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# MECHANISMS AND CONSEQUENCES OF REGULATING NEURABIN AND SPINOPHILIN'S INTERACTION WITH THE TUMOR SUPPRESSOR PROTEIN p140CAP

by

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*To Aditya Bikram Malhotra, the man who makes me feel like I can do anything!*

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# ABBREVIATIONS

6-OHDA: 6-hydroxydopamine

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ARF: Alternative reading frame

CaMKII: Calcium/calmodulin-dependent kinase II

CDK5: Cyclin-dependent kinase 5

CDKN2A: Cyclin-dependent kinase inhibitor 2A

Crk: proto-oncogene c-Crk

CSK: C-terminal SRC kinase

DA: Dopamine

DOCK180: Dedicator of cytokinesis (~180kDa)

FAK: Focal adhesion kinase

GPCR: G protein-coupled receptor

GST: glutathione S-transferase

HEK: Human embryonic kidney

IP: Immunoprecipitation

LTD: Long-term depression

LTP: Long-term potentiation

MSN: Medium spiny neuron

NMDAR: N-methyl-D-Aspartate Receptor

PD: Parkinson's Disease

PKA: cAMP-dependent protein kinase A

PKC: Protein Kinase C

PP: Protein phosphatase

PP1: Serine/threonine protein phosphatase 1

PSD: Post-synaptic density

Rac1: Ras-related C3 botulinum toxin substrate 1

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFK:  SRC family of protein tyrosine kinases

SH2:  SRC homology 2

SH3:  SRC homology 3

SRC:  Neuronal proto-oncogene tyrosine-protein kinase SRC

SRCIN1:  SRC inhibitor kinase 1

# ABSTRACT

Author: Kaur, Harjot. MS
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Glioblastoma is the most aggressive type of brain cancer with very poor prognosis. Due to the lack of understanding of underlying mechanisms, there are no anti-invasive clinical therapeutics available. SRC terminal kinase (SRC) is a tumorigenic protein that is highly expressed in glioblastoma samples. SRC inhibitor kinase 1 (SRCIN1), also known as p140Cap is a negative regulator of SRC. Silencing SRCIN1 results in increased tumor invasion. Our lab has discovered two novel scaffolding proteins Spinophilin (Spn) and neurabin (Nrb) that bind to SRCIN1. They may play a role in regulating SRCIN1 activity, as well as its downstream effects that ultimately decrease SRC's tumorigenic activity. Spn and Nrb are two scaffolding proteins that are heavily expressed in the central nervous system. Spn knockout mice develop more tumors, indicating that Spn acts as a tumor suppressor protein, although the mechanisms of Spn's anti-tumor properties are not well understood. Spn and Nrb are PP1 targeting proteins that target PP1 to other substrates, resulting in dephosphorylation and alteration of function. We found that PP1 increases Spn association with SRCIN1, but decreases Nrb association with SRCIN1, indicating that the two proteins might have opposite effects to balance the activity of p140Cap. We also found that cyclin-dependent kinase 5 (CDK5) phosphorylates and regulates the association of these scaffolding proteins with the tumor suppressor protein, p140Cap. Understanding these mechanisms provides insight into new therapeutic targets that may ultimately decrease SRC activity and its tumorigenic and invasive properties.

# CHAPTER 1. INTRODUCTION

## 1.1 Signaling Proteins

Cell signaling is ubiquitously required for normal biological function. Signaling pathways are a part of almost every biological function and exhibit a great deal of diversity. There are thousands of molecules involved in various pathways; however, the interactions between these molecules are largely regulated by two different kinds of molecules – protein kinases and protein phosphatases. Protein kinases phosphorylate proteins by adding a phosphate group whereas protein phosphatases balance kinase activity by dephosphorylating or removing a phosphate group from the protein. The phosphorylation status of a protein can regulate that protein's function.

### 1.1.1 Serine Threonine Protein Kinases – PKA, SR1, Cdk5

There are over 350 serine/threonine kinases encoded by the mammalian genome (Cohen, 2002). Here we will introduced three kinases that are highly abundant in the central nervous system. Protein Kinase A (PKA) is a tetramer consisting of two regulatory subunits and two catalytic subunits. PKA is also known as cyclic AMP-dependent protein kinase because it is activated in the presence of cAMP. Four cAMP molecules bind to the PKA molecule causing a conformational change in the regulatory subunits. The regulatory subunits then dissociate, exposing the two activated catalytic subunits. These catalytic subunits are able to phosphorylate serine or threonine residues on multiple different protein substrates (Tumham, 2016).

Cyclin-dependent kinase 5 (CDK5) is another serine/threonine kinase. It is a monomeric cyclin dependent kinase that requires association with proteins such as p35, a regulatory partner, for activation (Paglini, 2001). CDK5 is a proline-directed kinase that phosphorylates serines and threonines immediately upstream of a proline residue. Although CDK5 is widespread, highest expression is seen in the nervous system (Tsai, 1993).

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine-specific protein kinase that is activated by the Ca2+/calmodulin complex. CaMKII is one

of the most abundant proteins in the mammalian forebrain making up 1-2% of total brain protein (Kennedy, 2006). It plays a role in various signaling cascades including memory and learning, and misregulation of CaMKII is linked to multiple neurological disorders, such as Alzheimer disease and Parkinson disease (Ghosh, 2015). Moreover, CaMKII is also found to be downregulated in cancer tissue, including pancreatic, leukemic and breast tumor cells (Kim, 2011).

### 1.1.2 Protein Phosphatase 1

Protein phosphatase 1 (PP1) is a serine/threonine phosphatase that controls various biological functions including glycogen metabolism, cell progression and neuronal function (Fong, 2000; Cohen, 2002). PP1 contains a catalytic subunit and a regulatory subunit. There are three genes that encode 4 different PP1 proteins. *PPP1CA*, *PPP1CB* and *PPP1CC* genes encode PP1α, PP1β, and two different PP1γ splice variants, PP1γ1 and PP1γ2, respectively (Wera, 1995). Throughout this thesis, PP1γ will refer to the γ1 isoform of PP1. Although different isoforms of PP1 share sequence identity, they have distinct tissue and subcellular distributions, suggesting differences in the way they are localized as well as cellular functions. Moreover, PP1 obtains target specificity by associating with a large array of regulatory subunits.

## 1.2 PP1 Scaffolding Proteins

While there are over 350 serine/threonine kinases encoded by the mammalian genome, the number of serine-threonine phosphatase catalytic subunits is only ~40. To enhance substrate targeting and specificity, phosphatases, such as PP1, utilize regulatory subunits (Cohen, 2002). These phosphatase regulatory subunits or scaffolding proteins play an important role in various signaling pathways by targeting PP1 to multiple different proteins. They act by tethering signaling molecules together and localizing them to specific areas of the cell (Terry-Lorenzo, 2002). In certain cases, they may also cause conformational changes in signaling proteins, resulting in either positive or negative regulation of the

pathway. Some examples of PP1 scaffolding proteins include spinophilin and neurabin (Allen, 1997; Stephens, 1999).

### 1.2.1 Spinophilin

Spinophilin, also known as Neurabin-2 is an 817 amino acid protein encoded by the *PPP1R9B* gene. It is an actin binding protein that is highly enriched in dendritic spines. Dendritic spines on postsynaptic neurons form points of contact with axon terminals and act as a signaling relay station. The plasticity of dendrites is crucial for normal brain functioning (Harms, 2006). Spine motility is regulated by the polymerization of actin, a major cytoskeletal component present in spines (Fischer, 1998). Spinophilin stabilizes actin structures in the spines by binding and bundling actin polymers (Satoh, 1998). Spinophilin's ability to bind to actin is fully contained within residues 1-154, otherwise known as the actin binding domain (Schuler, 2008). This interaction between spinophilin and actin is regulated by phosphorylation of spinophilin's actin binding domain by PKA (Hsieh-Wilson, 2003), cyclin-dependent kinase-5 (Futter, 2005) and calcium/calmodulin-dependent kinase II (Grossman, 2004). Spinophilin also acts as a regulatory subunit of PP1 catalytic subunits. Immunoprecipitations of spinophilin from rat brains extracts showed a preferential binding to PP1γ1 and PP1α and a significantly weaker association with PP1β. (Terry-Lorenzo, 2002). Via the PDZ domain, spinophilin targets PP1 to glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolpropionate (AMPA and N-methyl-D-aspartate (NMDA) receptors (Terry-Lorenzo, 2002), thereby modulating their activity and trafficking through regulation of their phosphorylation state (Terry-Lorenzo, 2002). The coiled-coil domain of spinophilin allows it to bind to the coiled-coil domains of other proteins, as well as to homodimerize (Kelker, 2007).

### 1.2.2 Neurabin

Neurabin is a scaffolding protein encoded by the *PPP1R9A* gene. Unlike spinophilin which is enriched in neurons but found in other tissues, neurabin is almost exclusively expressed in neurons (Oliver, 2002). Additionally, neurabin is also expressed in lamellipodia of growing neurons, indicating its role in axonal growth (Nakanishi, 1997). Neurabin also shares structural similarities and about 65% of overall sequence identity with Spinophilin

(Schuler, 2008). Both of these scaffolding proteins have an F-actin binding domain, PP1-binding domain, PDZ domain and a coiled coil domain. Neurabin also has an additional sterile alpha motif (SAM) domain in its C-terminus. The highest levels of sequence identity between neurabin and spinophilin is found in the PDZ domains (86%), the PP1 binding domains (81%), and the coiled-coil domains (63%). Neurabin also acts as an F-actin binding protein and provides structural stability as well as plasticity to dendritic spines. The actin binding domain contains residues 1-144 and is sufficient for binding to F-actin. Like spinophilin, neurabin binds PP1 via its PP1 binding domain (residues 426-502). PP1 has been known to dephosphorylate and regulate AMPA and NMDA receptors (Yan, 1999). The AMPA and NMDA glutamatergic receptors in the post-synaptic density are phosphorylated by PKA and CaMKII (Snyder, 1998). Like spinophilin, The PDZ domain of neurabin residues 502-594 is responsible for their direct interactions with larger proteins such as the AMPA and NMDA receptors via binding to their C-termini (Kelker, 2007). The coiled coil domain residues 658-827 allows neurabin to bind to other neurabin molecules and to coiled-coil domains of other proteins, including spinophilin. The SAM domain of neurabin ranges from residues 970-1056 and its function is not well understood. We used the neurabin isoform 4, which has all the same domains as the canonical neurabin isoform with the exception of SAM. Isoform 4 has an alternative insert sequence at the C-terminal domain.

## 1.3 Glutamate receptors and neuronal signaling in the brain

Normal brain function is dependent upon appropriate cell signaling which occurs at the synapse, a small space between the axon of a signal-transmitting neuron and a dendrite (or a dendritic spine) of a signal-receiving neuron. Dopaminergic neurons in the substantia nigra and glutamatergic neurons in the motor cortex project to the striatum and form synapses with dendritic spines of the medium spiny neurons (MSNs).

Once the synapse is formed, proper machinery in the pre- and post-synaptic neurons is needed for normal signal transduction, which underlies appropriate synaptic connectivity. The machinery that is essential for signal transduction is a protein dense specialization in

the tip of the spines referred as to the postsynaptic density (PSD) (Hausser, Spruston, & Stuart, 2000). MSN dendritic spines were first described by Ramón y Cajal (Cajal, 1888). These small protrusions play a significant role in proper synaptic connectivity since they serve as the main site to receive presynaptic input by significantly increasing the overall dendrite surface area (Gray, 1959).

After being released from the pre-synaptic neuron, glutamate will bind to its receptors on the dendritic spines of MSNs. The two types of ionotropic glutamate receptors localized to dendritic spines on striatal MSNs are α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and N-methyl-D-aspartate receptor (NMDAR). Glutamate binding activates these receptors allowing $Na^+$ and/or $Ca^{2+}$ influx into the cell, and thereby activating $Ca^{2+}$-dependent processes. Dopamine (DA) has the ability to regulate this glutamate signal. There are two classes of DA receptors that reside on two different populations of striatal MSNs: the DA D1R-containing, or direct pathway MSNs, and the DA D2R-containing, or indirect pathway MSNs (Strange, 1993). The $D_1$ family of receptors that activate the direct pathway are $G_{\alpha s}$-protein-coupled and upon ligand binding activate adenylyl cyclase which in turn activates PKA signaling. Conversely, the $D_2$-family of DA receptors are found on indirect pathway striatal MSNs, are $G_{\alpha i}$-coupled, and inhibit adenylyl cyclase, which blocks downstream PKA signaling (Stoof & Kebabian, 1984). The direct pathway is responsible for initiating movement and the indirect pathway is responsible for inhibiting spontaneous, unintentional movements. The balance between direct and indirect pathways is essential for normal motor control and aberrancies in either pathway can lead to different symptoms of Parkinson Disease (PD) (Albin, Young, & Penney, 1989; Frank, Seeberger, & O'Reilly R, 2004).

Loss of nigral dopaminergic projections causes alterations in the functionality of striatal MSNs (German, Manaye, Smith, Woodward, & Saper, 1989), such as modulation of normal PKA signaling (Nishi et al., 2008), as well as the appearance of motor deficits associated with Parkinson disease (Albin et al., 1989; Rodriguez-Oroz et al., 2009; Starr, 1995). These changes occur because at the molecular level, DA depletion alters the functionality of PSD proteins. PSD proteins are an essential and critical part of normal synaptic communication. Absence of one or more of these synaptic proteins may affect how other proteins interact with each other and lead to changes in normal synaptic function

and connectivity. One of the proteins that has altered functionality following DA depletion is the NMDAR. NMDARs are glutamate receptors that underlie long-term potentiation (LTP) and/or long-term depression (LTD), electrophysiological properties of neurons that play a role in learning and memory (Malenka & Bear, 2004). DA depletion affects LTP and LTD in both PD patients and animal models of PD. Specifically, 6-hydroxydopamine (6-OHDA) lesions of the substantia nigra in rats lead to loss of LTD (Calabresi, Maj, Pisani, Mercuri, & Bernardi, 1992; Ingham, Hood, Taggart, & Arbuthnott, 1998). However, the molecular changes that occur in NMDAR function that link to these pathological changes in motor learning and memory observed in animal models of PD are yet to be fully understood.

### 1.3.1 NMDAR function and localization

Spinophilin and Neurabin are major PP1 targeting proteins in the postsynaptic density. One of the major targets of these PP1 targeting proteins are ionotropic glutamate receptors. Glutamatergic synapses regulate most of the excitatory neurotransmission in the mammalian brain and play a critical role in mediating functional neuronal connectivity. Glutamate released from presynaptic axons activates several glutamate-gated ion channels on postsynaptic cells including AMPARs, NMDARs, and kainate receptors, which get their names from their specific response to pharmacological agents (Dingledine, Borges, Bowie, & Traynelis, 1999; Hollmann & Heinemann, 1994). It has been shown that dysfunction of these receptors is associated with multiple neurological and psychiatric disorders, including Parkinson disease, Alzheimer disease, and schizophrenia (S. Cull-Candy, Brickley, & Farrant, 2001; Waxman & Lynch, 2005).

NMDA receptors are a major class of glutamate receptors that are blocked by a $Mg^{2+}$ ion in their inactive state. Despite binding a glutamate molecule, an NMDA receptor remains closed due to the $Mg^{2+}$ block. Once surrounding AMPA receptors are activated, the cell becomes depolarized, pushing out the $Mg^{2+}$ block and opening up the NMDA channel. These receptors have several subunits and three families of genes (*Grin1*, *Grin2* and *Grin3*) that encode three families of proteins (GluN1, GluN2, and GluN3) (S. Cull-Candy et al., 2001). Studies show that NMDARs are tetramers in which two GluN1 subunits assemble with two GluN2 and/or one GluN2 and one GluN3 subunit. GluN1

subunits are obligatory subunits necessary for functional expression of the NMDA receptors (S. G. Cull-Candy & Leszkiewicz, 2004). According to biochemical, electrophysiological and crystallographic analysis, a GluN1/ GluN2 heterodimer is the functional unit in tetrameric NMDARs (Furukawa, Singh, Mancusso, & Gouaux, 2005).

The NMDAR is a voltage-sensitive glutamate receptor, which is blocked by extracellular $Mg^{2+}$ ion under resting membrane potential. The blocking $Mg^{2+}$ ion acts as a switch that allows for $Ca^{2+}$ influx upon membrane depolarization together with binding of glutamate to the GluN2 subunit and a co-agonist, such as glycine, to the GluN1 subunit (Erreger, Chen, Wyllie, & Traynelis, 2004).

A number of studies have shown that GluN2 and GluN3 subunits connote specific electrophysiological properties to the NMDARs (S. G. Cull-Candy & Leszkiewicz, 2004). As a result, variability in NMDAR subunit composition is an important factor to regulate NMDAR function. According to previous studies, subunit composition of NMDARs is developmentally regulated (Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994). The GluN1 subunit is the product of a single gene, which can be alternatively spliced, and normally is found ubiquitously throughout the brain. In contrast, GluN2 subunits (GluN2A-D) are encoded by four different genes and their expression patterns depend on the developmental stage and brain region. The expression patterns of GluN2A and GluN2B throughout the brain are relatively broad, with a parallel decrease in GluN2B and increase in GluN2A expression. However GluN2C and GluN2D have a more restricted expression, with GluN2C expression in cerebellum starting later in development and GluN2D being expressed early in development mostly in the brainstem and in thalamic and hypothalamic nuclei (Monyer et al., 1994). Endogenous NMDARs normally only contain GluN1 and GluN2 subunits, with GluN3 subunits only incorporated in a subpopulation of NMDARs and exhibiting decreased channel conductance properties (S. G. Cull-Candy & Leszkiewicz, 2004).

From a structural standpoint, NMDAR subunits contain several domains including a long extracellular N-terminal domain, a membrane-spanning domain, a pore loop, and a subunit-dependent, variable length intracellular C-terminal domain. The C-terminal “Tail” domain is the most variable region when comparing the various NMDAR subunit sequences. The Tail region is known to regulate receptor interactions with various

intracellular proteins. These protein-protein interactions are important for proper trafficking and localization of NMDARs to membranes. Additionally, different subunits of the NMDAR can couple receptors to various cytosolic signaling complexes. For instance, GluN2B interacts with various proteins such as SynGAP (Kim, Dunah, Wang, & Sheng, 2005) and an active form of CaMKII (Colbran et al., 1997), which leads to differing forms of synaptic plasticity (Barria & Malinow, 2005). Furthermore, the tail region of NMDARs is subject to various post-translational modifications such as phosphorylation, which can directly modulate protein activity, interactions, localization, and mobility.

### 1.3.2 NMDAR phosphorylation

Direct phosphorylation of ionotropic glutamate receptors plays a very integral role in regulating channel conductance, function, and receptor localization at synapses (Lee, 2006). NMDA receptor subunits are phosphorylated at serine/threonine residues by multiple kinases, including PKA, protein kinase B (PKB), protein kinase C (PKC), CDK5, CaMKII, and casein kinase II (CKII) (Mammen, Kamboj, & Huganir, 1999; Roche et al., 1994). In addition to kinases and phosphatases, *per se*, phosphorylation of synaptic proteins that modulate kinase or phosphatase targeting also regulate NMDAR phosphorylation (Lan et al., 2001; Sigel, Baur, & Malherbe, 1994; Zheng, Zhang, Wang, Bennett, & Zukin, 1999).

PKC has multiple effects on NMDAR function, including increasing the opening rate and upregulating NMDAR surface expression, which in turn regulates NMDAR activity (Lan et al., 2001; W. Y. Lu et al., 1999). PKA also plays a role in mediating NMDAR function by enhancing the amplitude of NMDAR-mediated excitatory postsynaptic currents (EPSCs) (Raman, Tong, & Jahr, 1996). Consistently, PKA activation seems to increase synaptic targeting of NMDA receptors (Crump, Dillman, & Craig, 2001) along with increasing calcium permeability of NMDARs (Skeberdis et al., 2006).

GluN1, the obligate subunit of the NMDAR, is also phosphorylated by various protein kinases (PK). Studies suggest that phosphorylation of serine 890 disrupts GluN1 clustering (Tingley et al., 1997) while serine 896 phosphorylation by PKC has no effect on clustering of GluN1. However, phosphorylation of S896 together with PKA phosphorylation of S897 contributes to increase in NMDA receptor surface localization (D. B. Scott, Blanpied, Swanson, Zhang, & Ehlers, 2001). GluN2A can be phosphorylated by

PKC, which leads to phosphorylation of S1291 and S1312 and potentiation of GluN2Acontaining NMDARs (Grant, Guttmann, Seifert, & Lynch, 2001; Jones & Leonard, 2005). Phosphorylation of GluN2A at S1416 by PKC decreases the GluN2A binding affinity to CaMKII (Gardoni et al., 2001). CDK5 is another PK that also phosphorylates GluN2A, which contributes to an increase in NMDA receptor activity (B. S. Li et al., 2001).

GluN2B constitutes most of the NMDARs in most brain regions early in development (S. Cull-Candy et al., 2001). While GluN2B expression is attenuated as the animal matures, it remains in hippocampus, cortex, striatum and other brain regions into adulthood. GluN2B containing NMDARs are located at both synaptic and extrasynaptic sites early in development. As development progresses, GluN2B becomes enriched at extrasynaptic sites (B. Li et al., 2002; Tovar & Westbrook, 1999). Moreover, GluN2B containing NMDA receptors have higher surface mobility compared to GluN2A containing NMDARs (Groc et al., 2006). Like GluN2A, GluN2B-containing receptors are also phosphorylated by PKC. Specifically, PKC phosphorylates GluN2B at S1303 and S1323, enhancing NMDA receptor currents. (Liao, Wagner, Hsu, & Leonard, 2001). Other studies show that S1303 of GluN2B is also a phosphorylation site for CaMKII (Omkumar, Kiely, Rosenstein, Min, & Kennedy, 1996). However, phosphorylation of Serine 1303 by CaMKII modulates NMDAR function in a different way from phosphorylation of PKC at the same site. Co-expression of CaMKII seemed to attenuate GluN1A/GluN2B currents by enhancing the extent of desensitization. Interestingly, CaMKII mediated phosphorylation of GluN2B-conatining NMDA receptors reduces desensitization at low intracellular $Cl^-$ (Tavalin and Colbran, 2017).

PKA also plays a role in GluN2B phosphorylation on S1166 (Murphy et al., 2014), which is critical in synaptic NMDAR function and $Ca^{2+}$ signaling in spines. Along with this PKA site, Y1472 (Zhang, Edelmann, Liu, Crandall, & Morabito, 2008), S1116 (Plattner et al., 2014) and S1284 are recently characterized phosphorylation sites that are either indirectly or directly phosphorylated by CDK5 and can modulate NMDAR function (W. Lu et al., 2015). Together, these data suggest that phosphorylation of NMDA receptor subunits plays an integral role in proper signaling as well as normal synaptic connectivity.

### 1.3.3 Hypotheses

Spinophilin is a scaffolding protein that binds and targets PP1 to other proteins. Spinophilin and PP1 have also been known to play a role in regulation of AMPA and NMDA glutamatergic receptors. Given the structural similarity and PP1-binding ability of neurabin and spinophilin, we hypothesize that neurabin has the ability to regulate AMPAR and NMDAR activity via PP1 targeting. Additionally, phosphorylation by kinases could possibly enhance or attenuate these interactions.

## 1.4 Cancer

### 1.4.1 Spinophilin in Cancer

Spinophilin was first suspected to play a role in cancer when genetic linkage studies linked it to breast cancer (Porter, 1993; Porter, 1994). Spinophilin knockout mice develop more tumors than wildtype mice. Samples from breast, colon and pancreatic cancer cells show decreased expression of spinophilin, indicating that spinophilin might be acting as a tumor suppressor (Ferrer, 2016). It is known that spinophilin interacts with T-cell lymphoma invasion and metastasis-inducing protein 1 (TIAM1) and is believed to play a role in regulating tumor growth (Buchsbaum, 2003). The exact mechanism of spinophilin's role in tumor growth or suppression is unknown. Spinophilin's interaction with other tumor suppressor proteins or oncogenes is also not well understood.

### 1.4.2 SRC and CSK function and localization

SRC family of kinases are oncogenes that exhibit high levels of activity in a number of human cancers. They have been known to regulate cell adhesion, invasiveness and motility in cancer cells and in tumor vasculature. Upon activation via integrin-adhesion, SRC kinase regulates cell growth, spreading and migration through increased phosphorylation of focal adhesion kinase (FAK) and other adaptor molecules such as p130Cas. Phosphorylated p130Cas recruits other proteins like Crk and DOCK180 that regulate Rac activation, which is crucial for actin cytoskeleton organization and cell motility (Chodniewicz and Kleme,

2004). Mice deficient in SRC, FAK or p130Cas exhibit impaired cell spreading and migration (Klinghoffer, 1999). Overexpressed SRC in transformed cells, on the other hand, results in increased reorganization of epithelial adhesion systems (Boyer, 1997). Increased levels of SRC have been discovered in various types of cancer tissues including glioblastoma (Stettner, 2005). Inhibiting SRC function results in decreased cell proliferation in breast cancer, prostate cancer and glioblastoma tissue (Jallal, 2007; Chang, 2008; Nam, 2006; Yamaguchi, 2005). SRC activation leads to downstream signaling through the RAS/mitogen-activated protein kinase (MAPK) and P13K pathways that have been shown to play an important role in tumor proliferation and invasion (Wong, 2007; Schlaepfer, 1999; Levy, 1986)

C-terminal SRC kinase (CSK) maintains SRC in its inactive state via phosphorylation of a negative-regulatory tyrosine residue (Y530). The binding of Y530 to SH2 domain results in folding of SRC into an inactive state (Ly, 2007). In order to be activated, Y530 residue is dephosphorylated by protein tyrosine phosphatase - $\alpha$; this results in unfolding of SRC into its active state (Egan, 1999). CSK can negatively regulate SRC and produce downstream anti-metastatic and anti-invasive effects. Overexpression of CSK in a mouse model of metastatic colon cancer decreased metastasis (Nakagawa, 2000).

### 1.4.3 p140Cap function and localization

p140Cap is a tumor suppressor protein that is encoded by the SRC inhibitor 1 (*SRCIN1*) gene. It is highly expressed in breast cancer cells such as MCF7, TRUD, MDA-MB-231 and MDA-MB-435. P140CAP directly associates with SRC and acts as a tumor suppressor protein by binding to C-terminal SRC kinase and SRC (Di Stefano, 2007). P140Cap binds to the SH2 domain of CSK and SH3 domain of SRC. Overexpression of p140Cap upregulates CSK activity and kinase-dead CSK induces a strong activation of SRC, suggesting that the kinase-dead CSK counteracts the ability of p140Cap to inhibit SRC activity. Silencing of CSK protein also leads in increased SRC phosphorylation in cells overexpressing p140Cap. This data supports the idea that p140Cap's negative regulation of SRC kinase occurs through activation of CSK kinase. P140Cap downregulates SRC and downstream pathways, therefore inhibiting breast cancer cell spreading, motility and invasion (Di Stefano, 2007). Additionally, it also localizes with actin stress fibers in

endothelial cells, suggesting its involvement in actin cytoskeletal organization (Di Stefano, 2004).

### 1.4.4 Hypothesis

Compared to mice with normal functioning spinophilin, spinophilin KO mice have a higher number of various types of tumors including glioblastoma. Analysis of glioblastoma tissue has revealed increased levels of SRC kinase. We hypothesize that spinophilin may be acting as a tumor suppressor by regulating the p140Cap → CSK → SRC pathway. Given neurabin's structural and domain similarity to spinophilin, we hypothesize that neurabin might be a novel cancer/tumor regulating protein. Moreover, spinophilin and neurabin's interaction with this pathway may be modulated by PP1 and serine/threonine kinases such as PKA, CDK5 and CaMKII.

# CHAPTER 2. METHODS

## 2.1 Generating DNA Constructs

### 2.1.1 Primer Design

Epitope-tagged forms of the synaptic proteins used in these studies were obtained by amplifying cDNAs for spinophilin, neurabin, GluN2B, PKAc, CDK5, the CDK5 activator, p35, CaMKII, SRCIN1, CSK, SRC, and the α(PP1α) as well as the γ1 isoform of PP1 (PP1γ). Templates used were: human spinophilin (Addgene plasmid #87122), human Neurabin (isoform 4; Uniprot ID: Q9ULJ8-4), human GluN2B (BC113618; Transomic Technologies), human PKAc – pDONR223-PPKACA, human CDK5 – pDONR223-CDK5, p35 – pDONR223-CDK5SR1 (PKAc, CDK5, and p35 were gifts from William Hahn & David Root (Johannessen et al., 2010) (Addgene plasmid #s 23495, 23699, and 23779), rat PP1γ1 (L.C. Carmody, A.J. Baucum, M.A. Bass, & R.J. Colbran, 2008), human PP1α, human SRCIN1, human CSK – pDONR223-CSK, and human SRC.

### 2.1.2 Templates

PCR primers for the above cDNAs containing attB sites and either Shine-Dalgarno and Kozak sequences (for production of C-terminal tagged proteins), or a stop codon (for N-terminal tagged proteins) were synthesized and ordered from Integrated DNA Technologies (Coralville, IA).

### 2.1.3 PCR Reactions

To create PCR products, PCR amplification was performed using Q5 DNA polymerase (New England Biolabs, Ipswich, MA), using manufacturer's protocol. The PCR conditions were as follows: 1) a 2-minute initial denaturation at 98° C, 2) a 30 second denaturation at 98° C, 3) a 10-second annealing reaction at a primer-specific gradient temperature, and 4) a 4-minute extension period at 72 degrees C. Steps 2-4 were repeated 38 times for standard reactions. Annealing temperature was varied using a gradient with multiple ranges according to specific melting points of oligonucleotides (gradient ranging from 50-70° C).

A final elongation step of 10 minutes was performed at 72° C. PCR products were mixed with 6x DNA loading dye (New England Biolabs) and separated on 1% agarose gel containing SYBR Safe (Lite Technologies, Carlsbad, CA). Electrophoresis was performed for 45 minutes – 1 hour at 80V. Amplification of the correct size DNA was confirmed by the appearance of a band of the appropriate size. Bands were subsequently excised from the gel and DNA was isolated using a DNA gel extraction kit (Zymo Research, Irvine, CA or ThermoFisher Scientific, Waltham, MA). DNA was generally eluted in Tris-EDTA (TE) elution buffer. The concentration was then quantified using the BioTek Cytation 3 system (BioTek Instruments Inc. Winooski, VT). All vectors were then sequenced verified (GENEWIZ, Inc., South Plainfield, NJ).

### 2.1.4 Gateway BP Cloning

The PCR product for each DNA construct was combined with donor vector (pDONR221) using BP Gateway cloning technology from Life Technologies. The PCR product (20-50fmol) was used in each reaction along with 150 ng of plasmid vector and BP Clonase II enzyme mixture. Reactions were performed for 1 hour to overnight at 25° C according to manufacturer's protocol. Mixtures were then treated with proteinase K at 37° C for 10 minutes. Transformation was then carried out by use of 10 μL of reaction mixture to transform NEB5α competent E. coli from New England Biolabs. Cells were plated on Luria broth (LB) agar with kanamycin antibiotic and incubated overnight at 37° C. Individual bacterial colonies were picked and placed into 10mL liquid LB cultures with kanamycin and incubated overnight at 37° C while shaking.

The next day, cells were centrifuged for 10 minutes at 4000 x g and lysed. The bacterial pellet was then lysed and DNA was purified using miniprep purification kits (Zymo Research or Thermo Scientific). The concentration of DNA was measured using the BioTek Cytation 3 system (BioTek Instruments, Inc.). A diagnostic digestion was performed using approximately 500 ng of resulting DNA using appropriate restriction enzymes and then separating on a 1 % agarose gel for validation of proper insertion. Gels were imaged on a Bio-Rad Gel Doc EZ (Bio-Rad Laboratories, Inc. Hercules, CA). Successful BP recombination was confirmed by appearance of appropriate bands and

samples were then further validated by sequencing (GENEWIZ, Inc). Sequence verified samples were used for LR recombination.

### 2.1.5 Gateway LR Cloning

LR recombination was used to generate proteins with different epitope tags that would be used for immunoprecipitation. For mammalian protein expression, pcDNA3.1 destination vectors with either HA, V5, myc or FLAG tags were used. LR reactions were performed using manufacturer's recommendations. Specifically, 150 ng of donor vector containing the intended DNA was incubated with 150 ng of appropriate destination vector, at 25° C for 1 hour. LR reaction was then followed by Proteinase K digestion at 37° C for 10 minutes. Competent NEB5a cells were transformed with 10 μL of the reaction mixture. The cells were then plated on LB-containing plates in the presence of ampicillin and incubated at 37° C overnight.

Bacterial colonies were excised after overnight growth and cultured in 10 mL liquid LB cultures with ampicillin. DNA was then extracted as described above and confirmed with a diagnostic digest. If appropriate DNA banding patterns were present, additional bacterial colonies were selected from the plate and were cultured in larger (50-250 mL) cultures for maxipreps (Zymo Research or Thermo Scientific). DNA was re-screened via restriction digestion. All original empty DNA vectors used in BP or LR cloning were modified from original vectors obtained from Life Technologies.

### 2.1.6 Mutagenesis PCR

In order to make DNA constructs with point mutations, QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used. Mutagenesis reactions were performed using Q5 DNA buffer, 1 μl DNA polymerase, 5 μM dNTPs and 10 ng of template DNA. The thermocycler settings for PCR reactions was as follows: 1) initial denaturation at 98°C for 2 minutes, 2) denaturation at 98°C for 45 seconds, 3) annealing reaction at a primer-specific temperature for 1 minutes, 4) elongation at 68°C for 15 minutes. Steps 2-4 were repeated 18 times, and excess template DNA was eliminated via DpnI digestion at 37°C for 2 hours. 3 μl of 6X loading dye was added to each sample and 20 μl of sample was loaded into a well on a 1 % agarose gel. After confirming the presence

of a high-intensity band, corresponding to the appropriate molecular weight was visualized, the remaining PCR product was transformed into competent DH5α-derived E. coli. Vectors. The presence of the desired point mutation was also sequence verified (GENEWIZ, Inc.)

## 2.2 Mammalian Protein Expression

Human embryonic kidney 293 FT cells (HEK293; LifeTechnologies) were used for mammalian protein expression. Cells were incubated and allowed to grow in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 584 mg/L L-glutamine, 1 mM Sodium Pyruvate, 100U/mL penicillin and 100 ug/mL streptomycin. 75 mm culture flasks were incubated at a constant 37° C and 5% CO2 (Panasonic Healthcare, Secaucus, NJ).

Prior to transfecting with DNA, cells were counted and distributed into 25 mm flat-bottomed culture flasks. Approximately 1,000,000 cells were plated into each flask and allowed to grow overnight. About 18-24 hours later, cells covered about 70-80% of the bottom of the flask indicating 70-80% confluency. At this point, cells were transfected with the appropriate DNA. DNA concentrations were adjusted based on protein expression. The amount of DNA used for various proteins in these experiments were: spinophilin (1 μg), neurabin (4 μg), $GluN2B_{TAIL}$ (1 μg), PKA (1 μg), CDK5 (1 μg), p35 (1 μg), CaMKII (1 μg), PP1α (1 μg), PP1γ1 (1 μg), SRCIN1 (1 μg), CSK (2 μg) and SRC (2 μg). The appropriate amount of DNA was added to 250 μL of serum-free DMEM in a 1.7mL microcentrifuge tube. A transfection reagent (Polyjet reagent SignaGen Laboratories Rockville, MD) was added to an additional 250 μL of serum-free DMEM in a separate 1.7mL microcentrifuge tube. The amount of transfecting reagent to be added was determined using a 3:1 volume: mass ratio (e.g. 3 μL of Polyjet for 1 μg of DNA vector). The serum-free DMEM containing the transfection reagent was then added to the tube containing DNA and incubated at room temperature for 15 minutes. The combined mixture was then gently added to a flask of HEK293 cells. These cells were incubated at 37°C and allowed to grow for overnight.

After a 24 hour incubation period, the flasks were processed at room temperature. In order to lyse cells and immunoprecipitate proteins, Tris-KCl lysis buffer was made just

prior to processing and stored on ice. The buffer consisted of 150 mL KCl, 1 mM DTT, 2 mM EDTA, 50 mM Tris-HCl pH 7.5, 1 % (v/v) Triton X-100, 20 mM NaF, 20 mM β-glycerophosphate, 20 mM NaVO3, 10 mM Na pyrophosphate, 1X Halt protease inhibitor cocktail). The media was aspirated and cells were washed with 5 mL of cold phosphate-buffered saline (PBS). PBS was immediately aspirated off and cells were lysed in 2 mL of KCl lysis buffer. The lysis buffer containing cells was transferred into a 2 mL microcentrifuge tube, and were sonicated at 25 % amplitude for 15 seconds at 4°C using a probe sonicator (Thermo Scientific). The mixture was then centrifuged at 13,000 x g for 10 minutes while maintaining a temperature of 4°C. The supernatant was transferred to a 1.7mL microcentrifuge and used for immunoprecipitation.

## 2.3 Immunoprecipitations

After centrifuging cell lysates, the supernatant was used for inputs and immunoprecipitations. For the inputs, 25 µl of 4X sample buffer (0.2 M Tris HCl pH 6.8, 40% glycerol, 0.1 M DTT, 8% SDS w/v, 0.04% bromophenol blue w/v in water) was added to a separate 75 µl sample of cell lysate. This mixture was vortexed and immediately stored at -20°C. In order to immunoprecipitate proteins, 500 µl of HEK293 cell lysate was transferred to a microcentrifuge tube and incubated with the appropriate immunoprecipitation antibody, while rotating at 4°C for approximately 1 hour. After 1-hour incubation period, 20 µl of protein G magnetic beads were added to each sample and incubated overnight while rotating at 4°C. The next day, these samples were magnetically separated and washed three times with IP wash buffer and stored in 50 µL of 2x sample buffer (4x sample buffer diluted 1:2 with Milli-Q water).

Antibodies used for immunoprecipitation were: rabbit monoclonal anti- spinophilin (E1E7R, 14136, Cell Signaling technology, INC.), goat polyclonal anti-spinophilin (A-20, SC14774, Santa Cruz Biotechnology, INC.), rabbit monoclonal anti-NMDAR2B (D15B3, 4212, Cell Signaling technology, INC.) goat polyclonal anti-V5 tag (A190-119A, Bethyl Laboratories, Inc.), rabbit polyclonal anti V5 tag (D3H8Q, 13202, Cell Signaling technology, INC.), goat polyclonal anti-HA tag (A190-107A, Bethyl Laboratories, Inc.

Montgomery, TX), goat polyclonal anti-Myc tag (A190-104A, Bethyl Laboratories, Inc. Montgomery, TX), and mouse polyclonal anti-PP1 (E-9, sc-7482, Santa Cruz Biotechnology, INC.).

## 2.4 SDS-PAGE and Western Blotting

Inputs and protein immunoprecipitates were used for western blotting. All samples were first heated at 65°C for 10 minutes. The immunoprecipitated samples were placed on a magnet to separate magnetic beads out of the protein suspension. 10 µL of each input and 20 µL of each immunoprecipitated sample was loaded onto a 26-well pre-cast Criterion 4-15% polyacrylamide gradient gel (Bio-Rad), a 15 well 4-15% Mini-Protein TGX polyacrylamide gradient gel (Bio-Rad), or a 1.5 mm hand-cast 10% polyacrylamide gel. The gels were then run at 75V for 15 minutes followed 175V for 1 hour.

Proteins were then transferred to a nitrocellulose membranes using the TransBlot Turbo system (Bio-Rad) at a voltage of 9V for 30 minutes. The nitrocellulose membrane containing the proteins was then placed in a blotting box and blocked using 5 % (w/v) nonfat dry milk in 0.1 % (v/v) Tween-20 in 1 M Tris-buffered saline pH 7.5 (TBST). This step was repeated three times, each lasting about 10 minutes. The membranes were then incubated with primary antibodies diluted in the same 5 % dry milk in Tween-20. The blots were allowed to shake overnight at 4°C. The following day, blots were again blocked with 5 % milk in Tween-20 for 10 minutes, three times. Next, the blots were incubated with secondary antibodies in darkness, at room temperature for 1 hour. After discarding the milk containing secondary antibodies, the blots were washed with Tris-buffered saline (TBS) three times, each wash being 10 minutes. The Odyssey imaging system was then used to perform fluorescence scans and Image Studio software (LiCor, Lincoln, NE) was used for data analysis.

Primary antibodies used were: a rabbit polyclonal anti-V5 (G-14, sc-83849, Santa Cruz Biotechnology, INC), a goat polyclonal anti-HA (A190-107A, Bethyl Laboratories, Inc. Montgomery, TX), a goat polyclonal anti-PP1γ (sc-6108 Santa Cruz Biotechnology), a mouse monoclonal anti-PP1 α (E-9, sc-7482, Santa Cruz Technology, INC), a rabbit

polyclonal anti phospho-NMDAR2B-Ser1284 (5355, Cell Signaling Technology) and a mouse monoclonal anti-Myc (9E10, sc-40, Santa Cruz Technology, INC).

Secondary antibodies used were: Alexa Fluor 790-conjugated AffiniPure Donkey Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, INC.), Alexa Fluor 790-conjugated AffiniPure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories, INC.), Alexa Fluor 790-conjugated AffiniPure Donkey Anti-Goat IgG (Jackson ImmunoResearch Laboratories, INC.), Alexa Fluor 680-conjugated donkey anti-Goat (Molecular Probes, Invitrogen detection technologies) and Alexa Fluor 680-conjugated donkey anti-Rabbit (Molecular Probes, Invitrogen detection technologies). Jackson ImmunoResearch antibodies were typically diluted 1:50000 in 5 % milk and Invitrogen antibodies were generally diluted 1:10000 in 5 % milk.

## 2.5 Statistical Analysis

After scanning blots on the Odyssey imaging system, Image Studio software was used to quantify integrated fluorescence intensities of bands. The strength of an association between two proteins was calculated by dividing the integrated fluorescence intensity for the co-immunoprecipitated protein by the integrated fluorescence intensity for the immunoprecipitated protein. In order to normalize for any differences in protein expression, the above value was divided by the input value for the co-immunoprecipitated protein.

$$\frac{\dfrac{\text{Intensity of co-IP protein in Precipitation}}{\text{Intensity of IP protein in Precipitation}}}{\text{Intensity of co-IP protein in Inputs}}.$$

A ratio of experimental/control was generated in order to average across multiple gels and transfections, with each transfection corresponding to a unique biological replicate. The N values for each individual experiment correspond to the number of unique biological replicates. To compare between groups, a one-column t-test was performed to compare the

experimental to a theoretical value of 1. *p value of <0.5;  **p value < 0.01;  *** p value < 0.001;  **** p value < 0.0001.

# CHAPTER 3. RESULTS

## 3.1 Neurabin – PP1 interaction

### 3.1.1 Neurabin binds to both isoforms of PP1 - PP1α and PP1γ in a heterologous cell system

Neurabin is a PP1 scaffolding protein that binds and targets PP1 to other proteins. To confirm this interaction in HEK cells, we transfected HEK293 cells with HA tagged neurabin and myc tagged PP1α or PP1γ. HA tagged neurabin was immunoprecipitated with an antibody against the HA tag. The samples were then analyzed with SDS-PAGE and western blotting. We blotted for neurabin using an HA antibody and for any co-immunoprecipitated PP1 using a myc antibody. There was a robust interaction seen between neurabin and both PP1α and PP1γ (Figure 1). Conversely, myc tagged PP1 was also immunoprecipitated and samples were blotted for co-precipitated neurabin. There was a strong reciprocal interaction noted, as suggested by the presence of co-precipitated neurabin. Additionally, there was no non-specific binding observed, further confirming this interaction.

### 3.1.2 PKA overexpression does not change neurabin association with PP1α and PP1γ

In order to study the effects of PKA overexpression on the interaction between neurabin and PP1, HEK293 cells were transfected with neurabin and PP1α or PP1γ, with and without overexpressed PKA. The amount of PP1 co-immunoprecipitated with neurabin in the presence of overexpressed PKA was normalized against the amount of PP1 co-immunoprecipitated with neurabin without overexpression. This was done by using the formula mentioned in the Methods section. For example,

$$PP1_{\text{PKA overexpression:}} \quad \frac{\text{Co-precipitated PP1/ Immunoprecipitated neurabin}}{PP1_{INPUTS}}$$

$$PP1_{CONTROL} \quad : \quad \frac{\text{Co-precipitated PP1/Immunoprecipitated neurabin}}{PP1_{INPUTS}}$$

To evaluate the significance of change caused by the experimental condition, we normalized the experimental condition back to the control condition to obtain a ratio of change in the experimental condition. For example,

$$\frac{PP1_{PKA\ OVEREXPRESSION}}{PP1_{CONTROL}}$$

Statistical analysis of the data collected from experiments looking at the effects of PKA overexpression on neurabin-PP1 association revealed a significant amount of variability. The data did not show a consistent trend and PKA overexpression did not seem to have a significant effect on neurabin's ability to bind PP1 (Figure 2).

### 3.1.3 CDK5 overexpression does not change neurabin association with PP1α, but increases association with PP1γ

In order to study the effects of CDK5 overexpression on the interaction between neurabin and PP1, HEK293 cells were transfected with neurabin and PP1α or PP1γ, with and without overexpressed CDK5 along with its activator, p35. Statistical analysis of the data collected from experiments looking at the effects of CDK5 overexpression on neurabin-PP1α association revealed a significant amount of variability. The data showed an increase in neurabin and PP1α association, but was not significant. However, there was a significant increase observed in neurabin-PP1γ association (Figure 3). These data suggest that CDK5 is able to phosphorylate neurabin or PP1γ, possibly increasing their interaction.

### 3.1.4 CaMKII overexpression decreases neurabin association with PP1α, but does not affect association with PP1γ

In order to study the effects of CaMKII overexpression on the interaction between neurabin and PP1, HEK293 cells were transfected with neurabin and PP1α or PP1γ, with and without overexpressed CaMKII. The effects of CaMKII overexpression seemed to be the opposite of what was seen with CDK5 overexpression. CaMKII significantly decreased the neurabin-PP1α association but had no effect on the neurabin-PP1γ association. In the presence of overexpressed CaMKII, the amount of PP1α co-precipitated with neurabin was

significantly lower than the amount of PP1α co-precipitated in the control (Figure 4), suggesting a decrease in the association.

## 3.2 Spinophilin – PP1 interaction

### 3.2.1 Spinophilin binds to both isoforms of PP1 - PP1α and PP1γ in a heterologous cell system

Spinophilin is a PP1 scaffolding protein that binds and targets PP1 to other proteins. To confirm this interaction in HEK cells, we transfected HEK293 cells with HA tagged spinophilin and myc tagged PP1α or PP1γ. HA tagged spinophilin was immunoprecipitated with an antibody against the HA tag. The samples were then analyzed with SDS-PAGE and western blotting. We blotted for spinophilin using an HA antibody and for any co-immunoprecipitated PP1 using a myc antibody. There was a robust interaction seen between spinophilin and both PP1α and PP1γ (Figure 5). Conversely, myc tagged PP1 was also immunoprecipitated and samples were blotted for co-precipitated spinophilin. There was a strong reciprocal interaction noted, as suggested by the presence of co-precipitated spinophilin. Additionally, there was no non-specific binding observed, further confirming this interaction.

### 3.2.2 PKA overexpression increases spinophilin association with PP1α, but not with PP1γ

In order to study the effects of PKA overexpression on the interaction between spinophilin and PP1, HEK293 cells were transfected with spinophilin and PP1α or PP1γ, with and without overexpressed PKA. In the presence of overexpressed PKA, the amount of PP1α co-precipitated with spinophilin was significantly higher than the amount of PP1α co-precipitated in the control (Figure 6), suggesting a increase in the association.

### 3.2.3 CDK5 overexpression increases spinophilin association with PP1α and PP1γ

In order to study the effects of CDK5 overexpression on the interaction between spinophilin and PP1, HEK293 cells were transfected with spinophilin and PP1α or PP1γ, with and without overexpressed CDK5. While overexpression of CDK5 decreased PP1 expression, there was an increase in the remaining PP1α and PP1γ that were co-precipitated with spinophilin (Figure 7), suggesting that phosphorylation of spinophilin or PP1 by CDK5 increases the association between the two proteins.

### 3.2.4 CaMKII overexpression does not change spinophilin association with PP1α and PP1γ

In order to study the effects of CaMKII overexpression on the interaction between neurabin and PP1, HEK293 cells were transfected with neurabin and PP1α or PP1γ, with and without overexpressed CaMKII. Overexpressed CaMKII caused an increase in the amount of PP1α co-precipitated with spinophilin and a decrease in the amount of PP1γ co-precipitated with spinophilin. However, neither of these changes were significant. (Figure 8).

## 3.3 Neurabin – GluN2B$_{TAIL}$

### 3.3.1 Neurabin binds to GluN2B$_{TAIL}$ of the NMDAR in a heterologous cell system

As previously discussed, neurabin is a PP1 targeting protein and PP1 is known to dephosphorylate and regulate the activity of glutamatergic AMPARs and NMDARs. Spinophilin, a structurally similar protein has been previously shown to interact with the GluN2B$_{TAIL}$ of the NMDA receptor (Salek et al., unpublished observations). In order to determine whether neurabin was able to target PP1 to the GluN2B$_{TAIL}$ of the NMDAR in a heterologous cell system, HEK293 cells were transfected with HA-Neurabin and V5-GluN2B$_{TAIL}$. After processing the cells, neurabin was immunoprecipitated with an antibody against the HA tag. The first lane serves as a control for neurabin, where neurabin is immunoprecipitated with the HA antibody but there is no GluN2B$_{TAIL}$ band present. The second lane serves as a control GluN2B$_{TAIL}$; although there is GluN2B$_{TAIL}$ present in the

inputs, it does not precipitate with the HA antibody in the absence of neurabin. In the third sample, neurabin and GluN2B$_{TAIL}$ were co-transfected. Upon immunoprecipitation of neurabin with the HA antibody, a strong GluN2B$_{TAIL}$ band was seen on the blot (Figure 9). These data suggest that GluN2B$_{TAIL}$ binds to neurabin, either directly or indirectly as a part of a complex.

### 3.3.2 Neurabin aa580-850 interacts with GluN2B$_{TAIL}$ in a heterologous cell system

We utilized isoform 4 of neurabin, which is larger than the canonical neurabin isoform (1098 vs 1253 AA). Isoform 4 does not contain the complete SAM domain and contains a unique amino acid sequence between residues 918 and 1184 (Figure 27). Neurabin has multiple domains that bind to different types of proteins. In order to determine which domain was interacting with GluN2B$_{TAIL}$, we made several fragments of the neurabin protein. We split up the protein into five fragments: neurabin AA1-301 (N1) consisting of the actin binding domain, neurabin AA280-600 (N2) consisting of the PDZ domain and the PP1 binding domain, neurabin AA580-850 (N3) consisting of the PDZ domain and two coiled-coil domains, neurabin AA830-1253 (N4) consisted of the isoform 4 insert domain and C-terminus and neurabin AA580-1253 (N5) consisted of PDZ, coiled-coil, isoform 4 insert domain, and C-terminus. Like the full-length neurabin protein, all fragments were HA-tagged.

Each fragment was individually co-transfected with V5-GluN2B$_{TAIL}$ in HEK cells. The fragment containing the actin binding domain (N1) was not expressed in cells, even at high DNA concentrations.

GluN2B$_{TAIL}$ was immunoprecipitated with an antibody against the V5 tag. Western blotting demonstrates that the PP1 binding domain (N2) does not co-precipitate with GluN2B$_{TAIL.}$ N3 (PDZ and coiled-coil domains) and N5 (PDZ, coiled-coil and insert domains) both co-precipitated with GluN2B$_{TAIL.}$ N4 (insert domain and C-terminus) does not co-precipitate with GluN2B$_{TAIL.}$ Moreover, there was an apparent equal association between N3-GluN2B$_{TAIL}$ and N5 - GluN2B$_{TAIL.}$ (Figure 10). These data suggest that the insert domain does not play an important role in this interaction and that the PDZ and coiled-coil domains are important in neurabin's binding to GluN2B$_{TAIL.}$ Future studies will need to narrow down which regions bind to GluN2B$_{TAIL}$

### 3.3.3 PKA overexpression increases neurabin association with GluN2B$_{TAIL}$

Our previous data showed that in some cases, PKA overexpression increased neurabin binding to PP1 whereas in some cases it decreased neurabin-PP1 association. Previous literature has shown that PKA phosphorylates neurabin in its PP1 binding domain and reduces its binding to the catalytic subunit of PP1 (McAvoy, 1999). In order to determine the effects of PKA on neurabin- GluN2B$_{TAIL}$ association, neurabin and GluN2B$_{TAIL}$ were co-transfected with and without overexpressed PKA. Western blot shows that the amount of GluN2B$_{TAIL}$ co-precipitated with neurabin was higher in the presence of overexpressed PKA (Figure 11).

### 3.3.4 CDK5 overexprsesion decreases neurabin association with GluN2B$_{TAIL}$

In experiments discussed above, we have seen that CDK5 overexpression increases neurabin's association with PP1γ. To determine the effects of CDK5 on neurabin's association with GluN2B$_{TAIL}$, neurabin and GluN2B$_{TAIL}$ were co-transfected with and without CDK5 (and its activator, p35). Based on preliminary data, CDK5 seemed to cause a drastic decrease in association (Figure 12). This is the opposite of the effect seen with PKA overexpression; however, the experiment will need to be repeated to determine significance.

## 3.4 Neurabin and Spinophilin interaction with SRCIN1 pathway

### 3.4.1 Neurabin and spinophilin interaction with SRCIN1 pathway

Previous studies identified a novel spinophilin interacting protein, called SNAP-25 interacting protein (SNIP) (Baucum, 2010). This protein is also known as p130CAS-associated protein, p140Cap, or SRC kinase signaling inhibitor 1 and is encoded by the *SRCIN1* gene. In order to validate if spinophilin associates with p140Cap and to determine if neurabin also interacts with this protein, HEK293 cells transfections were utilized. For control conditions, cells were transfected with HA-neurabin, HA-spinophilin and V5-p140Cap independently. Neurabin and spinophilin were also co-transfected with p140Cap.

After processing the cells, neurabin and spinophilin were immunoprecipitated using antibodies against the HA tag. Using the same antibody to precipitate both proteins limited antibody variability. p140Cap was immunoprecipitated with antibody against V5 tag. HA and V5 antibodies were used to blot these proteins. Inputs indicate presence of desired protein(s) in each lane. On the HA-IP blot, there is a band seen around 140kDa, indicating co-immunoprecipitation of p140Cap along with both neurabin and spinophilin (Figure 13). Conversely, a reciprocal interaction was detected when blotting the V5-immunoprecipitates. Together these data suggest an association between spinophilin or neurabin and p140Cap.

### 3.4.2 Neurabin does not directly interact with SRC, but spinophilin does

As previously discussed, SRC is an oncogenic protein that is negatively regulated by p140Cap. Neurabin and spinophilin's ability to bind to p140Cap suggests that they might be able to indirectly regulate this cancer pathway. We also wanted to explore the possibility of neurabin and spinophilin directly regulating SRC, as opposed to p140Cap-mediated regulation. The first step was to determine whether there is an association between the scaffolding proteins and SRC.

In order to determine whether the scaffolding proteins neurabin and spinophilin directly bind to SRC, HEK293 cell transfections were carried out. For control conditions, cells were transfected with HA-neurabin, HA-spinophilin and myc-SRC independently. Neurabin and spinophilin were also co-transfected with SRC. After processing the cells, SRC was immunoprecipitated using antibody against the myc tag. HA and myc antibodies were used to blot these proteins. Inputs indicate presence of desired protein(s) in each lane. On the myc-IP blot, there is a spinophilin band observed, indicating its co-immunoprecipitation with SRC (Figure 14). There is no neurabin band present, suggesting that it did not co-immunoprecipitate with SRC. This lack of an interaction could be due to lack of binding or a low expression of neurabin. Future studies will need to further elucidate if neurabin binds to SRC.

## 3.5 Neurabin – p140Cap

### 3.5.1 Neurabin aa580-600 may be sufficient for interaction with p140Cap

After establishing that neurabin binds to p140Cap, we wanted to determine which domain of neurabin was responsible for interaction with p140Cap. Previously listed fragments of neurabin – N1 (aa1-301), N2 (aa280-600), N3 (aa580-850), N4 (aa830-1253) and N5 (aa580-1253) were individually co-transfected with myc-p140Cap. p140Cap was immunoprecipitated using an antibody against the myc tag. N3, N4 and N5 fragments co-precipitated with p140Cap (Figure 15). The fact that N3 and N4 were both able to bind to p140Cap suggests that the overlapping region of aa830-850 may be sufficient for binding with p140Cap. This theory would have to be tested by creating a neurabin fragment of aa830-850 and co-transfecting it with p140Cap.

### 3.5.2 PKA overexpression does not change neurabin association with p140Cap

In order to study the effects of PKA overexpression on the interaction between neurabin and p140Cap, HEK293 cells were transfected with neurabin and p140Cap, with and without overexpressed PKA. Statistical analysis of the data collected from experiments looking at the effects of PKA overexpression on neurabin-p140Cap association revealed a significant amount of variability (Figure 16). Data were inconsistent and PKA overexpression did not have a significant effect on neurabin binding to p140Cap.

### 3.5.3 CDK5 overexpression decreases neurabin association with p140Cap

In order to study the effects of CDK5 overexpression on the interaction between neurabin and p140Cap, HEK293 cells were transfected with neurabin and p140Cap, with and without overexpressed CDK5. Results revealed a significant decrease in the amount of p140Cap co-precipitated with neurabin (Figure 17). These data suggest that CDK5 is able to bind and/or phosphorylate neurabin or p140Cap in a way that makes it less likely for the two proteins to interact.

### 3.5.4 CaMKII overexpression does not change neurabin association with p140Cap

In order to study the effects of CaMKII overexpression on the interaction between neurabin and p140Cap, HEK293 cells were transfected with neurabin and p140Cap, with and without overexpressed CaMKII. Data was variable and there was no significant effect on neurabin's ability to bind p140Cap (Figure 18).

### 3.5.5 PP1α and PP1γ overexpression decrease neurabin association with p140Cap

In order to study the effects of PP1α and PP1γ overexpression on the interaction between neurabin and p140Cap, HEK293 cells were transfected with neurabin and p140Cap, with and without overexpressed PP1α and PP1γ. Results revealed a decreased association between neurabin and p140Cap in the presence of either PP1α or PP1γ. (Figure 19). While the mechanism by which PP1 decreases this association is unclear, one possible explanation is that PP1-dependent dephosphorylation of neurabin or p140Cap leads to a decreased association between these proteins.

## 3.6 Spinophilin – p140Cap

### 3.6.1 PKA does not change spinophilin association with p140Cap

In order to study the effects of PKA overexpression on the interaction between spinophilin and p140Cap, HEK293 cells were transfected with spinophilin and p140Cap, with and without overexpressed PKA. Results revealed that PKA had no effect on the association of spinophilin with p140Cap (Figure 20).

### 3.6.2 CDK5 overexpression decreases spinophilin association with p140Cap

In order to study the effects of CDK5 overexpression on the interaction between spinophilin and p140Cap, HEK293 cells were transfected with spinophilin and p140Cap, with and without overexpressed CDK5. Results revealed a significant decrease in the amount of p140Cap co-precipitated with spinophilin (Figure 21). These data suggest that

CDK5 is able to bind and/or phosphorylate spinophilin or p140Cap in a way that makes it less likely for the two proteins to interact.

### 3.6.3 CaMKII overexpression does not change spinophilin association with p140Cap

In order to study the effects of CaMKII overexpression on the interaction between spinophilin and p140Cap, HEK293 cells were transfected with spinophilin and p140Cap, with and without overexpressed CaMKII. Data demonstrated a trend for decreased association but did not reach significance (Figure 22). These data did not show a significant effect on spinophilin's ability to bind p140Cap.

### 3.6.4 PP1α overexpression increases spinophilin association with p140Cap, but PP1γ has no effect

In order to study the effects of PP1α and PP1γ overexpression on the interaction between neurabin and p140Cap, HEK293 cells were transfected with spinophilin and p140Cap, with and without overexpressed PP1α and PP1γ. The data from experiments looking at the effects of PP1α overexpression on spinophilin-p140Cap association revealed a consistently significant increase in the amount of p140Cap co-precipitated with spinophilin (Figure 23). Overexpression of PP1γ did not seem to have a significant effect on spinophilin-p140Cap association.

### 3.6.5 SpinophilinF451A has decreased binding to p140Cap, compared with spinophilinWT in presence of overexpressed PP1α and PP1γ

Based on previous experiments, we have established that CDK5 overexpression enhances spinophilin's interaction with PP1α, overexpression of PP1α enhances spinophilin-p140Cap interaction. CDK5 overexpression with endogenous PP1α attenuates spinophilin's interaction with p140Cap. In order to determine the role of spinophilin-PP1 binding, a mutant form of spinophilin was used. Spinophilin F451A is a mutant from of the protein that has decreased binding to PP1. If the enhanced spinophilin-p140Cap interaction is a result of direct spinophilin-PP1 binding, then the wildtype spinophilin protein should have increased p140Cap binding with PP1 overexpression than the mutant

spinophilin which will not be able to bind to PP1 despite the overexpression. Preliminary data suggest that in the presence of overexpressed PP1, wildtype spinophilin binds more p140Cap than the mutant spinophilin (Figure 24).

### 3.6.6 SpinophilinF451A has increased binding to p140Cap, compared with spinophilinWT in presence of overexpressed CDK5

CDK5 overexpression increases spinophilin-PP1 binding. Overexpressed PP1α increases spinophilin-p140Cap interaction. CDK5 significantly decreases spinophilin-p140Cap interaction. To determine the role of spinophilin-PP1 binding in this effect, the mutant Spinophilin F451A was used. In the presence of overexpressed CDK5, normal spinophilin would have increased PP1 binding and that theoretically, that should result in increased spinophilin-p140Cap interaction as opposed to spinophilin F451A, which would not be able to bind the same amount of PP1 as normal spino. We hypothesized that the mutant form would have bind less p140Cap than spinophilin WT.

However, preliminary data suggests that more p140Cap is co-precipitated with spinophilin F451A than WT spinophilin (Figure 25). This does not support the hypothesis that higher spinophilin-PP1 binding is linked to higher spinophilin-p140Cap binding.

# CHAPTER 4. DISCUSSION

## 4.1 Neurabin and Spinophilin play a role in regulation of NMDAR

As previously discussed, neurabin and spinophilin are both PP1 targeting proteins and PP1 is known to dephosphorylate and regulate the NMDAR. Spinophilin's interaction with the

$GluN2B_{TAIL}$ of the NMDAR has been previously studied in the laboratory (Salek et al., unpublished observations) but the role of neurabin in regulation of the NMDAR is largely unknown. Serine/threonine kinases such as PKA and CDK5 phosphorylate spinophilin and/or NMDAR and alter the association of these proteins. Neurabin is a structurally similar protein that is phosphorylated by PKA in its PP1-binding region. We have discovered that neurabin is also able to associate with $GluN2B_{TAIL}$ region of the NMDAR (Figure 9) Moreover, we have found that the fragment of neurabin ranging aa580-850, containing the PDZ and coiled-coil domains binds with $GluN2B_{TAIL}$ (Figure 10). Previous studies with spinophilin and $GluN2B_{TAIL}$ have shown that overexpression of the serine/threonine kinase PKA increases spinophilin-$GluN2B_{TAIL}$ association, whereas another serine/threonine kinase CDK5 decreases this association. A similar trend was seen with neurabin and $GluN2B_{TAIL.}$ Overexpression of PKA increased the amount of $GluN2B_{TAIL}$ binding to neurabin (Figure 11). Preliminary data also showed a decrease in association with overexpression of CDK5 (Figure 12).

These data suggest that the interaction between neurabin and $GluN2B_{TAIL}$ is altered by the kinases PKA and CDK5. PKA could be phosphporylating neurabin or $GluN2B_{TAIL}$ to increase the association, while CDK5 could be phosphorylating neurabin or $GluN2B_{TAIL}$ at other sites, resulting in a decrease in association.

## 4.2 Spinophilin plays a role in regulation of tumor suppressor protein, p140Cap

Spinophilin's role in cancer first came to light when genetic linkage studies linked it to breast cancer (Porter, 1994). Spinophilin was later found to be downregulated in cancer tissue obtained from breast, prostate and brain tumors (Jallal, 2007; Chang, 2008; Nam, 2006; Yamaguchi, 2005). Spinophilin is known to directly associate with the tumor suppressor $p14^{ARF}$ (ARF), an alternative reading frame protein product of the cyclin-dependent kinase inhibitor 2A (CDKN2A) protein (Sherr, 2006). As previously discussed, spinophilin acts as a regulatory subunit of PP1. PP1$\alpha$ has been identified as the protein phosphatase that dephosphorylates phosphorylated retinoblastoma protein (pRb) and is associated with growth arrest response (Nelson, 1997; Berndt, 2004).

Hyperphosphorylated form of the Rb protein is predominant during the growth phase of cells, and PP1α activity is downregulated at this time. Through this mechanism, PP1 is able to control the cell cycle. Loss of spinophilin reduces *PPP1CA* levels, thereby maintaining higher levels of phosphorylated pRb. This results in an increase in p53 activity; however, in the absence of p53, reduced levels of spinophilin increase tumorigenic properties of cells. Therefore, spinophilin acts as a p53 loss-dependent tumor suppressor (Carnero, 2012). Partial or total loss of spinophilin has been seen in breast tumors, which strongly correlated with loss of activity of tumor suppressor protein p53 as well (Carnero, 2012). Spinophilin also interacts with doublecortin, an actin binding protein that is also involved in subcellular targeting of PP1 (Tsukada, 2003). Spinophilin enhances PP1-mediated dephosphorylation of doublecortin at PSer297 (Bielas, 2007). Spinophilin knockout mice have increased levels of PSer297-doublecortin in the brain and as a result, have abnormal spine formation (Feng, 2000). Doublecortin is a microtubule binding protein that is responsible for growth arrest at the G2-M phase in cell cycle of glioma cells and localization of doublecortin-spinophilin-PP1 complex into the cytosol of glioma cells leads to anti glioma effects via blocking of mitosis (Santra, 2006; Santra, 2009). Glioblastoma and neuroblastoma models are of particular interest when it comes to regulation of tumor suppressor proteins because spinophilin (and neurabin) have highest expression levels in the central nervous system.

Glioblastoma (GBM) is the most aggressive type of brain cancer with very poor prognosis. The adhesion and signaling mechanisms underlying GBM are poorly understood, and as a result there are no effective anti-invasive clinical therapies. Spinophilin binds to the cytoplasmic domain of β8 integrin in GBM cells and regulates cell invasion. GBM cells lacking spinophilin showed increased numbers of invadopodia and enhanced extracellular matrix degradation (Cheerathodi, 2016).

Another protein that is upregulated in various types of cancers including glioblastoma is SRC (Stettner, 2005). Inhibiting SRC function results in decreased cell proliferation in breast cancer, prostate cancer and glioblastoma tissue (Jallal, 2007; Chang, 2008; Nam, 2006; Yamaguchi, 2005). SRC activation leads to downstream signaling through the RAS/mitogen-activated protein kinase (MAPK) and P13K pathways that have been shown to play an important role in tumor proliferation and invasion (Wong, 2007; Schlaepfer,

1999; Levy, 1986). SRC is negatively regulated by C-terminal SRC kinase (CSK) and p140Cap . Silencing of p140Cap results in increased cell spreading, migration rate and SRC activity. Additionally, upregulation of p140Cap results in increased activation of CSK, leading to inhibition of SRC and downstream signaling. (Di Stefano, 2007).

p140Cap has been previously studied in epithelial tumor cells where it regulates tumor progression (Damiano, 2010). It also plays a role in normal synapse formation and maintenance (Jaworski, 2009). p140Cap has several serine and tyrosine residues that makes it receptive to tyrosine kinases as well as serine/threonine kinases. Phosphorylation of p140Cap by these kinases can affect its interactions with other molecules via post translation modification (Daniele, 2013). Spinophilin is a PP1 targeting protein that can target PP1 to substrates, resulting in dephosphorylation of proteins at serine/threonine residues. This dephosphorylating activity can negate the effects of phosphorylation by kinases.

We have discovered that spinophilin binds to p140Cap (Figure 13) suggesting that spinophilin's tumor suppression properties may be mediated, in part, via its interaction with p140Cap. The interaction between these two proteins is a critical one and it is important to understand how this interaction is regulated by kinases and phosphatases discussed earlier. While overexpression of PKA or CaMKII does not have a significant effect on this interaction, CDK5 overexpression drastically ***decreases*** the amount of p140Cap binding to spinophilin (Figure 21). Overexpression of PP1$\alpha$, a dephosphorylating protein (without CDK5 overexpression) ***increases*** p140Cap binding to spinophilin (Figure 23). CDK5 ***increases*** PP1$\alpha$ binding to spinophilin (Figure 7).

Spinophilin F451A is a mutant from of the protein that has decreased binding to PP1. Therefore, given the above that PP1 increased spinophilin binding to p140Cap and that CDK5 increased PP1 binding to spinophilin, we wanted to determine if spinophilin binding to PP1 is required for the CDK5-dependent decrease in spinophilin/p140Cap association. Preliminary data reveals that in the presence of overexpressed CDK5, the amount of p140Cap binding to Spino F451A is higher than the amount of p140Cap binding to Spino WT (Figure 24). Therefore, PP1 binding to spinophilin may be important in regulating the association of spinophilin with p140Cap.

As previously mentioned, neurabin is a scaffolding protein that is structurally similar to spinophilin and has similar binding partners (Kelker, 2006). Given the similarities, we explored the possibility of its association with p140Cap.We have established that neurabin also binds to p140Cap (Figure 13). Moreover, co-transfection of individual neurabin fragments/domains with p140Cap revealed that neurabin aa580-850 and neurabin aa850-1253 are both able to bind to p140Cap. This suggests that p140Cap may be binding to the overlapping region of aa830-850. This would have to be further confirmed by showing co-immunoprecipitation of neurabin aa830-850 with p140Cap.

Although PKA and CaMKII did not significantly change neurabin's interaction with p140Cap, CDK5 overexpression consistently caused a drastic ***decrease*** in the amount of p140Cap binding to neurabin (Figure 17). Additionally, PP1 – a dephosphorylating protein also caused a ***decrease*** in amount of p140Cap binding to neurabin. This trend was seen with overexpression of both PP1$\alpha$ and PP1$\gamma$ (Figure 19). CDK5 overexpression ***increases*** PP1 association with neurabin (Figure 3).

These data suggest that CDK5 could be phosphorylating neurabin and p140Cap, as well as PP1 to alter association. Conversely, the dephosphorylating effects of PP1 could be altering the association between neurabin and p140Cap, via dephosphorylation of either one or both of these proteins.

# CHAPTER 5. CONCLUSION

Glioblastoma (GBM) is the most aggressive type of brain cancer with very poor prognosis. Due to the lack of understanding of underlying mechanisms, there are no effective anti-invasive clinical therapies. Previous studies have shown that spinophilin directly plays a role in regulating cell invasion and in GBM cells (Cheerathodi, 2016). On the other hand, SRC activation leads to downstream signaling through the RAS/MAPK pathway to increase tumor proliferation and invasion (Wong, 2007). Morever, silencing of p140Cap (negative regulator of SRC) results in increased tumorigenesis and invasion.

Our data has discovered spinophilin and neurabin as two novel proteins that interact with the tumor suppressor protein p140Cap. Additionally, we have also discovered that CDK5 phosphorylates these proteins and alters their interaction. Spinophilin has been previously known to inhibit PP1 and its dephosphorylating properties (Kelker, 2006; Ragusa, 2010). PP1 also had an interesting effect on the interaction between these proteins; it increased the interaction between spinophilin and p140Cap and decreased the association between neurabin and p140Cap. These data suggest a possibility of different phosphorylation and dephosphorylation sites on neurabin and spinophilin that are targeted by the CDK5 and PP1. Further studies will have to done in order to determine the downstream effects of these interactions on SRC. Understanding the mechanisms that increase or decrease SRC activity can help us therapeutically target proteins that may ultimately decrease SRC activity and associated tumorigenesis and invasion.

# FIGURES

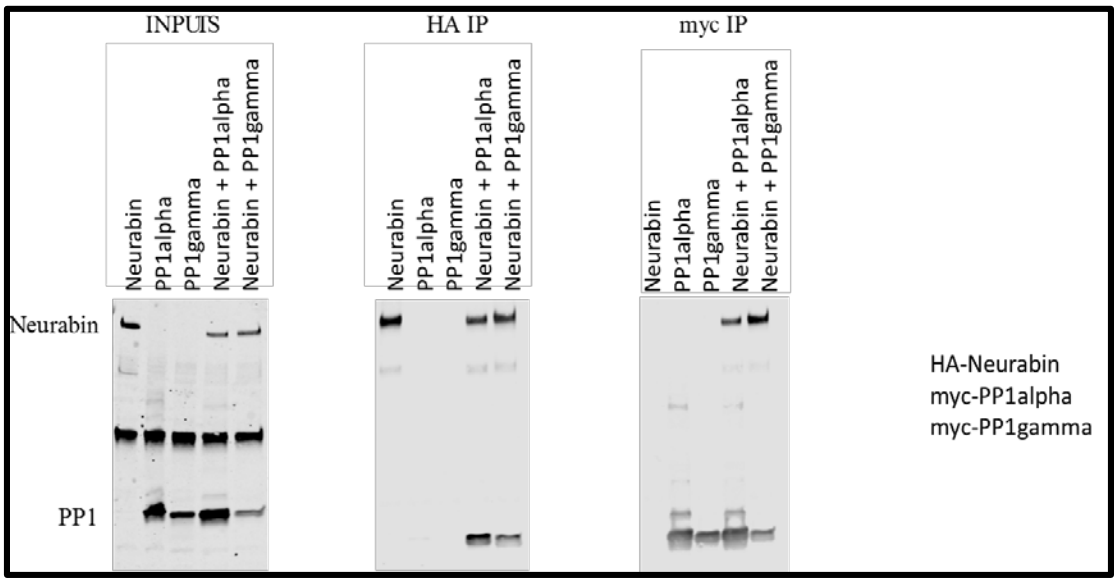



Figure 1 Neurabin interacts with PP1α and PP1γ in HEK293 cells.

HEK293 cells were transfected with HA-Neurabin and myc-PP1. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5 tag. Western blot results show an association between these two proteins.

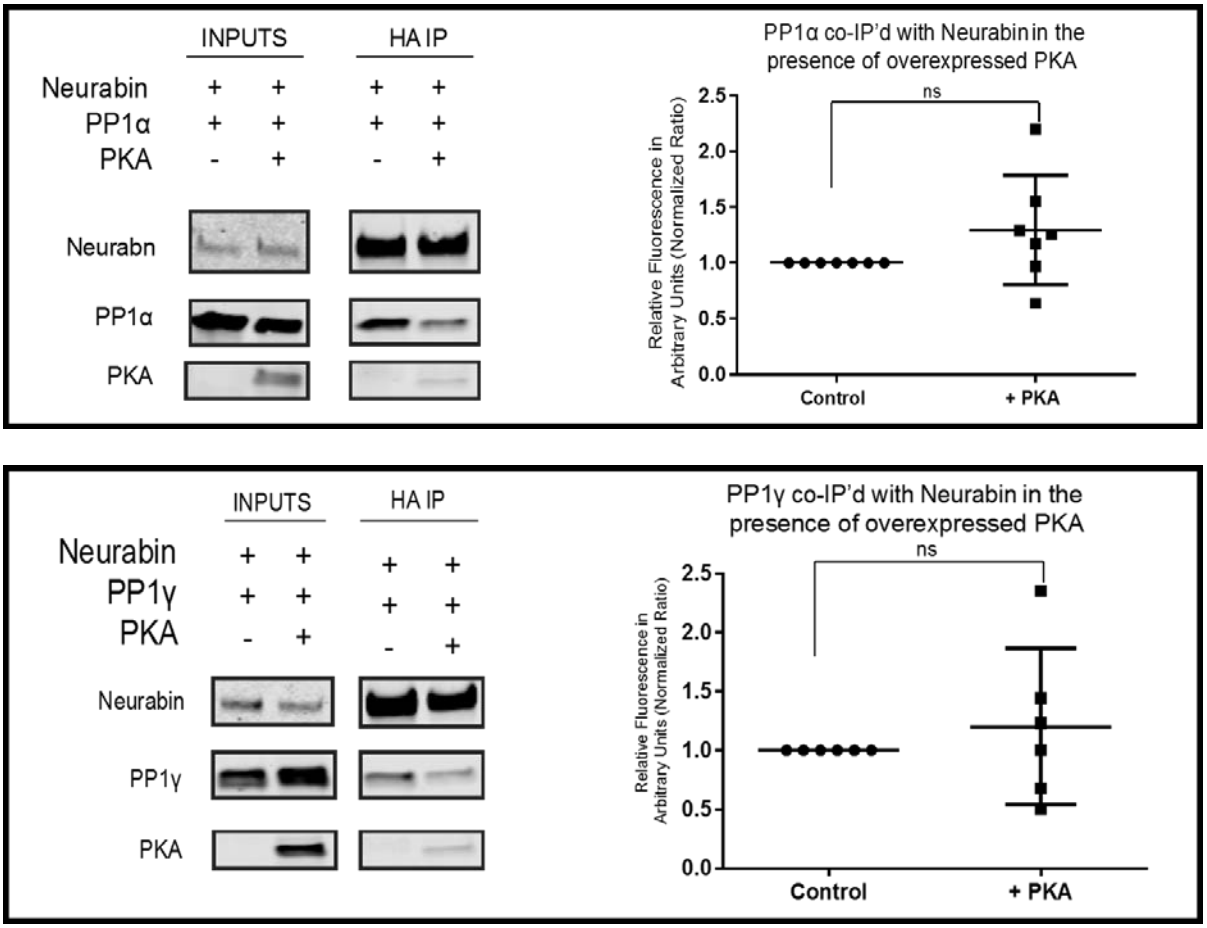



Figure 2 Neurabin interaction with PP1α and PP1γ is not significantly changed by PKA

HEK293 cells were transfected with HA-Neurabin and myc-PP1, with and without overexpression of myc-PKA. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or myc-tag. Western blot results show that the association of neurabin with PP1α or PP1γ is unchanged in the presence of overexpressed PKA.

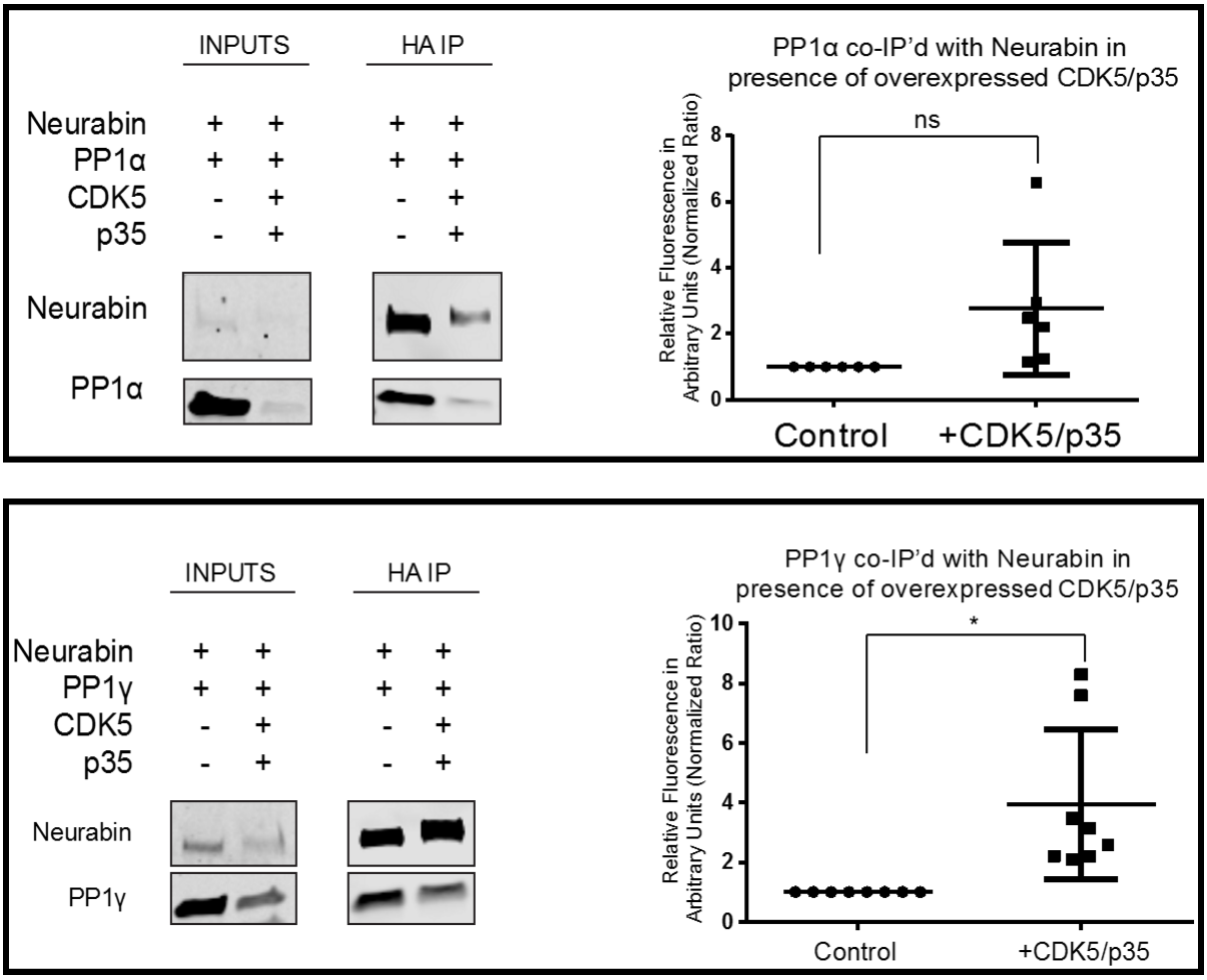



Figure 3 Neurabin interaction with PP1α is not significantly changed with overexpression of CDK5 but neurabin interaction with PP1γ is increased by CDK5

HEK293 cells were transfected with HA-Neurabin and myc-PP1, with and without overexpression of Flag-CDK5 and myc-P35. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or myc-tag. Western blot results show that neurabin association with PP1α is unchanged, while neurabin association with PP1γ is increased in the presence of overexpressed CDK5 and its activator myc-P35.

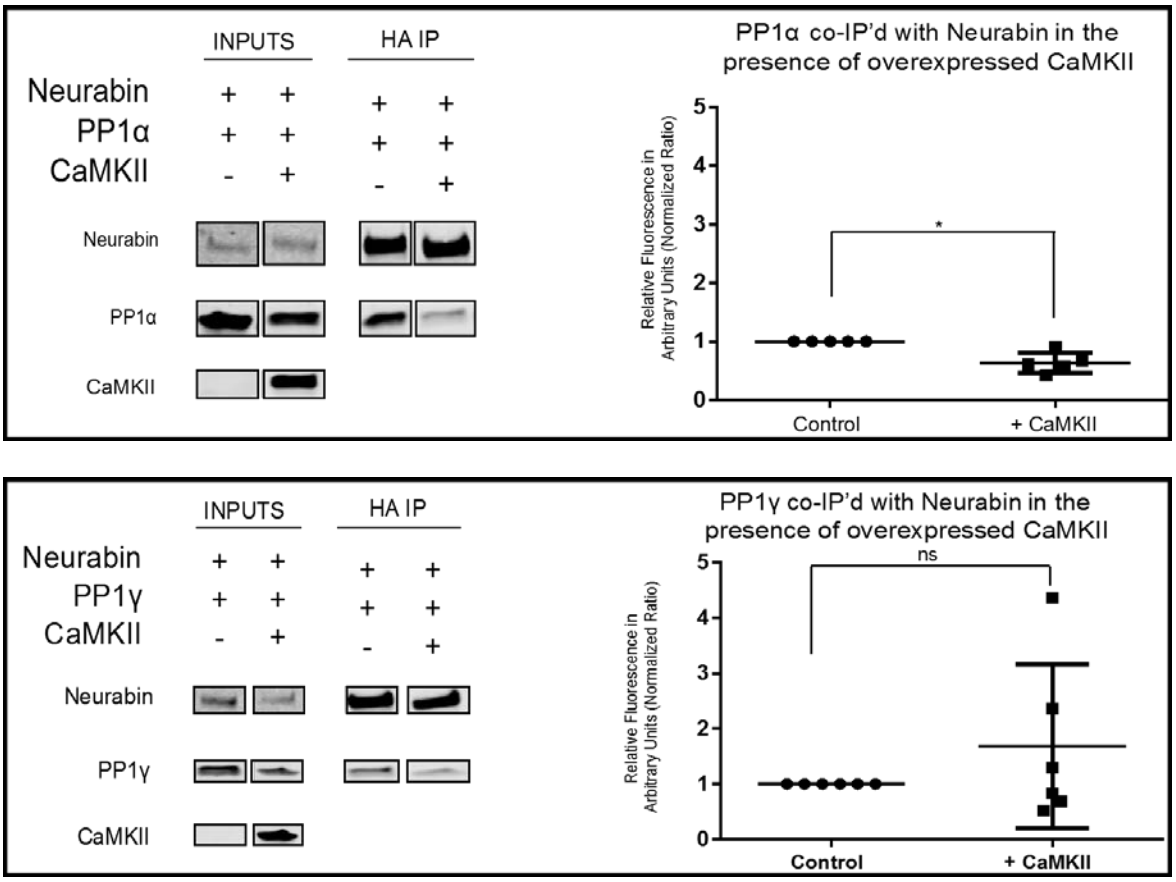



Figure 4 Neurabin interaction with PP1α is significantly decreased with overexpression of CaMKII but neurabin interaction and PP1γ is not significantly changed with overexpression of CaMKII

HEK293 cells were transfected with HA-Neurabin and myc-PP1, with and without overexpression of myc-CaMKII. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or myc-tag. Western blot results show that neurabin association with PP1α is decreased, while neurabin association with PP1γ is unchanged in the presence of overexpressed CaMKII.

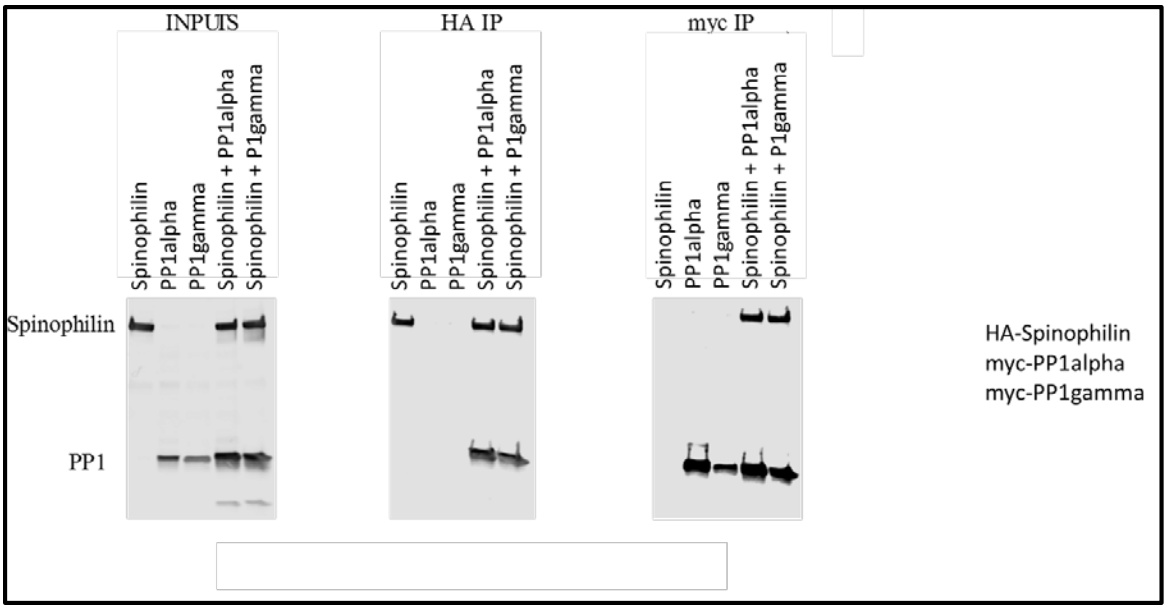



Figure 5 Spinophilin binds to PP1α and PP1γ in HEK cells

HEK293 cells were transfected with HA-Spinophilin and myc-PP1. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5 tag. Western blot results show an association between these two proteins.

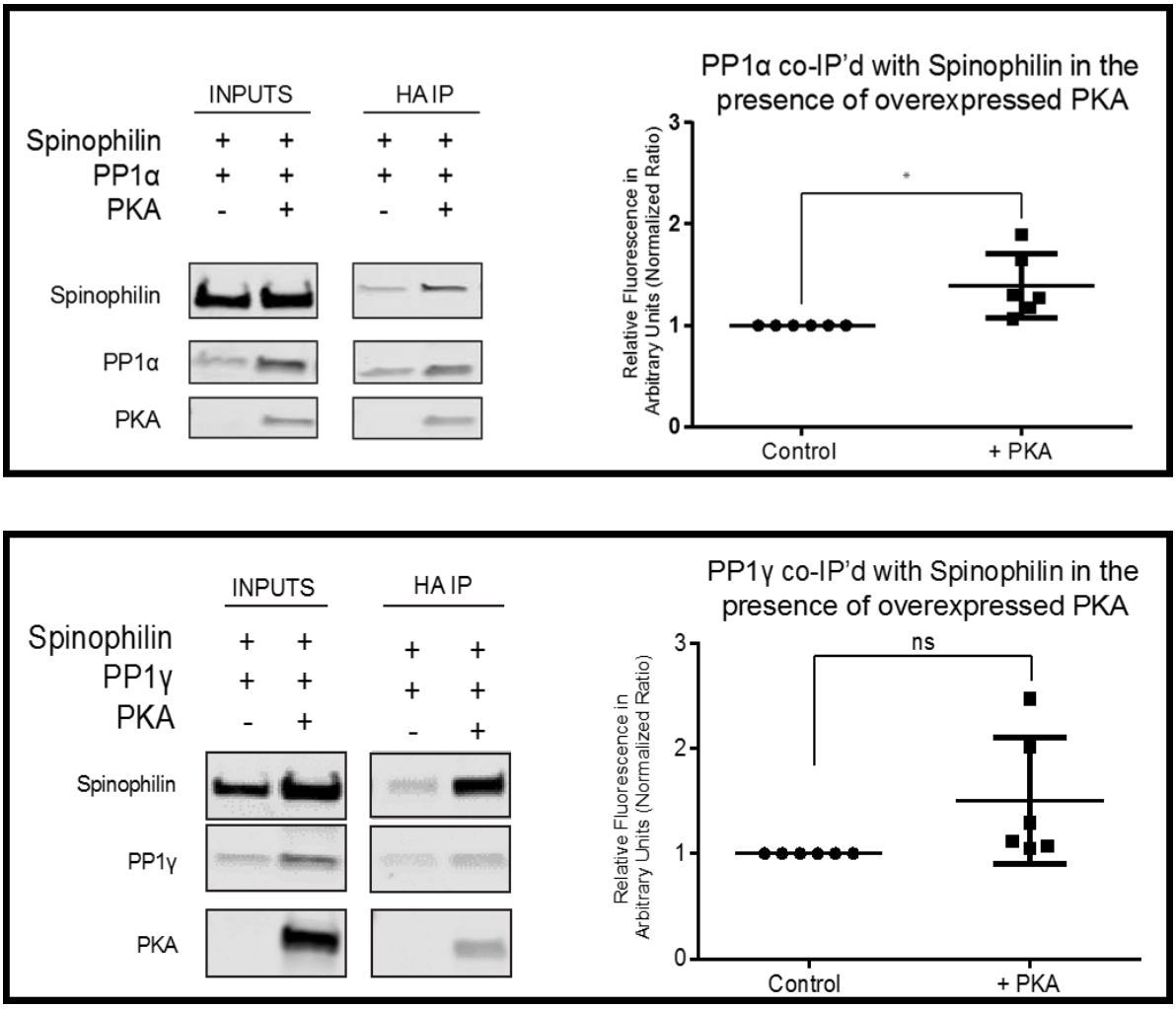
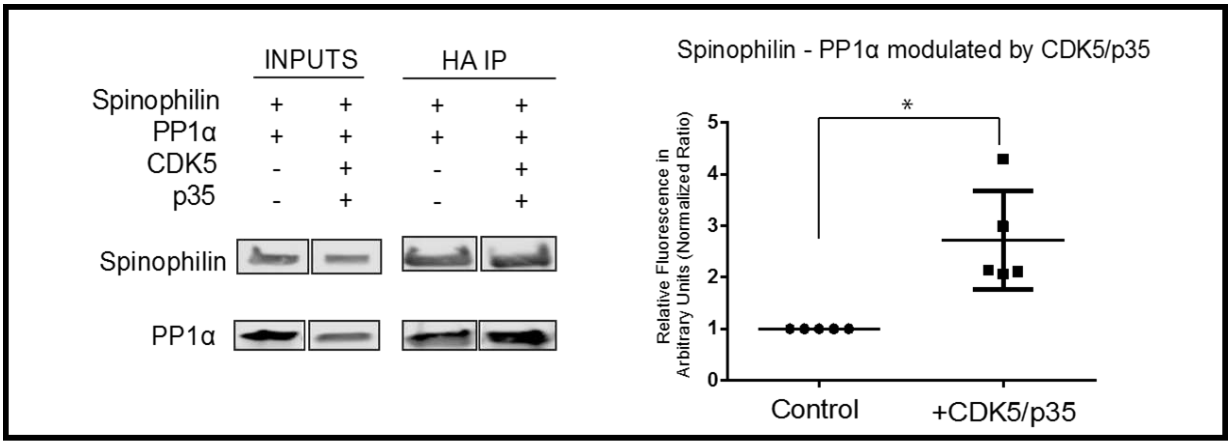



Figure 6 Spinophilin interaction with PP1α is significantly increased with overexpression of PKA, but spinophilin interaction with PP1γ is not significantly changed with overexpression of PKA

HEK293 cells were transfected with HA-Spinophilin and myc-PP1, with and without overexpression of myc-PKA. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or myc-tag. Western blot results show that spinophilin association with PP1α is increased, while spinophilin association with PP1γ is unchanged in the presence of overexpressed PKA.



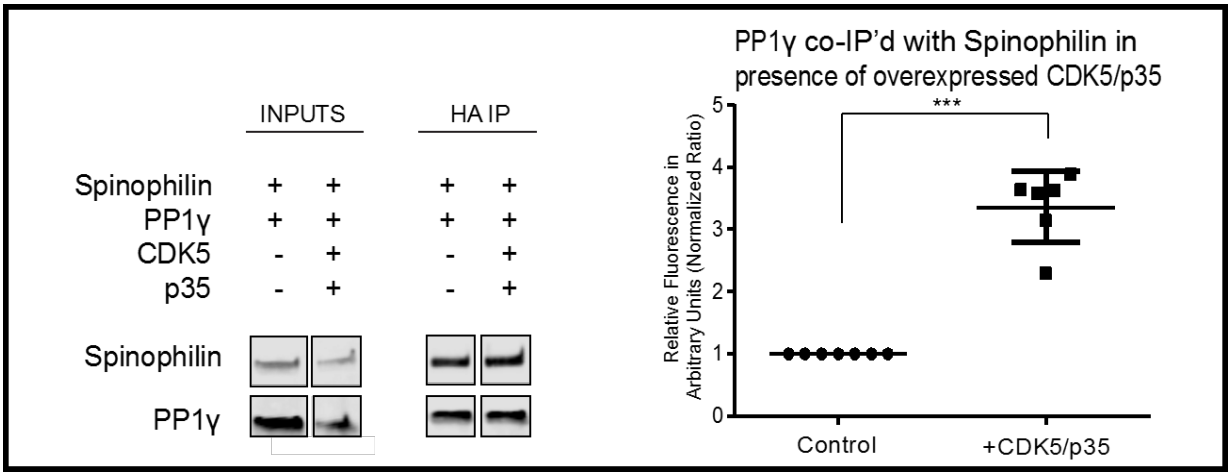




Figure 7 Spinophilin interaction with PP1α and PP1γ is significantly increased with overexpression of CDK5

HEK293 cells were transfected with HA-Spinophilin and myc-PP1, with and without overexpression of FLAG-CDK5 and myc-P35. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or myc-tag. Western blot results show that spinophilin association with PP1α and PP1ψ is increased with overexpression of CDK5, and its activator p35.

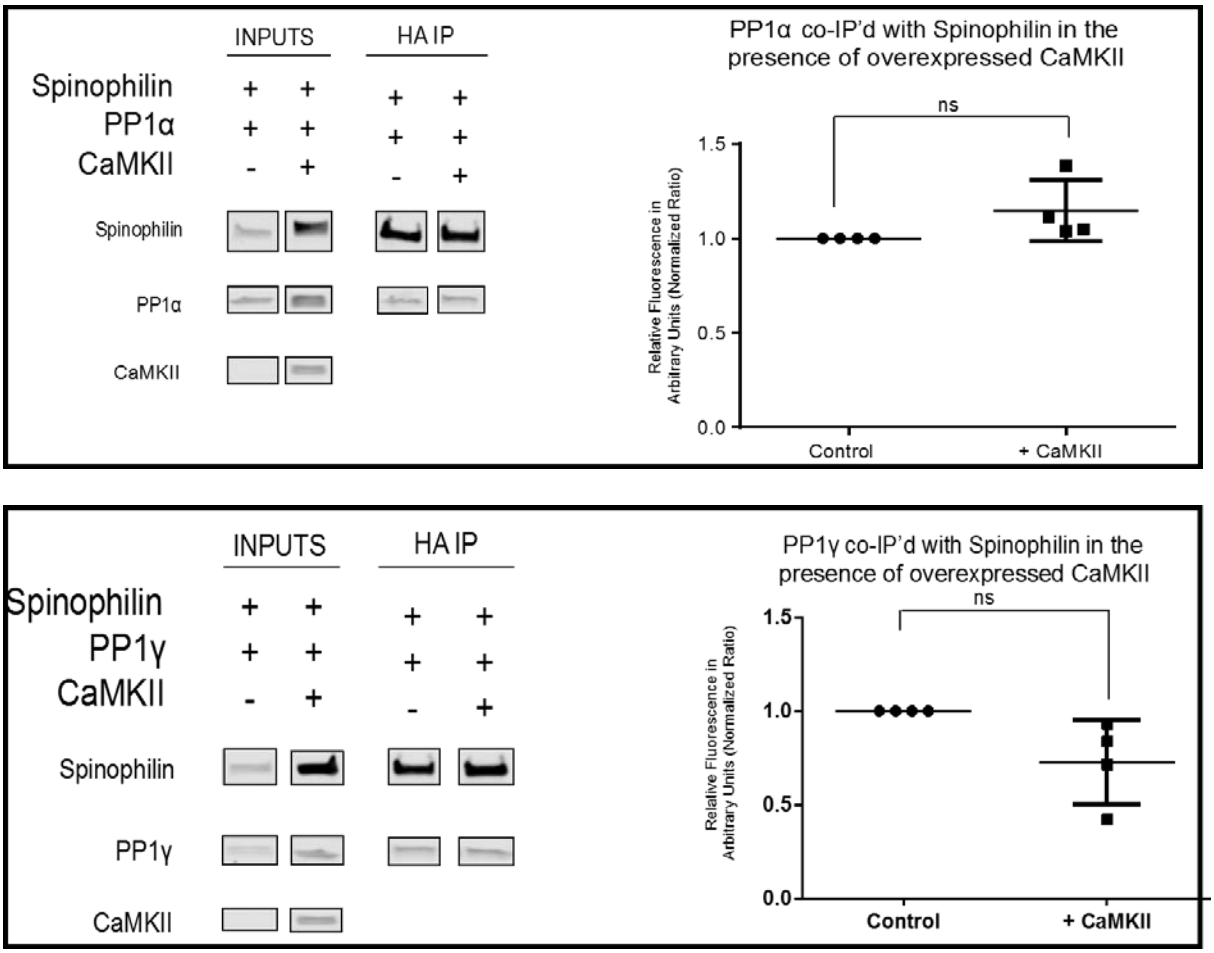



Figure 8 Spinophilin interaction with PP1α and PP1γ is not significantly changed with overexpression of CaMKII

HEK293 cells were transfected with HA-Spinophilin and myc-PP1, with and without overexpression of myc-CaMKII. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or myc-tag. Western blot results show that spinophilin association with PP1α and PP1γ is not significantly changed in the presence of overexpressed CaMKII.

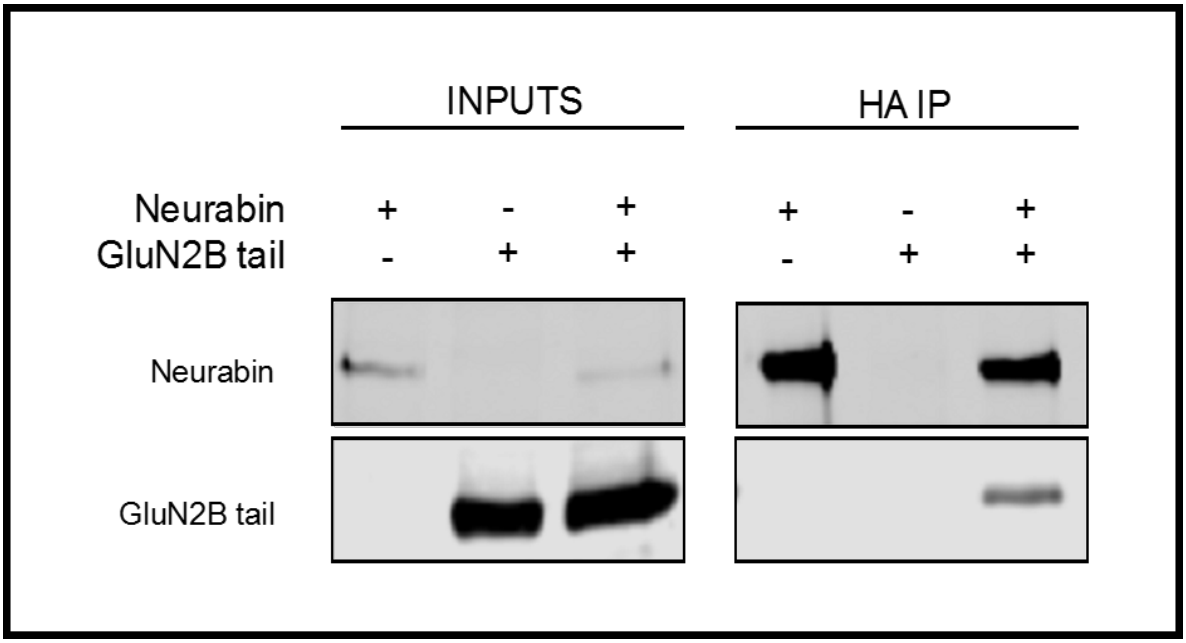



Figure 9 Neurabin binds to GluN2B$_{TAIL}$ of the NMDAR

HEK293 cells were transfected with HA-Neurabin and V5-GluN2B$_{TAIL}$. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5-tag. Western blot results show that Neurabin and GluN2B$_{TAIL}$ interact in HEK293 cells.

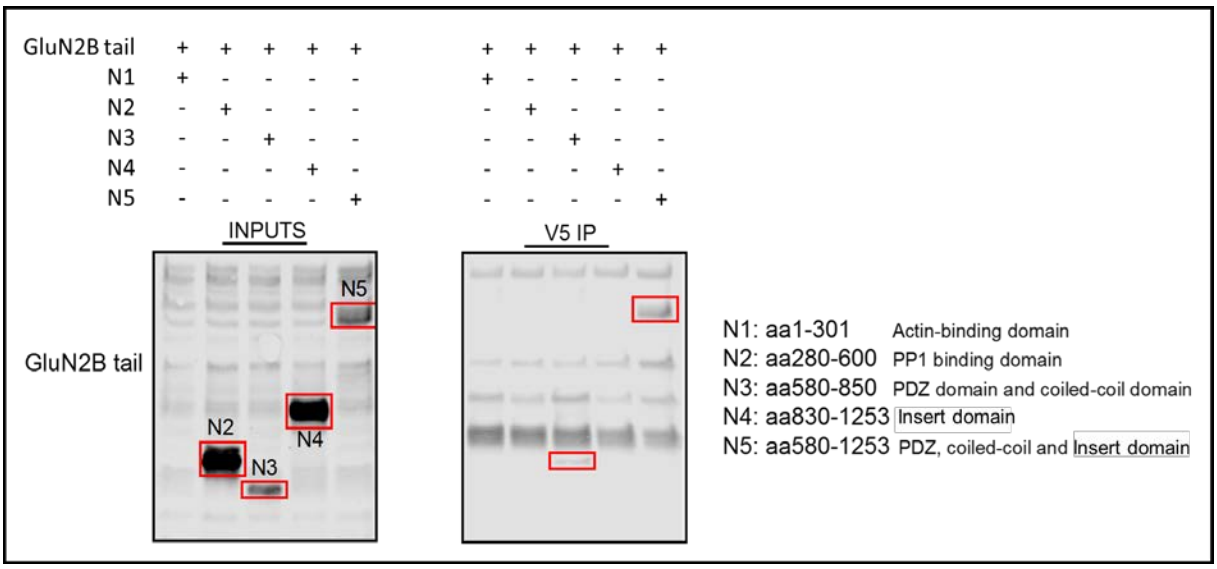



Figure 10 Neurabin aa580-830 are required for GluN2B$_{TAIL}$ interaction.

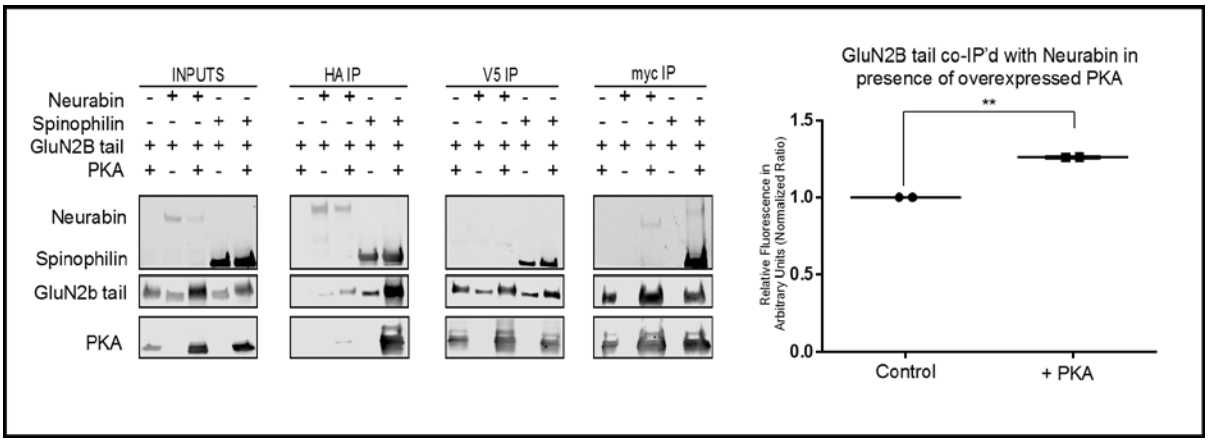



Figure 11 Neurabin interaction with GluN2B$_{TAIL}$ is increased by PKA overexpression

HEK293 cells were transfected with HA-Neurabin and V5-GluN2B$_{TAIL}$, with and without overexpression of myc-PKA. Immunoprecipitations were performed with antibodies raised against HA; immunoblots were performed with antibodies raised against either HA or V5-tag. Western blot results show that the neurabin association with GluN2B$_{TAIL}$ is significantly increased in the presence of overexpressed PKA.

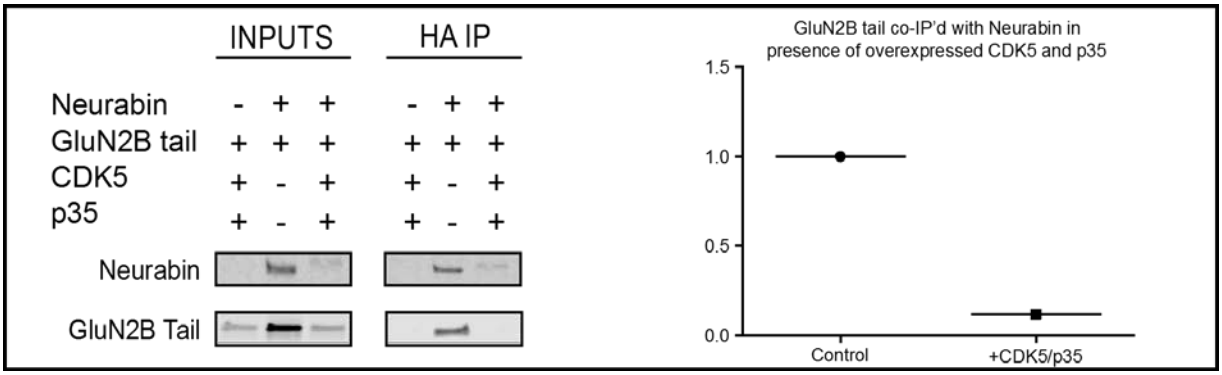



Figure 12 Neurabin interaction with GluN2B$_{TAIL}$ appears to be decreased with overexpression of Cdk5

HEK293 cells were transfected with HA-Neurabin and V5-GluN2B$_{TAIL}$, with and without overexpression of FLAG-CDK5 and myc-P35. Immunoprecipitations were performed with antibodies raised against HA; immunoblots were performed with antibodies raised against either HA or V5-tag. Western blot results show that neurabin association with GluN2B$_{TAIL}$ is decreased in the presence of overexpressed CDK5, and its activator P35.

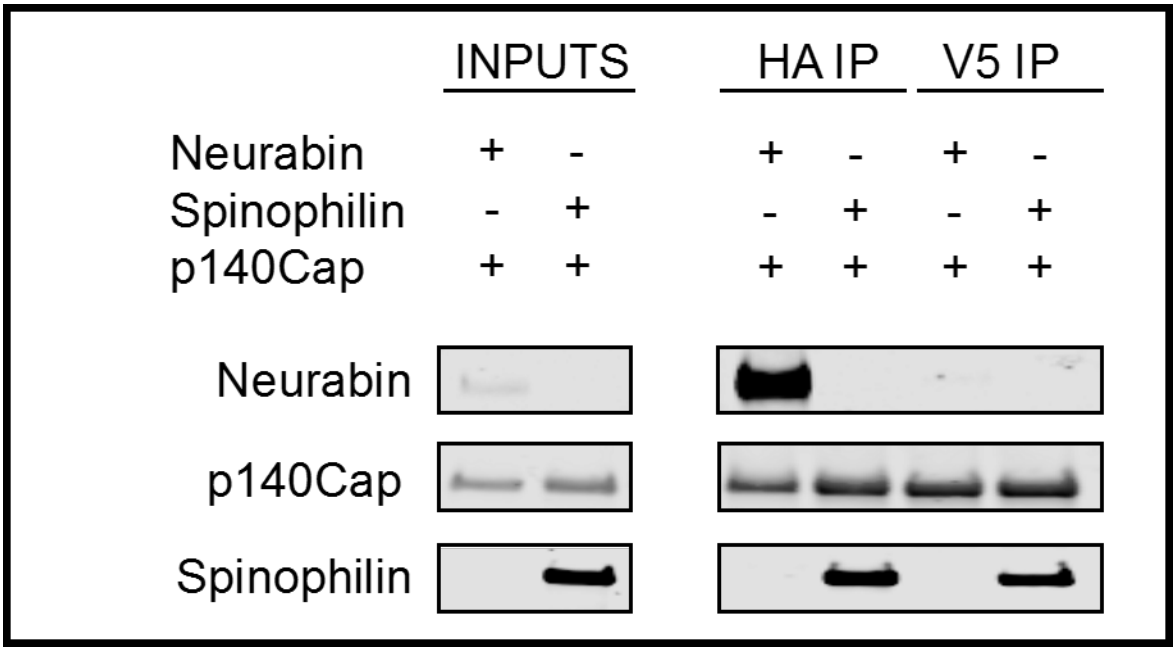



Figure 13 Neurabin and Spinophilin interact with tumor suppressor protein p140Cap

HEK293 cells were transfected with HA-Neurabin or HA-Spinophilin and V5-p140CAP. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5-tag. Western blot results show that there is an association between Neurabin and p140Cap, and Spinophilin and p140Cap.

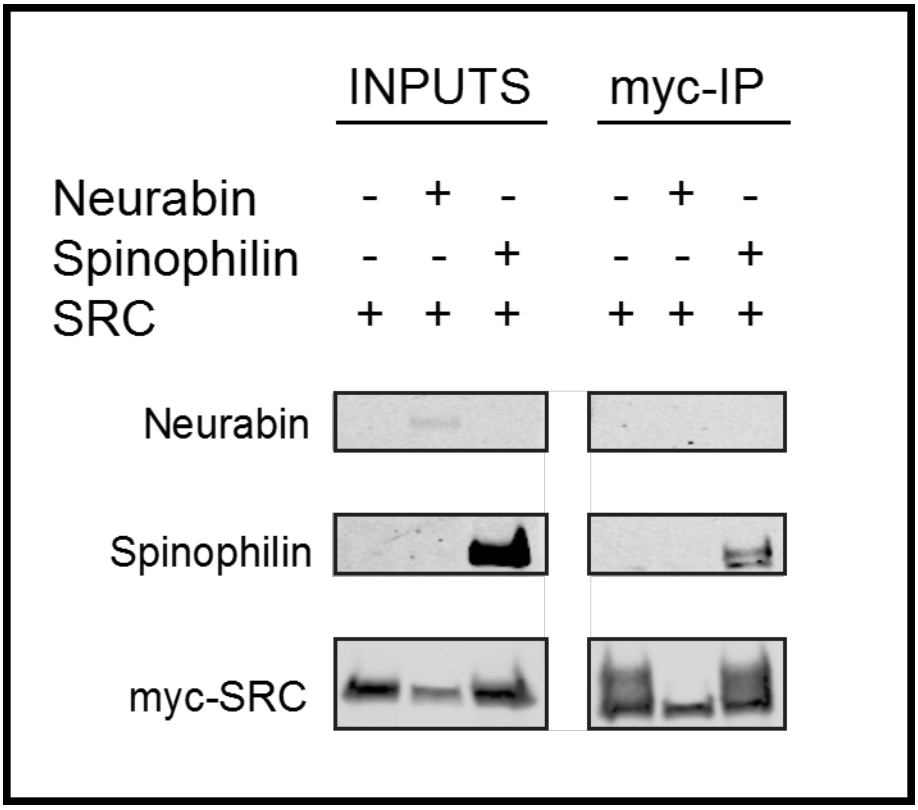



Figure 14 Neurabin does not directly interact with SRC, but Spinophilin does.

HEK293 cells were transfected with HA-Neurabin or HA-Spinophilin and myc-SRC. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or myc-tag. Western blot results show that there is an association between Neurabin and SRC, but there is a direct association between Spinophilin and SRC.

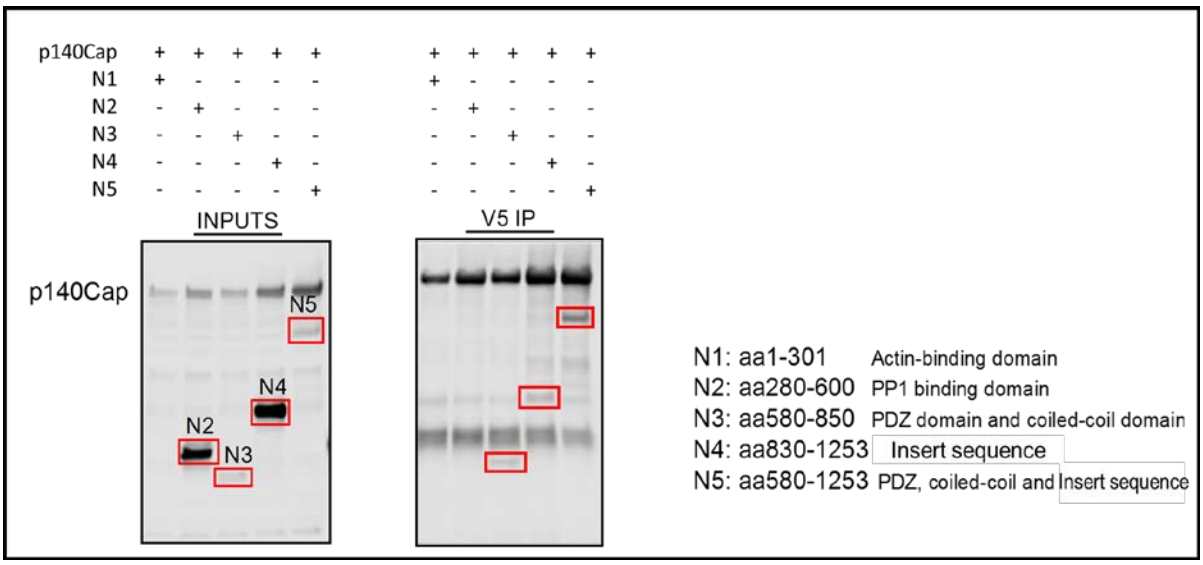



Figure 15 Neurabin aa580-850 and 830-1253 interact with p140CAP

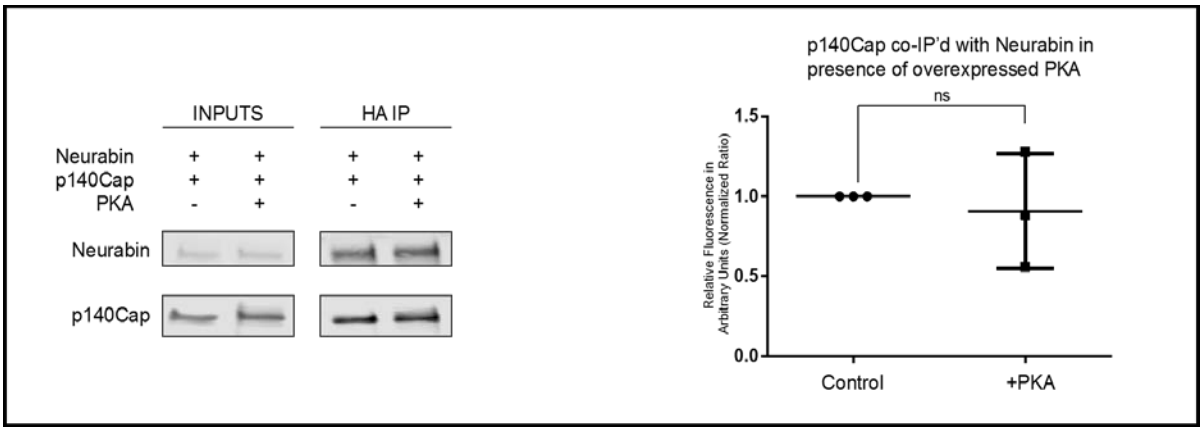



Figure 16 Neurabin interaction with p140CAP is not significantly changed by PKA

HEK293 cells were transfected with HA-Neurabin V5-p140Cap, with and without overexpression of myc-PKA. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5. Western blot results show that overexpression of PKA does not change neurabin association with p140Cap.

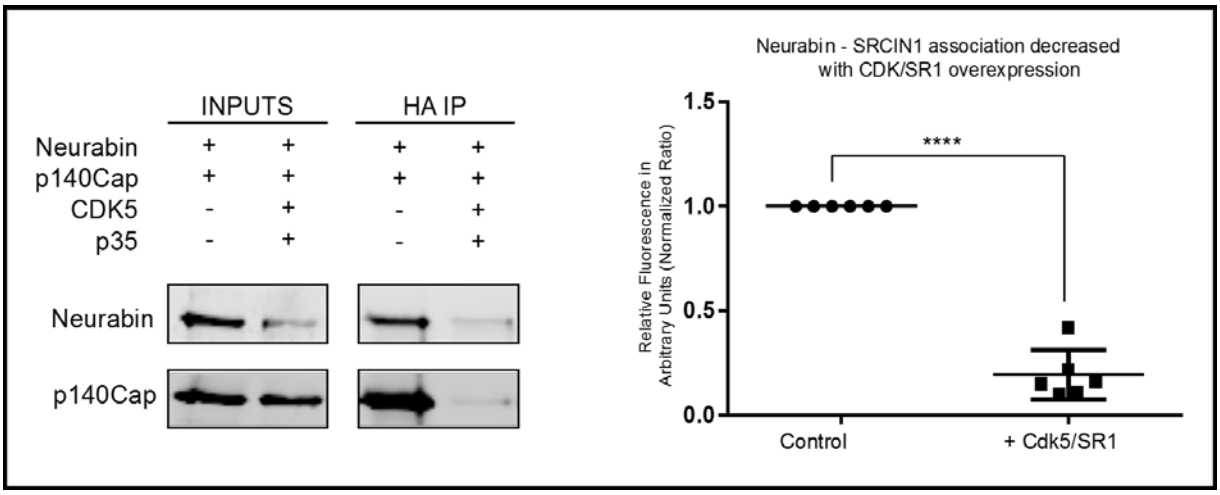



Figure 17 Neurabin interaction with p140CAP is significantly decreased by Cdk5

HEK293 cells were transfected with HA-Neurabin V5-p140Cap, with and without overexpression of FLAG-CDK5, and its activator myc-P35. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5. Western blot results show that overexpression of CDK5 and P35 decreases neurabin association with p140Cap

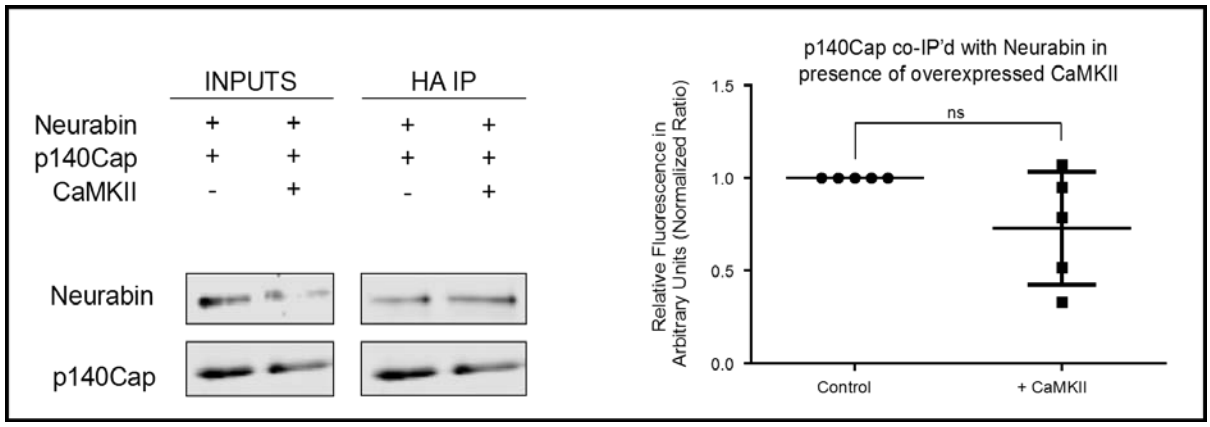



Figure 18 Neurabin interaction with p140CAP is not significantly changed by CaMKII

HEK293 cells were transfected with HA-Neurabin V5-p140Cap, with and without overexpression of myc-CaMKII. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5. Western blot results show that overexpression of CaMKII does not change neurabin association with p140Cap.

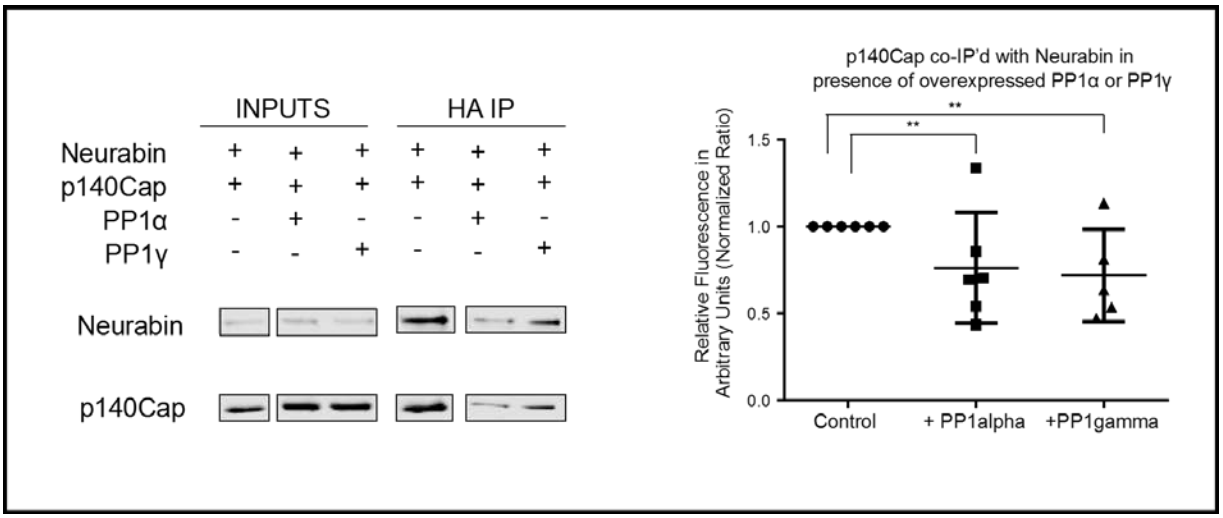



Figure 19 Neurabin interaction with p140CAP is decreased with PP1α and PP1γ overexpression

HEK293 cells were transfected with HA-Neurabin V5-p140Cap, with and without overexpression of myc-PP1α or myc-PP1γ. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or myc. Western blot results show that both overexpression of PP1α and overexpression of PP1γ decrease neurabin association with p140Cap.

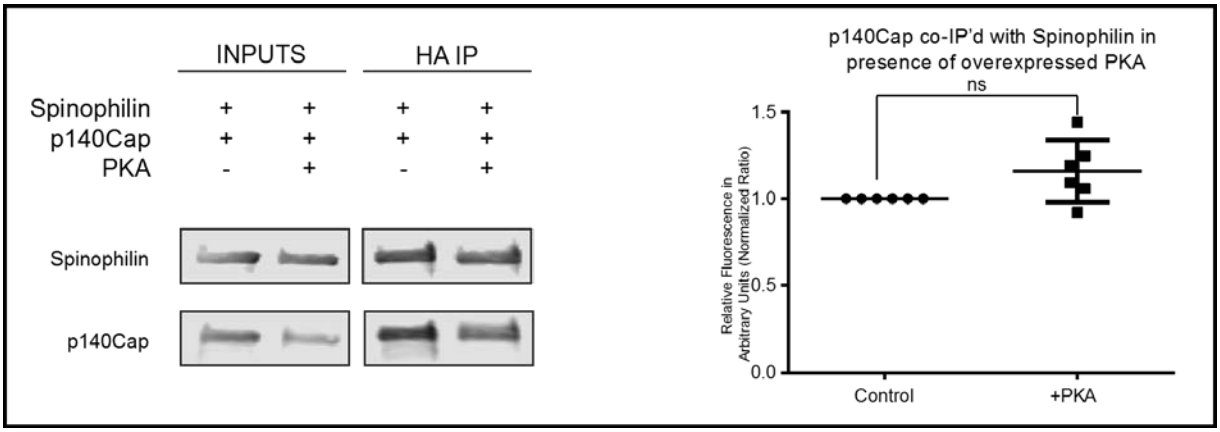



Figure 20 Spinophilin interaction with p140CAP is not significantly changed by PKA

HEK293 cells were transfected with HA-Spinophilin and V5-p140Cap, with and without overexpression of myc-PKA. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5. Western blot results show that overexpression of PKA does not change spinophilin association with p140Cap.

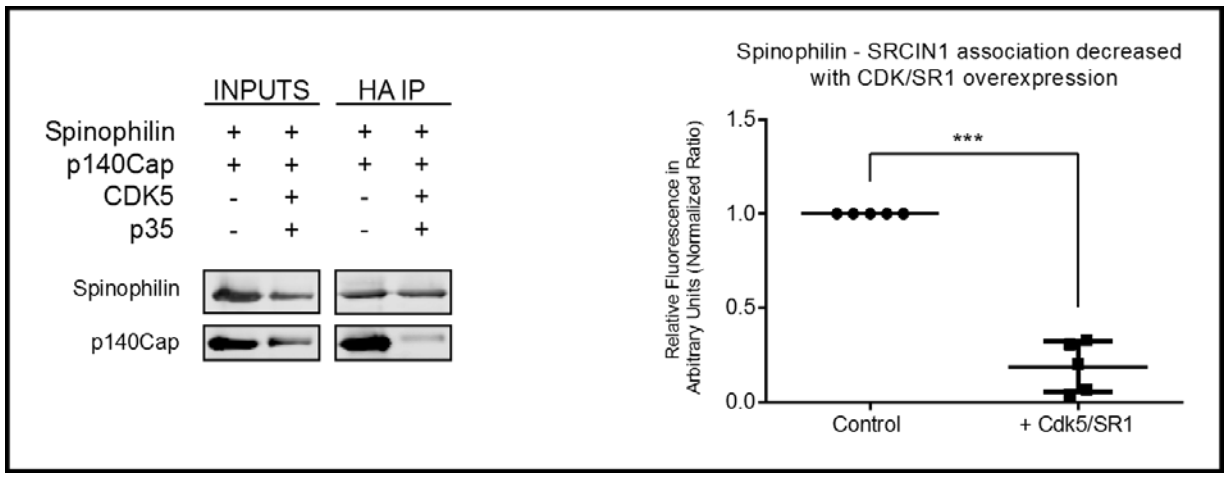



Figure 21 Spinophilin interaction with p140CAP is significantly decreased by Cdk5

HEK293 cells were transfected with HA-Spinophilin and V5-p140Cap, with and without overexpression of FLAG-CDK5 and its activator myc-P35. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5. Western blot results show that overexpression of CDK5 and P35 decreases spinophilin association with p140Cap.

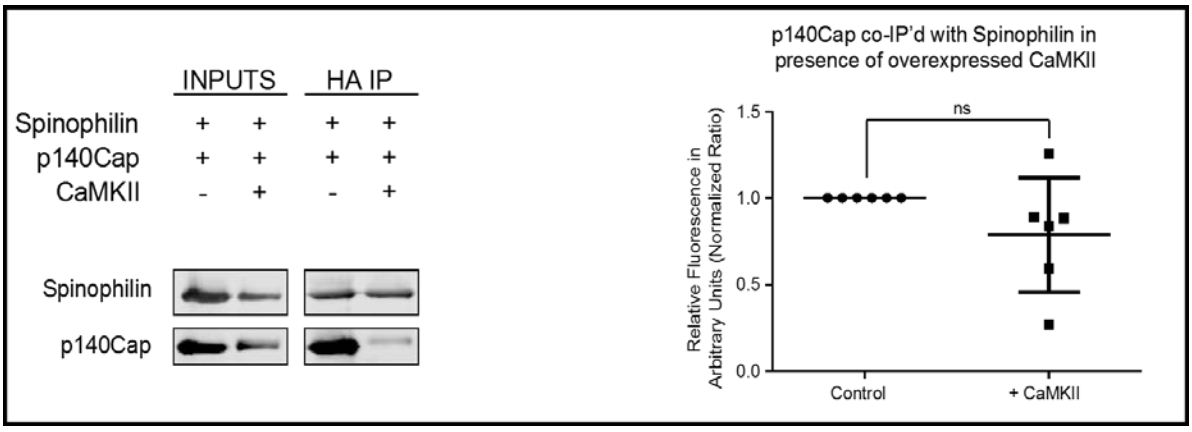



Figure 22 Spinophilin interaction with p140CAP is not significantly changed by CaMKII overexpression.

HEK293 cells were transfected with HA-Spinophilin and V5-p140Cap, with and without overexpression of myc-CaMKII. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5. Western blot results show that overexpression of CaMKII does not change spinophilin association with p140Cap.

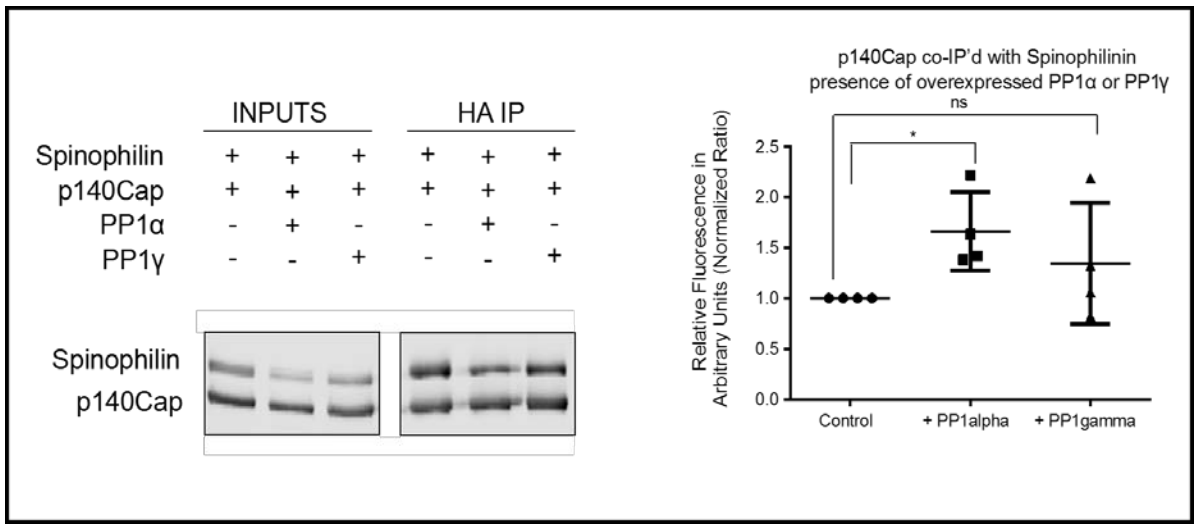



Figure 23 Spinophilin interaction with p140CAP is increased with overexpression of PP1α, but not significantly changed with overexpression of PP1γ.

HEK293 cells were transfected with HA-Spinophilin and V5-p140Cap, with and without overexpression of myc-PP1α or PP1γ. Immunoprecipitations and immunoblots were performed with antibodies raised against either HA or V5. Western blot results show that overexpression of PP1α increases spinophilin association with p140Cap, while overexpression of PP1γ does not change spinophilin association with p140Cap.

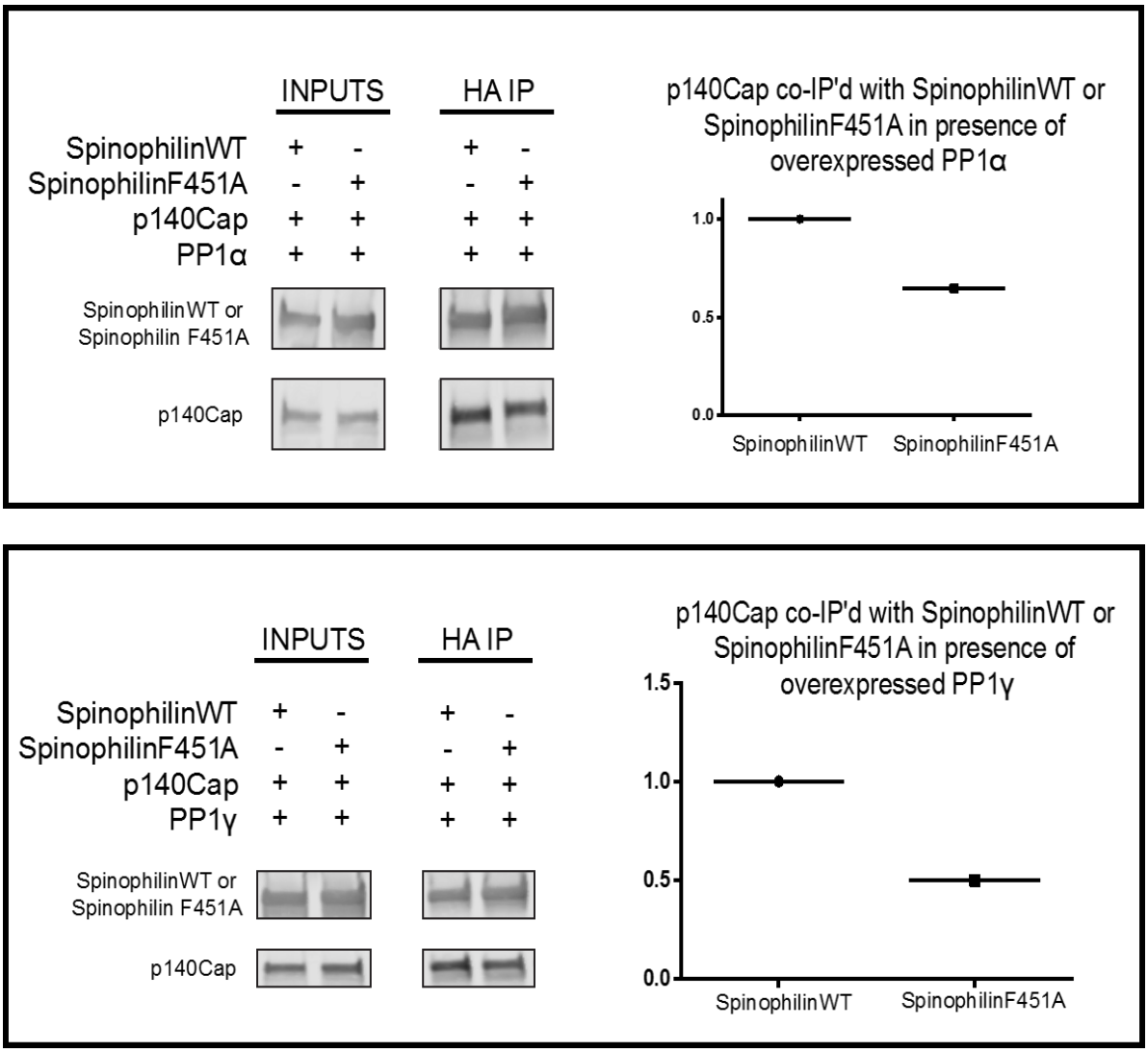



Figure 24 SpinophilinF451 has decreased binding to SRCIN1 in presence of overexpressed PP1α and PP1γ

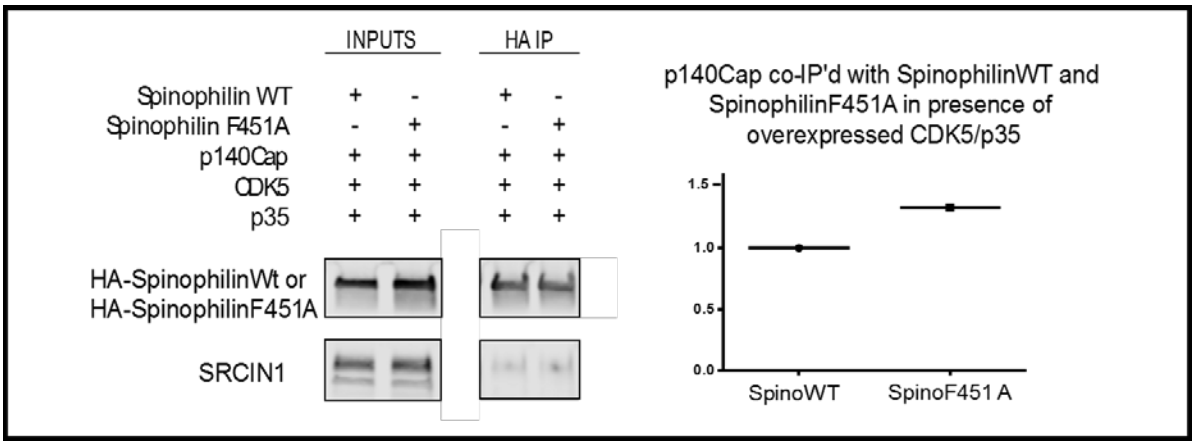



Figure 25 SpinophilinF451 has increased binding to SRCIN1 in presence of overexpressed Cdk5

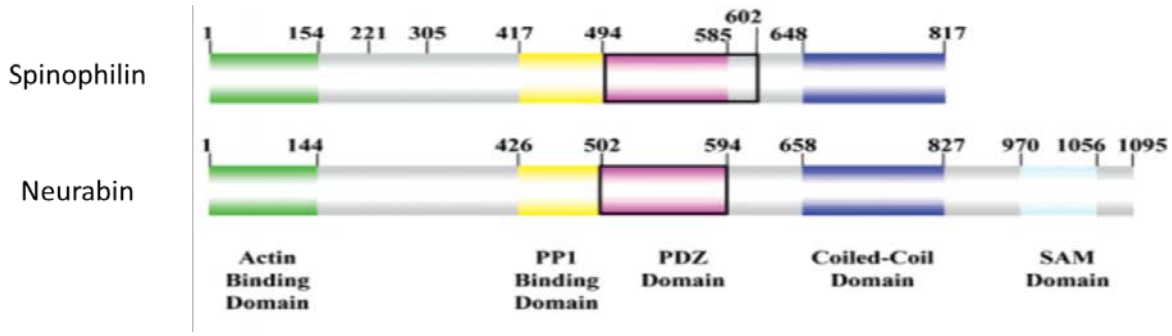



Figure 26 Neurabin and spinophilin domain structure (Kelker, 2006)

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CLUSTAL O(1.2.4) multiple sequence alignment


sp|Q9ULJ8-4|NEB1_HUMAN      MLKTESSGERTTLRSASPHRNAYRTEFQALKSTFDKPKSDGEQKTKEGEGSQQSRGRKYG   60
sp|Q9ULJ8|NEB1_HUMAN        MLKTESSGERTTLRSASPHRNAYRTEFQALKSTFDKPKSDGEQKTKEGEGSQQSRGRKYG   60
                            ************************************************************

sp|Q9ULJ8-4|NEB1_HUMAN      SNVNRIKNLFMQMGMEPNENAAVIAKTRGKGGHSSPQRRMKPKEFLEKTDGSVVKLESSV   120
sp|Q9ULJ8|NEB1_HUMAN        SNVNRIKNLFMQMGMEPNENAAVIAKTRGKGGHSSPQRRMKPKEFLEKTDGSVVKLESSV   120
                            ************************************************************

sp|Q9ULJ8-4|NEB1_HUMAN      SERISRFDTMYDGPSYSKFTETRKMFERSVHESGQNNRYSPKKEKAGGSEPQDEWGGSKS   180
sp|Q9ULJ8|NEB1_HUMAN        SERISRFDTMYDGPSYSKFTETRKMFERSVHESGQNNRYSPKKEKAGGSEPQDEWGGSKS   180
                            ************************************************************

sp|Q9ULJ8-4|NEB1_HUMAN      NRGSTDSLDSLSSRTEAVSPTVSQLSAVFENTDSPSAIISEKAENNEYSVTGHYPLNLPS   240
sp|Q9ULJ8|NEB1_HUMAN        NRGSTDSLDSLSSRTEAVSPTVSQLSAVFENTDSPSAIISEKAENNEYSVTGHYPLNLPS   240
                            ************************************************************

sp|Q9ULJ8-4|NEB1_HUMAN      VTVTNLDTFGHLKDSNSWPPSNKRGVDTEDAHKSNATPVPEVASKSTSLASIPGEEIQQS   300
sp|Q9ULJ8|NEB1_HUMAN        VTVTNLDTFGHLKDSNSWPPSNKRGVDTEDAHKSNATPVPEVASKSTSLASIPGEEIQQS   300
                            ************************************************************

sp|Q9ULJ8-4|NEB1_HUMAN      KEPEDSTSNQQTPDSIDKDGPEEPCAESKAMPKSEIPSPQSQLLEDAEANLVGREAAKQQ   360
sp|Q9ULJ8|NEB1_HUMAN        KEPEDSTSNQQTPDSIDKDGPEEPCAESKAMPKSEIPSPQSQLLEDAEANLVGREAAKQQ   360
                            ************************************************************

sp|Q9ULJ8-4|NEB1_HUMAN      RKELAGGDFTSPDASASSCGKEVPEDSNNFDGSHVYMHSDYNVYRVRSRYNSDWGETGTE   420
sp|Q9ULJ8|NEB1_HUMAN        RKELAGGDFTSPDASASSCGKEVPEDSNNFDGSHVYMHSDYNVYRVRSRYNSDWGETGTE   420
                            ************************************************************

sp|Q9ULJ8-4|NEB1_HUMAN      QDEEEDSDENSYYQPDMEYSEIVGLPEEEEIPANRKIKFSSAPIKVFNTYSNEDYDRRND   480
sp|Q9ULJ8|NEB1_HUMAN        QDEEEDSDENSYYQPDMEYSEIVGLPEEEEIPANRKIKFSSAPIKVFNTYSNEDYDRRND   480
                            ************************************************************

sp|Q9ULJ8-4|NEB1_HUMAN      EVDPVAASAEYELEKRVEKLELFPVELEKDEDGLGISIIGMGVGADAGLEKLGIFVKTVT   540
sp|Q9ULJ8|NEB1_HUMAN        EVDPVAASAEYELEKRVEKLELFPVELEKDEDGLGISIIGMGVGADAGLEKLGIFVKTVT   540
                            ************************************************************

sp|Q9ULJ8-4|NEB1_HUMAN      EGGAAQRDGRIQVNDQIVEVDGISLVGVTQNFAATVLRNTKGNVRFVIGREKPGQVSEVA   600
sp|Q9ULJ8|NEB1_HUMAN        EGGAAQRDGRIQVNDQIVEVDGISLVGVTQNFAATVLRNTKGNVRFVIGREKPGQVSEVA   600
                            ************************************************************

sp|Q9ULJ8-4|NEB1_HUMAN      QLISQTLEQERRQRELLEQHYAQYDADDDETGEYATDEEEDEVGPVLPGSDMAIEVFELP   660
sp|Q9ULJ8|NEB1_HUMAN        QLISQTLEQERRQRELLEQHYAQYDADDDETGEYATDEEEDEVGPVLPGSDMAIEVFELP   660
                            ************************************************************
```

Figure 27A. AA 1-660 sequence alignment of canonical isoform of neurabin and isoform 4 used for experiments


Figure 28B AA 1-660 sequence alignment of canonical isoform of neurabin and isoform 4 used for experiments

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