-tagged binding domain from the Rac effector PAK (GST-PBD) is used to detect the active form of Rac from lysates of GFP- and GFP-Asef2-expressing cells (Figure 22A). As expected, expression of wild-type Asef2 caused a significant increase in the level of active Rac (Figure 22B). Quantification showed that the amount of active Rac was increased approximately 8-fold in GFP-Asef2-expressing cells compared with control cells expressing GFP (Figure 22C). In contrast, GFP-Asef2-S106A expression caused an approximately 80% decrease in active Rac compared to expression of GFP-Asef2 (Figure 22B,C), suggesting that phosphorylation of Asef2 at S106 promotes its GEF activity towards Rac. Expression of GFP-Asef2-S106D resulted in an approximately 2-fold increase in active

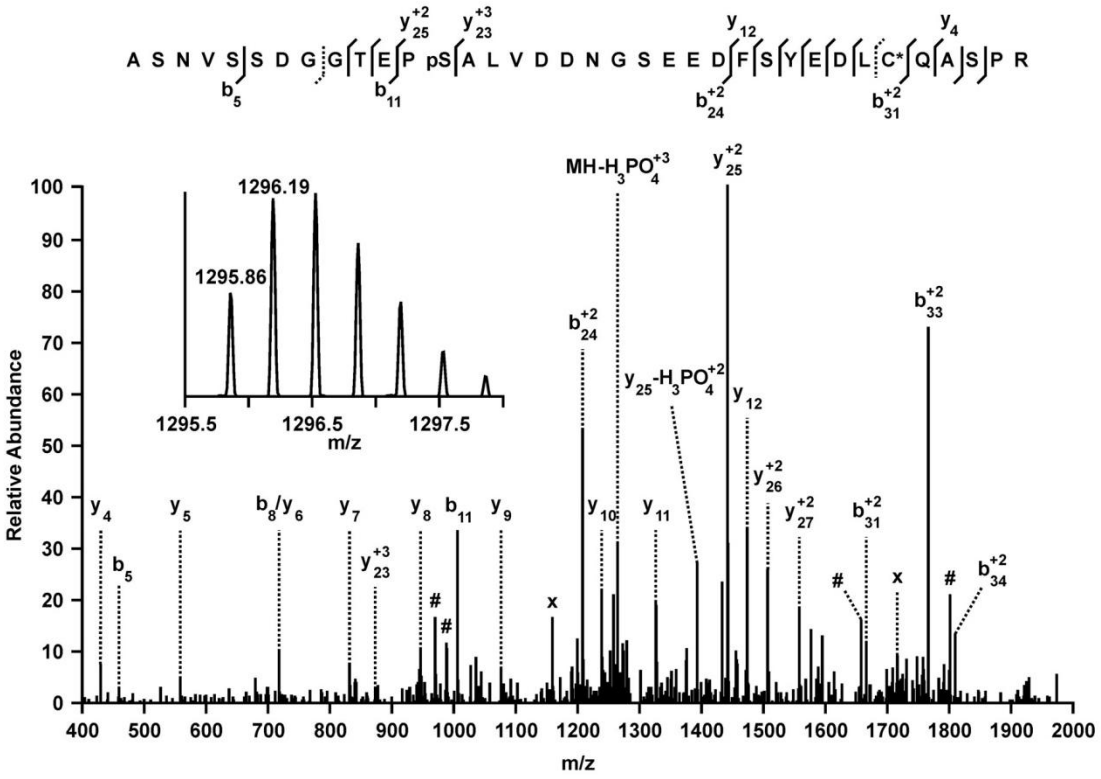


Figure 21. MS and MS/MS spectra for an Asef2 phosphorylated peptide. Data-targeted MS/MS scan of *m/z* 1295.86 in the Trypsin^{Org} sample, corresponding to the triply charged (inset) phosphopeptide ^{94}ASNVSSDGTEP**pS**ALVDDNGSEEDFSYEDLC*QASPR^{129}. All backbone cleavages (b and y ions) observed are marked on the sequence (top), and additional ions are labeled in the spectrum. Sequence positions for b_8 and y_6 are shown with dashed markers (top) due to isobaric *m/z* values of 718.3. "C*" denotes a carbamidomethyl-modified cysteine. "#" indicates ions corresponding to $-H_2O$ from b_{11} (2), b_{31}^{+2}, and b_{34}^{+2}, respectively. "X" denotes ions corresponding to $-H_3PO_4$ from b_{24}^{+2} and b_{33}^{+2}, respectively.

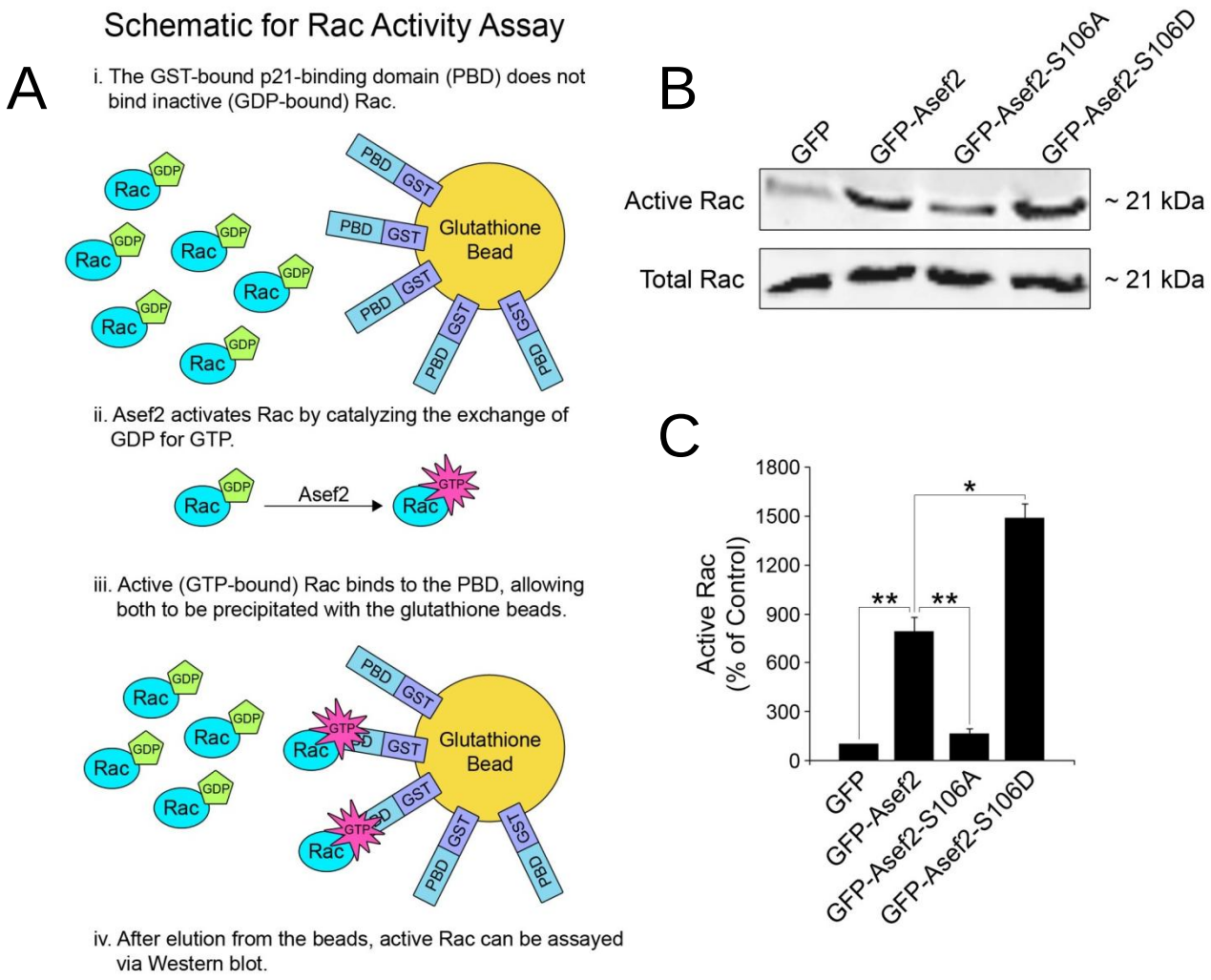


Figure 22. Phosphorylation of S106 stimulates Asef2 GEF activity. *A*, Schematic depicting the protocol used to detect active Rac (Rac activity assay), which was modified from Kraus et al.(Knaus et al. 2007) *B*, HT1080 cells were co-transfected with FLAG-Rac1 cDNA and either GFP, GFP-Asef2, GFP-Asef2-S106A, or GFP-Asef2-S106D, and active Rac was subsequently pulled down from lysates from these cells. The amount of total Rac is shown as a control. *C*, Quantification of the amount of active Rac from 3-7 separate experiments is shown. Error bars represent s.e.m. *, p = 0.007, **, p = 0.001.

Rac compared to expression of GFP- Asef2 (Figure 22B,C). Therefore, these results point to S106 as an important phosphorylation site in Asef2 that mediates its ability to activate Rac.

S106 phosphorylation regulates cell migration – We have previously shown that Asef2 promotes the migration of HT1080 cells plated on fibronectin via active Rac (Bristow et al. 2009). In this study, we demonstrate that phosphorylation of S106 is critical for Asef2-mediated activation of Rac. This led us to hypothesize that S106 phosphorylation of Asef2 plays a role in regulating cell migration. To test this hypothesis, we transfected HT1080 cells with either GFP, GFP-Asef2, GFP-Asef2-S106A, or GFP-Asef2-S106D cDNAs, then plated the cells on fibronectin-coated dishes, and assessed cell migration using live-cell imaging. Migration data were generated by tracking individual cells and were used to calculate the migration speed. Figure 23A shows the individual tracks of GFP-, GFP-Asef2-, GFP-Asef2-S106A-, and GFP-Asef2-106D-expressing cells. The migration paths of GFP-Asef2-expressing cells were significantly longer than those of control cells expressing GFP. Quantification showed an approximately 1.3-fold increase in migration speed in GFP-Asef2-expressing cells compared to those expressing GFP (Figure 23B). Intriguingly, mutation of serine 106 to alanine abolished this increase in migration (Figure 23B). The migration speed of GFP-Asef2-S106A-expressing cells was significantly decreased compared to cells expressing GFP-Asef2, suggesting that S106 phosphorylation is important for Asef2-promoted cell migration. All of the GFP-tagged proteins were expressed at similar levels (Figure 23C), indicating that changes in migration speed were not due to differential protein expression. Expression of the phosphomimetic S106D mutant resulted in an increase in migration speed compared to that observed with GFP expression (Figure 23B). However, the migration speed of cells expressing GFP-Asef2-S106D was not significantly different than the migration speed of GFP-Asef2-expressing cells. This result is somewhat unexpected, given the additional increase in Rac activity detected in the

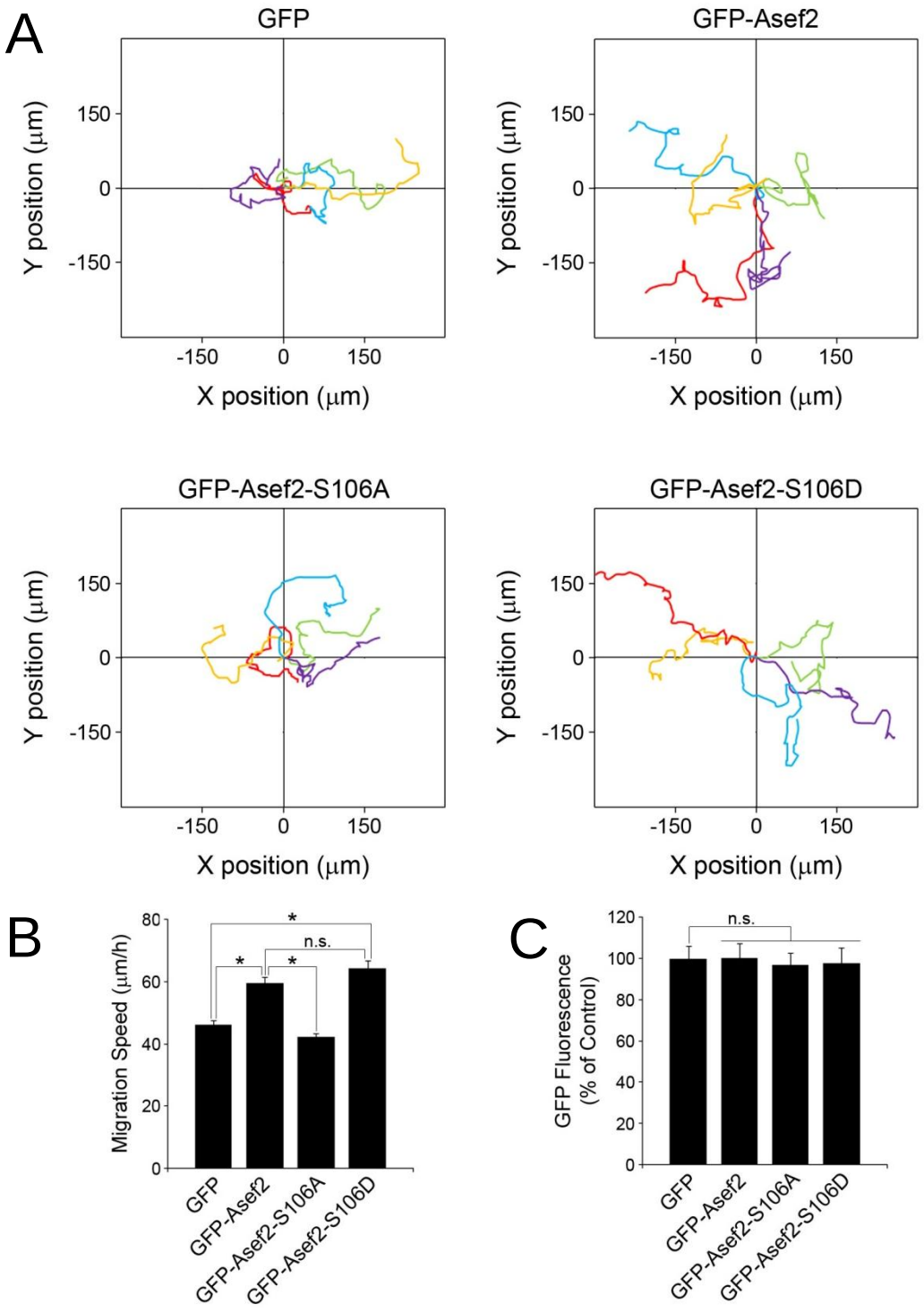


Figure 23. Phosphorylation of S106 is critical for Asef2-promoted cell migration. *A*, HT1080 cells expressing GFP, GFP-Asef2, GFP-Asef2-S106A, or GFP-Asef2-S106D were plated on fibronectin-coated dishes and imaged using time-lapse microscopy. The migration of individual cells was tracked and analyzed. Wind-Rose plots depicting the migration tracks for individual cells are shown. *B*, Migration speed was quantified for GFP-, GFP-Asef2-, GFP-Asef2-S106A-, and GFP-Asef2-S106D-expressing cells. Error bars represent s.e.m. for 66-159 cells from 4-9 independent experiments (*, $p < 0.001$). *C*, Quantification of the fluorescence intensity in cells transfected with the indicated cDNAs shows that all the constructs were expressed at comparable levels. Error bars represent s.e.m. for 69-76 cells from 3 separate experiments. For panels B and C, "n.s." denotes no statistically significant difference.

GFP-Asef2-S106D-expressing cells (Figure 22B,C). The expression of wild-type Asef2 may be sufficient to maximally stimulate Asef2 signaling, at least in terms of promoting cell migration. Specifically, the high level of active Rac resulting from wild-type Asef2 expression could be adequate to saturate downstream signaling; thus, a further increase in active Rac, such as that caused by GFP-Asef2-S106D expression, would not yield a higher migration speed. Consistent with this, a previous study showed that expression of constitutively-active Rac did not cause a further increase in migration speed compared to that observed with wild-type Rac expression (Pankov et al. 2005). Nevertheless, these results underscore the importance of S106 phosphorylation in regulating Asef2-mediated cell migration.

Phosphorylation of S106 modulates adhesion turnover – Because the ability of cells to migrate efficiently is dependent on the proper assembly and disassembly of their adhesions (adhesion turnover), and because Asef2-Rac signaling plays an important role in regulating adhesion dynamics (Rottner et al. 1999; Webb et al. 2004; Bristow et al. 2009), S106 phosphorylation may affect migration by modulating adhesion turnover. Therefore, we co-expressed mCherry-paxillin, a well-characterized adhesion marker, with GFP, GFP-Asef2, GFP-Asef2-S106A, or GFP-Asef2-S106D in HT1080 cells and analyzed adhesion turnover using an adhesion turnover assay that we previously developed (Webb et al. 2004; Bristow et al. 2009). In this assay, mCherry-paxillin-containing adhesions from these cells were imaged using time-lapse microscopy (Figure 24A), and the change in fluorescence intensity in individual adhesions was used to calculate $t_{1/2}$ values for adhesion assembly and disassembly. Cells expressing GFP-Asef2 exhibited an approximately 50% decrease in the $t_{1/2}$ values for adhesion assembly and disassembly compared to GFP-expressing cells (Figure 24B); this suggests that adhesions in GFP-Asef2-expressing cells turn over more quickly, resulting in faster cell migration speeds (Bristow et al. 2009). Conversely, the $t_{1/2}$ values for both adhesion assembly and disassembly

were significantly larger in GFP-Asef2-S106A-expressing cells compared to those cells expressing GFP-Asef2 (Figure 24B). These data are consistent with the slower cell migration speed that was observed in GFP-Asef2-S106A-expressing cells (Figure 23B), further emphasizing the importance of phosphorylation of this residue for efficient cell migration. Expression of the S106D mutant, on the other hand, resulted in $t_{1/2}$ values that were comparable to those observed with GFP-Asef2 expression. Collectively, these results suggest that the phosphorylation of Asef2 at S106 promotes faster adhesion turnover, which is critical for proficient cell migration.

Conclusions

Asef2 is emerging as an important GEF in modulating cellular processes, such as migration and adhesion dynamics; however, the mechanisms that regulate the activity and function of Asef2 are currently not well understood. In this study, we identified six phosphorylation sites in Asef2 by LC-MS/MS analysis. We demonstrate that phosphorylation of one of these sites, S106, which is located in the ABR domain, is important for modulating Asef2 GEF activity as well as for Asef2 function in cell migration and adhesion turnover. Four of the other detected phosphorylation sites (S5, S26, S78, and T217) are congregated toward the N-terminus of Asef2, and it is possible that they contribute to Asef2 regulation and/or function as well. Indeed, the N-terminal location of these phosphorylation sites puts them in a potential position to regulate the autoinhibitory state of Asef2, because this region of the protein contains the ABR-SH3 module, which maintains Asef2 in an autoinhibited, inactive state (Hamann et al. 2007). The sixth phosphorylation site (T617) is located in the C-terminus of Asef2. The C-terminus associates with the ABR-SH3 module to maintain Asef2 in an autoinhibitory state (Hamann et al. 2007). Thus, phosphorylation within this region could also affect Asef2 GEF activity. Furthermore, the C-terminus of Asef2 is involved in mediating protein-protein

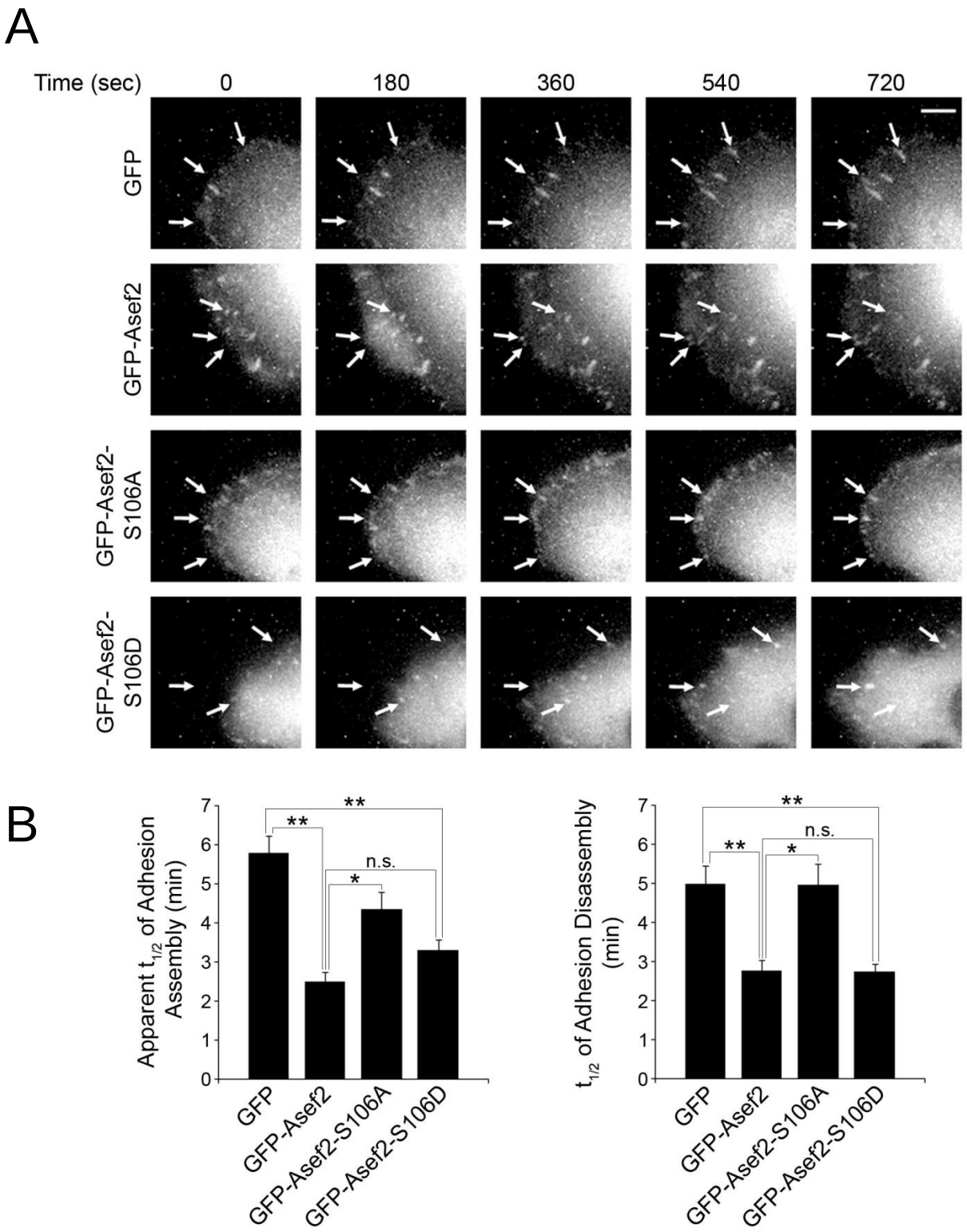


Figure 24. S106 phosphorylation regulates adhesion turnover. *A*, HT1080 cells were co-transfected with mCherry-paxillin cDNA and either GFP, GFP-Asef2, GFP-Asef2-S106A, or GFP-Asef2-S106D cDNAs and were subsequently used in adhesion turnover assays. Time-lapse images show adhesions that are assembling and disassembling at the leading edge of migrating cells (arrows). Bar=5 μm. *B*, Quantification of the apparent $t_{1/2}$ of adhesion assembly and the $t_{1/2}$ of adhesion disassembly for transfected cells is shown. Error bars represent s.e.m. for 34-70 adhesions, which were analyzed in 11-22 cells from 3-6 independent experiments (*, $p < 0.005$, **, $p < 0.001$). "n.s." denotes no statistically significant difference.

interactions; for example, Asef2 interacts with the actin-binding protein spinophilin, via this region (Sagara et al. 2009). Phosphorylation of T617 could be involved in regulating this association or other protein-protein interactions. Future studies are needed to determine the significance of these phosphorylation sites on Asef2 activity and function.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions and working models

We have shown that the guanine nucleotide exchange factor Asef2 promotes the formation of dendritic spines and synapses. In rat hippocampal neurons, Asef2 is found at sites of synaptic contact. Knockdown of Asef2 expression reduces spine and synapse density, while exogenous expression of Asef2 increases the number of spines and synapses. Asef2's role in spine development is dependent on its GEF activity, as mutations in its DH (GEF activity) domain that inhibit Rac activation abolish Asef2-mediated spine and synapse formation. Furthermore, knockdown of Rac blocks the Asef2-dependent development of spines. We have also found that the scaffold protein spinophilin, which has previously been shown to interact with Asef2 (Sagara et al. 2009), regulates Asef2's effect on spine and synapse density. Knockdown of spinophilin abolishes the increase in spines and synapses in Asef2-expressing cells. Also, in neurons exogenously expressing Asef2 and spinophilin, Asef2 co-localizes with spinophilin in spines. These results point to a model in which spinophilin targets Asef2 to sites of potential spine growth (Figure 25). Activation of Asef2 at these sites causes localized activation of Rac, which promotes actin polymerization and eventually the outgrowth of new spines. As the spines mature, spinophilin could continue to promote Asef2-dependent Rac activation to modulate actin dynamics within the spine head. The structural plasticity of the spines, therefore, could play a role in regulating synaptic transmission and, ultimately, the storage of information as memory.

We have also investigated the role of phosphorylation in regulating Asef2's activity. Previous work has demonstrated that Asef2 promotes cell migration through the activation of

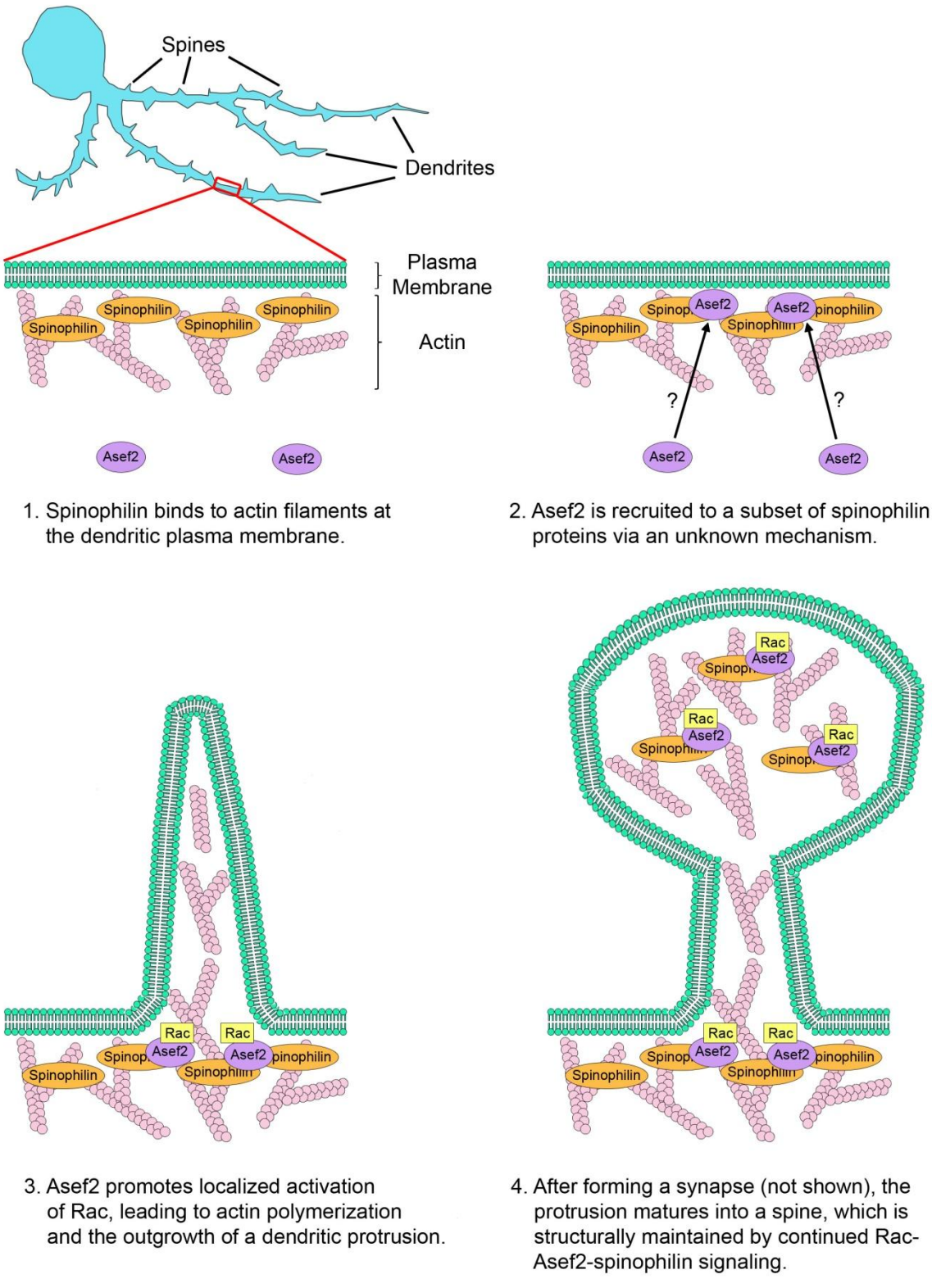


Figure 25. Model of Asef2-dependent dendritic spine formation.

Rac (Bristow et al. 2009). In collaboration with the McLean lab, we have identified six putative phosphorylation sites in Asef2 using a mass spectrometry approach. Five of these sites cluster around the N-terminus, while a single phosphorylation site is in the C-terminus. Because the N-terminus regulates the autoinhibition of Asef2 (Hamann et al. 2007; Kawasaki et al. 2007), the phosphorylation sites found in this area could mediate Asef2 activity. We mutated one of the phosphorylation sites, serine 106 in the APC-binding region of Asef2, to either disrupt or mimic phosphorylation. Loss of phosphorylation at this site abolished the Asef2-dependent activation of Rac; the phosphomimetic mutant, on the other hand, greatly enhanced Asef2's GEF activity. We also found that phosphorylation of serine 106 is important for cell migration, as inhibition of phosphorylation at this site blocked the increase in cell migration speed that is observed in cells expressing wild-type Asef2. Expression of the phosphomimetic mutant did not cause a further increase in cell migration speed, suggesting that there is a plateau level for Rac activation. Finally, we observed that loss of phosphorylation at serine 106 slowed the turnover of adhesions compared to wild-type Asef2. This result underscores the effect on cell migration speed because, if the cell cannot not assemble and disassemble adhesions rapidly, it will not move quickly. Therefore, phosphorylation of Asef2 – including at serine 106 – represents a novel mechanism for regulating its activity in migrating cells (Figure 26). While we do not know the specific mechanism for Asef2 phosphorylation, it is possible that it is phosphorylated after the initial activation by APC. Phosphorylation could maintain Asef2 in an "open" conformation so that it can perform its GEF function. Then, dephosphorylation could cause a conformational change that returns Asef2 to its autoinhibited state. Further work is needed to elucidate the effects of phosphorylation on Asef2's structure.

Future Directions

We have shown that Asef2 promotes the formation of dendritic spines in DIV11 hippocampal neurons. One future study is to investigate Asef2's role in early stages of neuronal

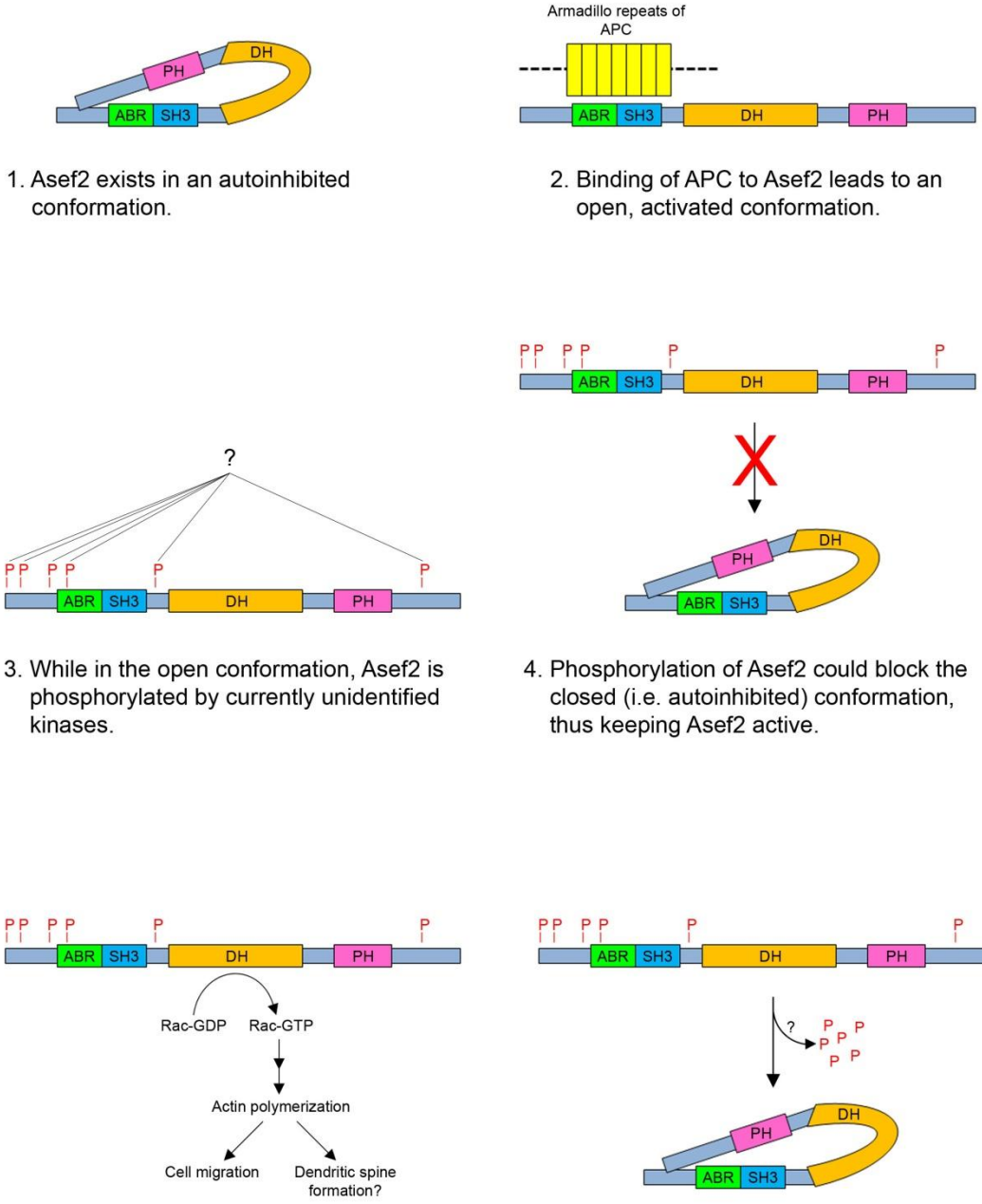


Figure 26. Model of Asef2 regulation via phosphorylation.

development, specifically its function in the formation of immature filopodia. These thin protrusions are hypothesized to be precursors to spines (Papa et al. 1995; Dailey and Smith 1996; Ziv and Smith 1996; Dunaevsky et al. 1999). Since Asef2 expression causes an increase in spine density in DIV11 neurons, we would expect that filopodia density would also be higher in Asef2-expressing neurons at an earlier time-point. Preliminary results in DIV7 neurons show that expression of Asef2 does promote the development of filopodia (Figure 27), which we define as dendritic protrusions that do not contact the presynaptic maker SV2 (Lin et al. 2013). This effect appears to be decreased in neurons expressing the DH domain-deficient mutant Asef2-K382A, but the difference is not statistically significant due to the low sample size (Figure 27). Therefore, Asef2's role in the formation of filopodia is likely dependent on its GEF activity. Future work is necessary to characterize the effect of Asef2 on filopodial dynamics. These structures are highly motile, which reflects their hypothesized role in searching for neurons to form synaptic contacts (Ziv and Smith 1996). So, it would be interesting to determine whether Asef2 regulates the speed of filopodia motility as well as the distance traveled by the filopodia within a specific range of time. Intriguingly, our preliminary data show that there is an increase in dendritic spine density in Asef2-expressing neurons, but not in Asef2-K382A-expressing neurons, compared to neurons expressing GFP at DIV7 (Figure 27). These results suggest that Asef2 hastens the maturation of dendritic protrusions so that they become spines at an earlier time-point. Also, this could suggest that Asef2 expression results in faster filopodia motility, which would enhance the probability of contacting a nearby neuron and of maturing into a dendritic spine. Therefore, Asef2 could regulate the generation of spines over multiple stages of neuronal development.

This dissertation focuses on Asef2's role dendritic spine formation, but nothing is known about the function of other Asef family members in this process. Collybistin is not likely to function in spines, which are sites of excitatory synaptic contact, since its predominant activity is

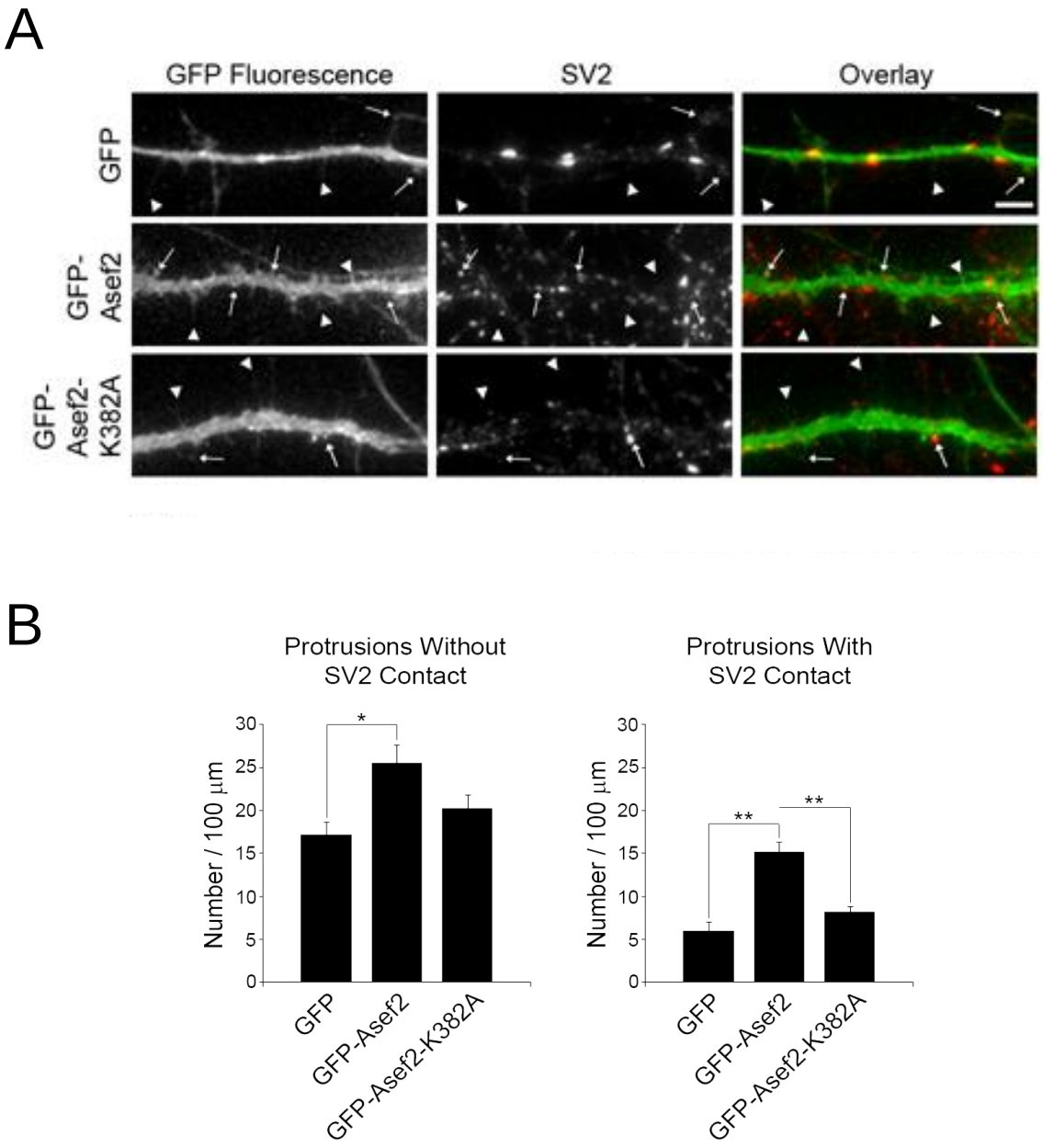


Figure 27. Asef2 promotes the formation of filopodia. *A*, DIV5 neurons were transfected with GFP, GFP-Asef2, or GFP-Asef2-K382A then fixed and stained for SV2 at DIV7. Arrowheads show protrusions not contacting SV2, while arrows show protrusions in contact with SV2. Bar, 5 μm. *B,* Quantification of protrusion density. Error bars represent s.e.m. for 15 dendrites per condition from one experiment. (*, $p = 0.007$, **, $p < 0.001$).

to target the scaffold protein gephyrin to inhibitory synapses (Kins et al. 2000). It is not known, however, if collybistin can interact with scaffold proteins associated with excitatory synapses – such as PSD95, Homer, or Shank (Boeckers 2006)– to regulate their clustering. Asef1 may play a role in dendritic spines, given its structural similarity to Asef2 (Hamann et al. 2007) (Figure 6). Therefore, it is possible that Asef1 and Asef2 could play overlapping roles in the development of spines and synapses. This could be tested by simultaneously expressing Asef1 and knocking down Asef2 (or vice versa); if these proteins functionally compensate each other, then knockdown of one should be rescued by expression of the other. Another possible future direction would be to analyze dendritic spine density in neurons from Asef1 $^{-/-}$, Asef2 $^{-/-}$, and Asef1 $^{-/-}$ Asef2 $^{-/-}$ mice (Kawasaki et al. 2009a). If Asef1 and Asef2 have redundant functions, then the simultaneous knockout of both proteins will have a greater effect on spine density than loss of either GEF individually. Given the importance of GEFs in regulating synaptic plasticity (Tolias et al. 2011), investigating how knockout of Asef1 and Asef2 affects synaptic transmission, including LTP and LTD, is an important future goal. While Asef1 and Asef2 could have similar roles in spines, they could be regulated via different mechanisms. For example, Asef1 does not interact with spinophilin (Sagara et al. 2009), so it could be targeted to sites of spine formation via a different scaffold protein. Also, differences in phosphorylation between Asef1 and Asef2 have been identified; for example, Asef1 was shown to be phosphorylated on tyrosine 94 by Src family kinases, while Asef2 did not exhibit any tyrosine phosphorylation under similar stimulation conditions (Itoh et al. 2008). This suggests a divergence in signaling between Asef1 and Asef2 that may influence their comparative effects on spine density. In addition, the differences in binding partners and post-translational modifications could imply that Asef1 and Asef2 are activated at distinct times and locations within the neuron.

Regarding the post-translational modification of Asef2, another important future study is to investigate the role of phosphorylation in mediating Asef2's effect on spine and synapse formation. We have shown that loss of phosphorylation at serine 106 – via mutation of this

residue to alanine – inhibits Asef2-dependent Rac activation, cell migration, and adhesion turnover. If phosphorylation is necessary for Asef2's promotion of spine development, then expression of the Asef2-S106A mutant should cause a decrease in spine and synapse density compared to expression of wild-type Asef2. It would also be interesting to investigate whether the phosphorylation state of Asef2 affects its ability to be targeted to spines by spinophilin. First, co-immunoprecipitation experiments could be performed to determine if Asef2-S106A binds less effectively to spinophilin than wild-type Asef2 does. Also, Asef2-S106A could be co-expressed with spinophilin in neurons; if phosphorylation of Asef2 is necessary for spinophilin-mediated targeting, then this mutant will not localize to spines. Another possibility is that spinophilin interacts with Asef2 before it becomes phosphorylated. In this case, the S106A mutant could still bind to spinophilin and be recruited to spines. Investigating these possibilities would shed light on the sequence of events in Asef2-mediated signaling, particularly whether Asef2 is phosphorylated before or after it is activated by APC. Furthermore, determining the functions of the other Asef2 phosphorylation sites (in both dendritic spine formation and cell migration) will be necessary to fully understand how differences in the relative levels of phosphorylation affect Asef2's GEF activity.

The link between Asef2 and spinophilin in dendritic spines further reinforces the importance of spinophilin as a scaffold for GEF signaling. Previous work has demonstrated that spinophilin interacts with Asef2 (Sagara et al. 2009) – and with other GEFs such as Tiam1 (Buchsbaum et al. 2003), Lfc (Ryan et al. 2005), and kalirin-7 (Penzes et al. 2001) – via its C-terminal region. This sets up the hypothesis that spinophilin acts as a signaling hub for GEFs, and that these GEFs could compete with each other for binding to spinophilin. Unfortunately, nothing is known about the specific regions within the C-terminus of spinophilin that are involved in the interaction with Asef2. Therefore, performing co-immunoprecipitation experiments using deletion mutants within spinophilin's C-terminus to pull down Asef2 will be beneficial for determining which regions within the C-terminus are critical. Preliminary data suggest that the

Asef2 interaction domain resides in the extreme C-terminal end of the protein (residues 741-818), since there is an almost complete loss of Asef2 binding with a deletion mutant lacking this region (Figure 28). Future work is necessary to determine if other regions are also necessary for the Asef2-spinophilin interaction. Furthermore, elucidation of a crystal structure of Asef2 in complex with spinophilin would greatly enhance our understanding of the interaction. The C-terminus of spinophilin contains a coiled-coil domain (Figure 6); however, the Asef2-spinophilin association is not thought to be mediated via coiled-coil interactions because Asef2 does not possess a coiled-coil domain (Sagara et al. 2009). Therefore, determining where Asef2 interacts with spinophilin will begin to clarify whether the Asef2-spinophilin association impedes spinophilin's interactions with other GEFs. The possibility of GEF competition for targeting by spinophilin represents a novel mechanism for spine formation.

The role of spinophilin itself in the regulation of spine development is not currently clear. While a previous study showed that knockout of spinophilin causes an increase in spines in young mice (Sagara et al. 2009), we have shown that knockdown of spinophilin inhibits spine formation (Figure 16). The discrepancy in the results could be explained by the fact that spinophilin is a scaffold for numerous (>25) proteins (Sarrouilhe et al. 2006). Therefore, complete loss of spinophilin could impact multiple signaling pathways, and the collective effect could result in aberrant spine formation. To better understand spinophilin's effect on spine density, we could express spinophilin in neurons and quantify spine density. Preliminary results show that expression of spinophilin causes an increase in dendritic protrusions (spines and filopodia could not be differentiated because the neurons were not stained for the synaptic marker SV2) (Figure 29). These results corroborate what we observed from the spinophilin knockdown experiments. Another important experiment is to determine if the spinophilin-mediated increase in spines is regulated by Asef2. To test this, we will co-express spinophilin with Asef2 shRNAs and quantify spine density. We expect that the increase in spine density

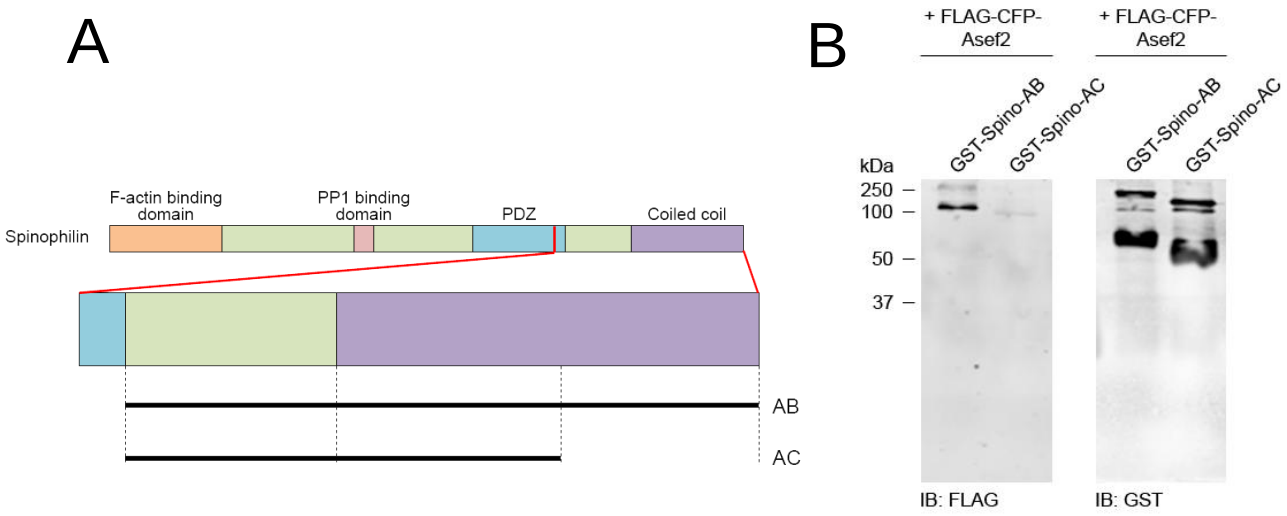


Figure 28. Interaction between Asef2 and spinophilin. *A*, Schematic for spinophilin C-terminal deletion mutants. *B*, HEK293 cells were co-transfected with FLAG-CFP-Asef2 and either GST-Spino-AB or GST-Spino-AC. The lysates were incubated with glutathione sepharose beads to pull down the GST constructs. Then, the lysates were assayed via Western blot for the amount of FLAG-CFP-Asef2 that was pulled down.

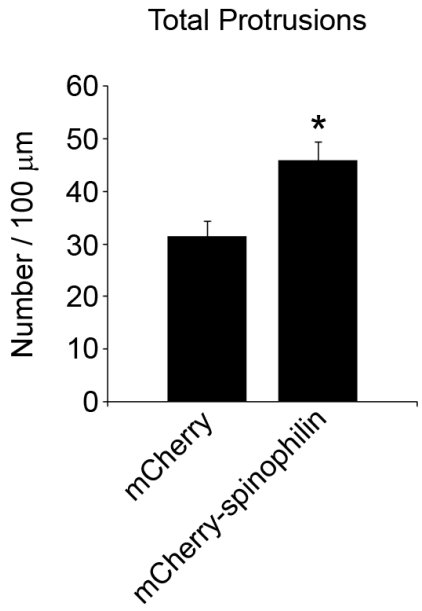


Figure 29. Spinophilin expression mediates the development of dendritic protrusions. Neurons were transfected with the indicated constructs, images were taken at DIV11, and the total number of protrusions (spines and filopodia) was quantified. Error bars represent s.e.m. for 10 dendrites from one experiment (*, $p < 0.03$).

caused by spinophilin will be attenuated when Asef2 is knocked down. A similar experiment would be to test if Rac activity is also integral for spinophilin-dependent spine development; these experiments will be important for supporting our model of a spinophilin-Asef2-Rac signaling pathway in spines. To further show that spinophilin's effect on spine formation is Asef2-dependent, we could express a C-terminal deletion mutant of spinophilin and assess spine density. We hypothesize that the C-terminal deletion mutant will not promote spine formation. Also, this mutant should be unable to localize Asef2 to spines; in fact, preliminary work suggests that Asef2's recruitment to spines is due to the C-terminus of spinophilin (data not shown). In all, these experiments are necessary to establish a definite link between spinophilin, Asef2, and Rac in the process of spine formation.

In order to further investigate the role of Asef2 and spinophilin in dendritic spines, it is important to look for other signaling proteins in this pathway. Unfortunately, there are very few known Asef2-interacting proteins. One intriguing candidate, however, is the microtubule-binding protein doublecortin (DCX); this protein stabilizes microtubules and has been implicated in a disorder called lissencephaly that is characterized by defective neuronal migration (des Portes et al. 1998; Gleeson et al. 1998). In neuronal precursor cells, Asef2 was shown to interact with a phosphomimetic mutant of DCX, and this mutant enhanced Asef2's GEF activity (Toriyama et al. 2012). Since phosphorylation of DCX releases it from microtubules and causes it to associate with actin (Schaar et al. 2004; Tanaka et al. 2004), Toriyama et al. hypothesized that phosphorylated DCX interacts with Asef2 and stimulates its activity towards Rac, thus promoting actin polymerization and migration of neuronal precursor cells (Toriyama et al. 2012). DCX has also been shown to interact with spinophilin and to co-localize with spinophilin in hippocampal neurons (Tsukada et al. 2003). This interaction – as well as DCX phosphorylation – promoted DCX's association with actin (Tsukada et al. 2005); dephosphorylation by PP1, which also interacts with spinophilin, reduces DCX binding to actin (Shmueli et al. 2006; Tsukada et al.

2006). Therefore, DCX function is tightly coordinated by its phosphorylation state and by its association with spinophilin.

Regarding the formation of spines, knockdown of DCX at DIV17 caused an increase in the density of dendritic filopodia, but had no effect on the density of spines (Cohen et al. 2008). This could suggest that DCX is necessary for the maturation of filopodia into spines. Alternatively, DCX could be involved in the retraction of spines and/or filopodia, so reduced expression of DCX would result in an increase in filopodia because these structures are not properly disassembled. Integration of DCX into the Asef2-spinophilin model of spine formation could represent an additional regulatory mechanism for Asef2 activation. Perhaps spinophilin regulates the phosphorylation state of DCX and, therefore, its ability to promote Asef2 activation. Spinophilin also acts as a scaffold for both proteins, so that it could bring DCX and Asef2 in close proximity and facilitate their interaction. Interestingly, DCX interacts with the C-terminus of spinophilin (Tsukada et al. 2003), which strengthens the hypothesis that spinophilin mediates the interaction between DCX and Asef2. Co-immunoprecipitation experiments would be useful to determine if these three proteins are associated in the same complex in neurons. Preliminary data show that the phosphorylation state of DCX regulates Asef2-dependent spine formation (Figure 30). While wild-type DCX inhibits Asef2's effect on spine and synapse density, the phosphomimetic mutant DCX-S47E promotes spine development in Asef2-expressing neurons (Figure 30). Importantly, this mutant also localizes to dendritic spines (Figure 30), which supports previous work showing that this mutant preferentially associates with actin (Schaar et al. 2004). Future studies could include co-expression of the S47E mutant with shRNAs targeting Asef2, which should block S47E-dependent spine growth. Another very important area of investigation is to determine the mechanism of Asef2 activation by DCX. If phosphorylated DCX promotes Asef2's GEF activity because of their concurrent association with spinophilin, then knockdown of spinophilin would reduce the interaction between phosphorylated DCX and Asef2, leading to lower levels of active Rac. Identifying the interaction

domains between Asef2 and DCX, and between Asef2 and spinophilin, would provide additional insight into the complex associations between these three proteins. The Asef2-DCX-spinophilin complex, therefore, is an exciting and novel putative regulator of dendritic spine and synapse formation.

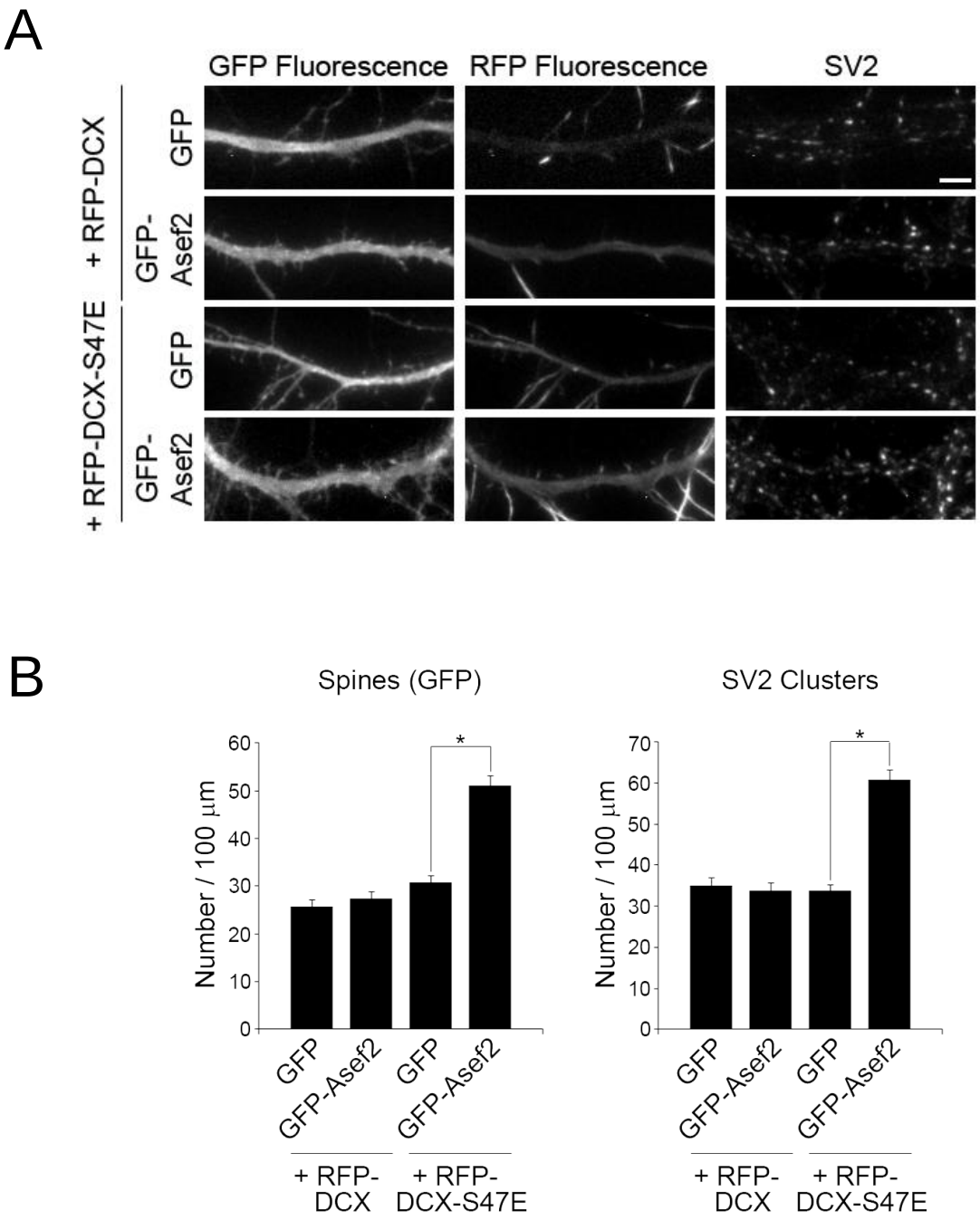


Figure 30. Phosphorylation of DCX promotes Asef2-dependent spine formation. *A*, Neurons were transfected with the indicated constructs and stained for SV2 at DIV11. Bar, 5 μm. *B,* Quantification of spine and synapse density. Error bars represent s.e.m. for 15 dendrites from one experiment (*, $p < 0.001$).

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