Identification and Validation of Novel Spinophilin-associated Proteins in Rodent Striatum Using an Enhanced ex Vivo Shotgun Proteomics Approach


Spinophilin regulates excitatory postsynaptic function and morphology during development by virtue of its interactions with filamentous actin, protein phosphatase 1, and a plethora of additional signaling proteins. To provide insight into the roles of spinophilin in mature brain, we characterized the spinophilin interactome in subcellular fractions solubilized from adult rodent striatum by using a shotgun proteomics approach to identify proteins in spinophilin immune complexes. Initial analyses of samples generated using a mouse spinophilin antibody detected 23 proteins that were not present in an IgG control sample; however, 12 of these proteins were detected in complexes isolated from spinophilin knock-out tissue. A second screen using two different spinophilin antibodies and either knock-out or IgG controls identified a total of 125 proteins. The probability of each protein being specifically associated with spinophilin in each sample was calculated, and proteins were ranked according to a $\chi^2$ analysis of the probabilities from analyses of multiple samples. Spinophilin and the known associated proteins neurabin and multiple isoforms of protein phosphatase 1 were specifically detected. Multiple, novel, spinophilin-associated proteins (myosin Va, calcium/calmodulin-dependent protein kinase II, neurofilament light polypeptide, postsynaptic density 95, $\alpha$-actinin, and densin) were then shown to interact with GST fusion proteins containing fragments of spinophilin. Additional biochemical and transfected cell imaging studies showed that $\alpha$-actinin and densin directly interact with residues 151–300 and 446–817, respectively, of spinophilin. Taken together, we have developed a multi-antibody, shotgun proteomics approach to characterize protein interactomes in native tissues, delineating the importance of knock-out tissue controls and providing novel insights into the nature and function of the spinophilin interactome in mature striatum.

Genomic sequencing has revealed the full repertoire of ~20,000 proteins that can be expressed in most mammals. Innate biochemical or enzymatic activities of many proteins are critical to their function, but these activities are often modified by interactions with other proteins. Moreover, many proteins have no known catalytic activity and are thought to serve structural roles in assembling protein complexes, greatly increasing the efficiency and fidelity of intracellular processes. Thus, systematic definition of protein interactomes promises tremendous insight into biochemical mechanisms underlying the functions of many proteins.

A prime example of the importance of protein-protein interactions for modifying biological function is the postsynaptic density (PSD), an actin-rich organelle localized to neuronal dendritic spines that contains receptors, kinases, phosphatases, and scaffolding proteins (1, 2). Dynamic changes in enzymatic activities and protein-protein interactions underlie changes in the size and shape of both PSDs and dendritic spines as well as the modulation of PSD-targeted neurotransmitter receptors that are critical for synaptic plasticity, learning, and memory. Furthermore, dendritic spine morphology and number are altered in many neurological disorders, including Parkinson disease (PD), Angelman syndrome, and fragile X syndrome (3–7).

Spinophilin (neurabin II) is an F-actin- and protein phosphatase 1 (PP1)-binding protein with no known catalytic function.
(8–10). It is highly expressed in brain and is localized to dendritic spines and PSDs where it plays a key role targeting PP1 to regulate synaptic plasticity, learning, and memory (11–14). Spinophilin associates with its homolog neurabin, which is also a PP1- and F-actin-binding protein that regulates synaptic plasticity and dendrite morphology (14–16). The interaction between spinophilin and the γ1 isoform of PP1 is enhanced in an animal model of PD (17), perhaps contributing to the altered phosphorylation of synaptic proteins, such as CaMKII and glutamate receptor subunits observed following dopamine (DA) depletion (18–20). DA depletion also decreases the number of dendritic spines on striatal medium spiny neurons (4, 5). Spine density is regulated by dynamic changes in the F-actin cytoskeleton, and spinophilin regulates dendritic spine density during development (21). Indeed, candidate protein or generic protein-protein interaction screens have identified many additional spinophilin-associated proteins (SpAPs) that modulate F-actin dynamics and/or cell morphology (22–27; for a review, see Ref. 28), consistent with the idea that spinophilin is an archetypical scaffolding protein. However, these interactions have mostly been characterized in vitro and/or following protein overexpression in cultured cells, and the inter-relationship of these interactions in vivo is largely unknown. Although the spinophilin interactome appears to dictate the biological roles of spinophilin, the composition of these complexes in the mature brain is poorly understood.

Co-immunoprecipitation is commonly used to confirm the biological relevance of specific bivalent protein-protein interactions in native tissues that were initially identified using generic molecular approaches, such as yeast two-hybrid screening. Prior studies combined this approach with mass spectrometry-based proteomics methods to more broadly characterize the composition of mammalian signaling complexes and the PSD interactome, such as the signalosome associated with synaptic N-methyl-D-aspartate receptors (29) and complexes associated with other PSD-enriched proteins (30). In addition, proteomics methodologies were used to identify over 1100 protein components of the PSD (30). Indeed, the potential for shotgun proteomics studies to provide novel insights into protein function in the brain is increasingly recognized (31). Moreover, computational approaches are being developed to identify potential protein-protein interactions (32). However, validation of specific interactions among the very large data sets of candidates typically identified using these approaches can be daunting. In addition, most proteomics analyses have relied on a single antibody to the target protein of interest with, at best, an unrelated non-immune IgG as a negative control, necessitating the use of very high quality antibodies.

We developed a systematic shotgun proteomics approach to define protein interactomes in a native tissue context. We used this approach to characterize the composition of spinophilin complexes isolated from rodent striatum and confirmed the association of multiple, novel SpAPs. Furthermore, we extensively characterized the interaction of two additional SpAPs, α-actinin and densin, using biochemical and imaging techniques. Our studies directly illustrate the importance of appropriate subcellular fractionation conditions, using multiple antibodies to the protein of interest, and the underappreciated, critical role of analyzing parallel samples prepared from knock-out (KO) animals. Thus, our findings demonstrate a methodological framework with key controls that can be broadly applied to characterizing protein interactomes, in addition to providing novel insights into the role of spinophilin in controlling synaptic signaling.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The following antibodies were used as indicated: spinophilin: mouse monoclonal antibody (BD Biosciences 612166; epitope mapping between rat spinophilin residues 238–348), rabbit polyclonal spinophilin antibody (Millipore 06-852; epitope mapping between rat spinophilin residues 286–390); goat polyclonal antibody (Santa Cruz Biotechnology SC-14774; epitope mapping between residues 50–100 of rat spinophilin); CaMKII: goat polyclonal antibody (33), mouse monoclonal CaMKIα antibody (Affinity Bioreagents/Thermo Fisher Scientific MA1-048); and mouse monoclonal CaMKIIβ antibody (Zymed Laboratories Inc./Invitrogen 13-9800); pan-α-actinin, rabbit polyclonal antibody (Santa Cruz Biotechnology SC-15339); densin; goat polyclonal antibodies 450 and 650 (34) and rabbit polyclonal antibody B2N (34); thousand and one amino acid kinase 1 (TAO1); rabbit polyclonal antibody (Bethyl Laboratories A300-524A); TAO3: rabbit polyclonal antibody (Bethyl Laboratories A300-536A); Tiam1: rabbit polyclonal antibody (Santa Cruz Biotechnology SC-872); P70S6 kinase: rabbit polyclonal antibody (Santa Cruz Biotechnology SC-230); doublecortin: rabbit polyclonal antibody (Cell Signaling Technology 4604); RasGrf1: rabbit polyclonal antibody (Santa Cruz Biotechnology SC-224); PP1γ: sheep polyclonal antibody (35); PSD-95 (NeuroMab 75-028); tyrosine hydroxylase (ImmunoStar 22941); neuramin: mouse monoclonal antibody (BD Transduction Laboratories 611088); myosin Va: rabbit polyclonal antibody (Sigma M4812); neurofilament light polypeptide: rabbit (Cell Signaling Technology 2837) and mouse (Santa Cruz Biotechnology SC-58559) antibodies; Myc: mouse monoclonal antibody (Vanderbilt Monoclonal Antibody Core); HA: mouse monoclonal antibody (Vanderbilt Monoclonal Antibody Core); and rabbit polyclonal antibody (Santa Cruz Biotechnology SC-805); and fluorescent secondary antibodies: donkey anti-mouse Alexa Fluor 546, donkey anti-β-actin Alexa Fluor 488, and donkey anti-goat Alexa Fluor 633 (Molecular Probes).

**Tissue Preparation and Subcellular Fractionation**—Mice were decapitated without anesthesia, brains were removed, and neostriata (referred to as striata) were rapidly dissected, frozen on dry ice, and stored at −80 °C. Two whole frozen mouse striata (one from each hemisphere; 20 mg of total tissue) were pooled and homogenized in 2 ml of an isotonic (150 mM KCl, 50 mM Tris-HCl, 1 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μM pepstatin, and 1 μM microcystin) or low ionic strength (2 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μM pepstatin, and 1 μM microcystin) buffer with no detergent in a Teflon-glass Wheaton tissue grinder with motorized plunger and incubated at 4 °C for 30–60 min. Samples were adjusted to 0.4–1 mg/ml total protein and then centrifuged at 9,000 × g at 4 °C for 10 min. Supernatants (S1) were saved for immunoprecipitation. The pellet (P1) was resuspended in 1 ml of isotonic or low ionic...
strength buffer containing 0.5% Triton X-100 (v/v; Sigma) in a micro-
centrifuge tube, and samples were then adjusted to a final volume of
2 ml. Samples were incubated at 4 °C for 30–60 min and then
centrifuged at 9,000 × g for 10 min. Supernatants (S2) were saved for
immunoprecipitation, and the P2 pellets were resuspended in 2 ml of
isotonic or low ionic strength buffer containing 1% Triton X-100 and
1% sodium deoxycholate (w/v; MP Biomedicals) and sonicated. Fol-
lowing incubation at 4 °C for 30 min, samples were then centrifuged
at 9,000 × g for 10 min, and the supernatants (S3) were saved for
immunoprecipitation. The final pellet (P3) was resuspended in 2 ml of
HEK293 cells (see below) prepared directly in low ionic strength buffer,
containing 0.5–1.0% Triton X-100 were immunoprecipitated essen-
tially as described (17). Antibody concentrations used were as fol-
lows: goat spinophilin, 4–8 μg/ml; rabbit α-actinin, 12 μg/ml; and appro-
imate non-immune IgG control of a similar concentration.
Co-immunoprecipitations—Solubilized extracts from 1) mouse stri-
atum prepared for fractionation studies (S1, S2, or S3) in isotonic or low
ionic strength buffer, 2) mouse whole brain homogenized in isotonic
(1% Triton X-100 and 1% sodium deoxycholate) or low ionic strength
(1% Triton X-100) buffer, or 3) rat or mouse striata or transected HEK293
cells (see below) prepared directly in low ionic strength buffer
containing 0.5–1.0% Triton X-100 were immunoprecipitated essen-
tially as described (17). Antibody concentrations used were as fol-
lows: goat spinophilin, 4–8 μg; rabbit spinophilin, 7.5–30 μg; mouse
spinophilin, 2.5–15 μg; rabbit α-actinin, 12 μg; and appropriate non-
immune IgG control of a similar concentration.
Western Blotting—Western blotting was done as described previ-
ously (18).

Proteomics and Mass Spectrometry Methods—Immune
complexes were fractionated by SDS-PAGE (10% acrylamide) and
stained with colloidal Coomassie Blue G-250 (Invitrogen). Each gel
was loaded with only one sample (and molecular weight markers) to
limit cross-contamination. Entire gel lanes, with or without prominent
IgG bands, were excised in two to three molecular weight ranges,
finely chopped, reduced with dithiothreitol, alkylated with iodoacet-
amide, and digested with trypsin. Tryptic peptides were resolved
using a reverse phase packed capillary tip (100 μm × 11 cm) packed with C18 resin (Jupiter C18, 5 μm, 300 Å; Phenomenex, Torrance, CA)
and a precolumn (100 μm × 6 cm) with the same resin using a frit
generated from liquid silicate Kasil 1 (36) essentially as described
previously (37). Mobile phase A was 0.1% formic acid; mobile phase
B was acetonitrile with 0.1% formic acid. The peptides were eluted
from the column using a 95-min protocol, including a 15-min wash
time (period 100% A for the first 10 min followed by a gradient of 98% A
at 15 min) at 1 µl/min to allow for solid phase extraction and removal
of residual salts followed by a 35-min linear gradient from 98% A to
75% A at 0.7 µl/min, then a steeper gradient to 10% A at 65 min,
and an isocratic phase at 10% A to 75 min. Fractions were analyzed by
tandem mass spectrometry using a ThermoFinnigan LTQ ion trap
mass spectrometer equipped with a Thermo MicroAS autosampler and
Thermo Surveyor HPLC pump, Nanospray source, and Xcalibur
2.0 instrument control software. MS/MS spectra of the peptides were
obtained using data-dependent scanning in which one full MS spec-
tra was followed by three MS/MS spectra.

The mscanvert tool from ProteoWizard (38) exported mass spectra
from the mzML v1.1 format. Intensity and m/z values were reported to
32-bit precision, and both MS and MS/MS scans were exported in
centroid format. MyrImatch 1.6.0 (39) identified peptides correspond-
ing to the MS/MS scans. Each scan was assessed as a singly charged
precursor if 90% of fragment intensity fell below the precursor m/z;
otherwise, the scans were identified under both doubly charged and
triply charged precursor assumptions. The sequence database for
immunoprecipitations in rat tissue included the IPI rat database (v3.66
with 39,677 sequences), the IPI mouse database (v3.66 with 56,791
sequences), and a database of 71 common contaminant sequences,
including five proteases, 42 Ig constant regions, 14 human keratins,
and 10 proteins from wool, cotton, and saliva. The sequence data-
base for immunoprecipitations in mouse tissue included all but the IPI
rat sequences. In both cases, all sequences were present in both
normal and reversed orientations to enable estimation of false dis-
covery rates. Each of the following modifications was allowed to be
present or absent on the corresponding residues: carbamidomethyl
Cys (+57), oxidized Met (+16), and pyro-Glu from N-terminal Gin
(+17). Only one end of each peptide was required to match an
expected trypsin cleavage site, and missed cleavages were not
counted. Precursor ions were required to fall within 1.25 m/z of
peptide average mass, whereas fragments were expected within 0.5
m/z of expected monoisotopic masses.

The IDPicker algorithm (version 2.5.0) filtered raw identifications,
conducted protein assembly, and produced tables of spectrum counts
for each experiment (40, 41). Immunoprecipitations from rat

tissue were assembled separately from those in mouse tissue. In all
cases, a peptide-spectrum match false discovery rate of 5% was
applied as a limit (estimated by doubling the numbers of reversed
matches and dividing by the total number of matches). Two distinct
peptide sequences were required of each protein for reporting. Par-
simony rules were in effect, grouping together indistinguishable se-
quences and reporting a minimal list of proteins. For spectral counting
(see below), peptides that match multiple proteins are included for
each protein they match. The original reports from IDPicker were
included in the supplemental material, and all data are publicly avail-
able. In all cases, immunoglobulins, keratin, and trypsin were ex-
cluded as valid interacting proteins.

Statistical Analysis and Ranking of Peptide Data—Spectral counts
for each protein from each antibody immunoprecipitation were used to
generate the probability that a more extreme distribution of identified
spectra between control (either IgG or KO) and experimental (wild type
tissue with spinophilin antibody) would be observed solely by chance.
For each antibody, Fisher’s exact test (42) computed a
p value based on the number of spectra matched to the protein in WT,
the number of spectra matched to the protein in KO (or IgG) controls,
the number of spectra not matched to the protein in WT, and the number of spectra
not matched to the protein in KO (or IgG) controls. These p values were
used to rank proteins identified searching both the rat and mouse IPI
databases that were detected using a mouse spinophilin antibody
and rat tissue (supplemental File RatTissueMouseAntibody.xls). Samples
immunoprecipitated from mouse striatum using the mouse, goat, or
rabbit (supplemental Tables S1, S2 and S3 respectively) antibodies
were searched with the mouse IPI database, and p values from
the individual immunoprecipitations are listed (supplemental File
MouseTissueAllAntibodies.xls).

Fisher’s method then combined the p values (from all five experi-
ments performed in mouse tissue) for the three antibodies into a χ²
degree of freedom, 4). A second set of calculations was
performed using only the rabbit and goat antibodies to generate a
χ²
degree of freedom, 3), and both values are given in
supplemental File MouseTissueAllAntibodies.xls. Only proteins
matching two or more peptides from a single immunoprecipitation are
listed in any of the tables. All computations were conducted in the R
statistical environment (43). Proteins containing more spectra in each
individual experimental immunoprecipitation compared with the cor-
responding control immunoprecipitation were placed into supple-
mental Table S2 and ranked by their
statistical environment (43). Proteins containing more spectra in each
individual experimental immunoprecipitation compared with the cor-
responding control immunoprecipitation were placed into supple-
mental Table S2 and ranked by their
tion compared with the experimental condition were placed into supplemental Table S3 and sorted by alphabetical order.

**Molecular Biology Constructs**—The Myc-tagged spinophilin construct containing cDNAs encoding the full-length rat protein in a pCMV4-myc vector was described previously (9), mCherry (-C1) vector was a kind gift from Dr. D. Piston (Vanderbilt University). A full-length GFP-densin construct (densin-FL–1–1542 Δ1291–1337), a C-terminal truncated GFP-densin construct missing the PDZ domain (ΔPDZ; Δ1291–1337 Δ1452–1542), a GFP-densin variant missing a larger C-terminal domain (densin-D43; Δ1291–1542), a GFP-densin variant that is missing a large internal fragment (densin-D23; Δ447–1487), a GST-densin construct (residues 1247–1542 Δ1291–1337; CTΔ), a GST-densin construct (residues 1247–1451 Δ1291–1337; MPD), and a His6-densin construct (residues 1247–1542; CTΔ) (see Fig. 4A) were described previously (34, 44). Densin-ΔPDZ and densin-D43 were inserted into pEGFPN3 vector between BglII and SacII sites. GST-spinophilin constructs in pGEX-2T or pGEX-4T were created essentially as described previously (9, 45). The spinophilin 1–300 construct forward primer was created with a BamHI restriction site (CGGGATCCCATGATGAAGACGGCCTC). The reverse primer was created with an EcoRI restriction site (CGGAATTCTTACACCGGTTTGATCTTGCGTAC). The α-actinin-2 cDNA (human) was a generous gift from Dr. Alan Beggs (Harvard University). The α-actinin-1 cDNA (human) was made by Dr. Carol Otey (46) and obtained from Dr. Donna Webb (Vanderbilt University). Full-length cDNAs were amplified by PCR using oligonucleotide primers containing EcoRI (5′) and Xhol (3′) and Smal (5′ and 3′) restriction enzyme sites for α-actinin-1 and α-actinin-2, respectively. PCR products were ligated into pGEX-4T-1 (Amersham Biosciences) with either purified His-tagged proteins or unpurified bacterial, over-

**RESULTS**

Quantification of Blots—For semiquantitative analysis of chemiluminescence, Western blots X-ray films exposed in a linear range were scanned using a flatbed scanner (Epson). Densitometric analyses were performed using NIH Image J. To quantify co-immunoprecipitations from control and DA-depleted striatum, immunoreactivity in the DA-depleted pellet was divided by immunoreactivity in the control pellet, and -fold changes in co-precipitating proteins were compared to 1

**Ex Vivo Spinophilin Complex Shotgun Proteomics**

-lysine for 5 min and subsequently washed five times in double distilled H2O and/or serum-containing DMEM. Cells were allowed to attach overnight and then transfected with DNA (2.5–3 μg) to express Myc-spinophilin, GFP-densin, and/or HA-α-actinin-2 using Lipofectamine LTX (Invitrogen) for 1–2 days. The medium was then removed, and cells were incubated in 1 ml of serum-free DMEM containing either 0.49% dimethyl sulfoxide (Pierce) or a differentiation medium (serum-free DMEM supplemented with 10 ng/ml fibroblast growth factor (Promega), 240 μM isobutylmethylxanthine (Sigma), 20 μM 12-O-tetradecanoylphorbol-13-acetate (Sigma), 48.6 μM forskolin (Sigma), and 5 μM DA (Sigma) (47). Cells were incubated for 12–14 h, washed in PBS (Invitrogen), and then fixed for 15 min at 37 °C in PBS containing 4% sucrose (EM Science) and 4% parafomaldehyde (Electron Microscopy Sciences). Autofluorescence was quenched by addition of 0.1% NH4Cl (Sigma) in PBS, and cells were permeabilized in 0.1% Triton X-100 in PBS. After blocking in 10% normal donkey serum for 60 min, cells were incubated in a 1:500–1:1,000 dilution of the appropriate primary antibody in PBS containing 2% normal donkey serum (Jackson Immunoresearch Laboratories) overnight, washed three times in PBS containing 0.1% Triton X-100, and then incubated in an appropriate secondary antibody conjugated to an Alexa Fluor (1:1,000–1:2,000). Cells were washed three times in PBS containing 0.01% Triton X-100, mounted on slides using Aqua Poly/ Mount (Polysciences, Inc.), and imaged using an LSM 510 META inverted confocal microscope (Zeiss) essentially as described previously (11). Transfected cells were visualized next to non-transfected cells, and each image channel was linearly adjusted so that background fluorescence from non-transfected cells was not detected. Images within each experiment were collected with minimal adjustment of the gain and laser power settings on the microscope. Z-stack images were acquired, and three-dimensional projections were made using Zeiss Image Browser software. Images of optical slices near the bottom of 9–12 undifferentiated or differentiated cells in two to three transfections from each transfection condition were thresholded automatically (48). Intensity correlation analysis was then used to compare normalized pixel intensities in each color channel, calculating an intensity correlation quotient (ICQ). Random overlap or mixed staining (ICQ ∼ 0), dependent staining (0 < ICQ ≤ 0.5), and segregated staining (0 > ICQ ≥ −0.5) can be assessed using this analysis (49). Thresholding and ICQ analysis were done using NIH Image J (downloaded from Wright Cell Imaging Facility with relevant analysis tools). Additional optical slices collected 1 and 2 μm above the initial slice were analyzed separately, yielding similar results (not shown). ICQ scores were compared by analysis of variance followed by Tukey post hoc test.
Western blotted for multiple synaptic proteins, revealing a dramatic impact of ionic strength on the solubilization properties of multiple dendritic proteins (Fig. 1A).

**Effect of Solubilization Conditions on Composition of Spinophilin Complexes**—Variable solubilization of spinophilin and other proteins by altering ionic strength, detergent concentration, and detergent type likely reflects differential effects on various protein-protein interactions. Immune complexes were isolated from each solubilized fraction by immunoprecipitation using a goat spinophilin antibody or a control, non-immune IgG. Western blotting revealed that PP1γ1 and neurabin were specifically associated with spinophilin irrespective of extraction condition (Fig. 1B). Neurabin and PP1γ1 were predominantly associated with spinophilin in the S1 fraction obtained using a low ionic strength buffer, whereas in isotonic buffer, both were predominantly associated with spinophilin in Triton/deoxycholate-solubilized extracts (Fig. 1B). PP1γ1 and neurabin were detected in spinophilin immune complexes isolated from striatum of 2–4-month old rats (adult) or 19-day-old (P19; an age that has high spinophilin expression) mice. Interestingly, other known SpAPs tested were not detected in the spinophilin complex (Fig. 2 and data not shown). Therefore, we decided to perform a shotgun proteomics study to identify putative SpAPs that are present in these complexes.

**Characterization of Spinophilin Complexes in Adult Rat Striatum**—Initial experiments used rat striatum because it is a larger tissue source than mouse striatum. A monoclonal mouse spinophilin antibody recognized a single band of the expected size in tissue extracts and specifically detected spinophilin immunoprecipitated by a second rabbit spinophilin antibody (Fig. 3A). Coomassie Blue staining detected multiple proteins in the spinophilin immune complex, including a major protein band that migrated at the apparent molecular mass of spinophilin (~116 kDa), that were not detected in an IgG control complex (Fig. 3B). Entire Coomassie-stained gel lanes from IgG and spinophilin precipitations (excluding IgG bands) were excised, macerated, digested with trypsin, and analyzed using mass spectrometry. The rat and mouse IPI databases (see “Experimental Procedures”) were searched, and 39 total proteins were detected by the presence of two or more peptides with 23 proteins specifically detected in spinophilin immunoprecipitates and 16 detected in both the spinophilin precipitates and the IgG control. Although neurabin and two PP1 isoforms (γ1 and α) were among the specific proteins detected, confirming our immunoblotting data, other known SpAPs were not detected (supplemental Table S1).

Among the more prominent proteins detected in the rat spinophilin complex (eight peptides) but not in the IgG control was TAO1, a microtubule affinity-regulating kinase kinase (also called MARKK) (50, 51). We confirmed the presence of...
TAO1 in spinophilin immune complexes isolated from rat striatum, hippocampus, and cortex by immunoblotting, whereas TAO3, a closely related family member, was not detected (Fig. 3C). Striatal dopamine depletion had no effect on TAO1 expression in rat striatum (Fig. 3D), but it significantly reduced the association of TAO1 with spinophilin immune complexes to 49 ± 4% of control levels (Fig. 3E). Probing TAO1 immune complexes using the mouse spinophilin antibody detected a protein that appeared slightly larger than the major spinophilin band detected in whole extracts (Fig. 3E). Dopamine depletion reduced the amount of co-precipitation of this protein with TAO1 to 59 ± 22% of control levels (Fig. 3E). However, TAO1 was not detected in immune complexes isolated using goat or rabbit spinophilin antibodies either by immunoblotting (not shown) or by proteomics analysis (supplemental Table S2). To further test the validity of this interaction, spinophilin was immunoprecipitated from striatum of WT and spinophilin KO mice (21). Spinophilin and PP1 as detected by Western blot are unchanged in 6-OHDA-lesioned rats. The ratios of lesion/intact from normalized input values (the mean ± standard error of the mean (error bars)) are compared with a theoretical value of 1 using column statistics. F, use of spinophilin KO tissue as a control (Fig. 4F), implying that it is a false positive and illustrating the value of using a KO tissue control.

Identification of Novel SpAPs Using Multiple Antibodies and KO Tissue Control—We then enhanced our proteomics strategy to identify proteins in immune complexes isolated using three different spinophilin antibodies, exploiting the availability of spinophilin KO tissue as a control (Fig. 4A). Each antibody detected an ~116-kDa protein in extracts from WT, but not KO, tissue with variable detection of nonspecific proteins (Fig. 3, B–D). Moreover, each antibody specifically immunoprecipitated a major ~116-kDa protein band as detected by Coomassie Blue staining (Fig. 3, E–G) but also immunoprecipitated a variable number of additional proteins in both WT and KO control extracts. Gel lanes containing spinophilin immune complexes isolated from both WT and KO tissue using all three antibodies were excised for proteomics analysis. Data from these analyses, as well as data obtained by analyzing independently isolated spinophilin complexes with an IgG control, were combined, used to search the mouse IPI database using the MyriMatch algorithm, and filtered to a
confident and minimal list of proteins using the IDPicker algorithm (see “Experimental Procedures”). We first compared the rat proteins identified using the mouse antibody and an IgG control (see above) to proteins identified in WT and KO immunoprecipitations using the same antibody. Notably, 12 of the 23 proteins specifically detected in rat spinophilin complexes using an IgG control were also present in immune complexes isolated from both WT and KO mouse striata (supplemental Table S1, shaded proteins), including TAO1, inositol trisphosphate receptor, and various subunits of the eukaryotic translation initiation factor 3. Approximately 50% of proteins identified as specific on the basis of an IgG control were eliminated by using a KO tissue control, demonstrating that KO tissue is critical for reducing false positives.

Because the mouse antibody generated numerous false positives and also only weakly detected spinophilin in mouse striatal extracts (data not shown), we next evaluated the proteins identified in complexes isolated from mouse striatum using goat or rabbit spinophilin antibodies with either an IgG or KO tissue control. Supplemental Tables S2 and S3 list the accession numbers, the total number of spectral counts, and unique (isoform-specific) peptide matches along with p values (see “Experimental Procedures”). Furthermore, the p values for proteins detected in a second, independent screen with both antibodies using an IgG control are given. All of these p values were then used to calculate χ² values, providing an estimate of the probability that the identification of each protein is specific (see supplemental File MouseTissueAllAntibodies.xls for a list of all proteins detected along with probability values). Proteins with more peptide matches in WT compared with KO are arranged in supplemental Table S2 based on the overall probability (χ² value) that there are more peptides in the experimental compared with the control condition. Proteins with equal or more spectral counts in the controls (KO or IgG) are given in supplemental Table S3 and are arranged alphabetically.

One advantage of IDPicker is that proteins with overlapping peptide matches are clustered. For example, multiple isoforms of protein phosphatase 1 were immunoprecipitated by all three antibodies. A total of six peptides across the three antibodies and two conditions (IgG control and KO control) matched to all isoforms (α, β, and the γ1/2 splice variants). However, one additional peptide matched PP1γ1γ2 and PP1α, five additional peptides uniquely matched PP1α, three additional peptides uniquely matched PP1γ1γ2, and one additional peptide uniquely matched PP1β. Superscript letters after the accession number indicate proteins placed in the same cluster by IDPicker because multiple proteins match to one or more peptides, but unique peptides confirm that both proteins are present in the sample. In contrast, asterisks with or without footnotes indicate that multiple protein accession numbers match to all of the peptides detected (e.g. PP1γ1 and PP1γ2). The first IPI accession number from the IDPicker analysis is listed with additional matching IPI accession numbers listed in the footnote.

![Figure 5: Validation of putative novel SpAPs using GST-spinophilin co-sedimentation assays.](image)

**Initial Validation of Novel SpAPs**—Our proteomics approach identified proteins in complex with spinophilin using an antibody-based approach. To test the validity of some of these hits, we used an independent, complementary, recombinant fusion protein-based approach. Solubilized extracts of mouse striatum were incubated with either GST alone or a set of three GST-spinophilin (GST-Sp) fusion proteins containing overlapping fragments spanning the entire amino acid sequence of the protein (Fig. 5A). Fusion proteins were isolated using glutathione-agarose, and the bound proteins were screened by Western blotting for proteins identified in the proteomics studies. We focused on several of the highest confidence protein hits based in part on the χ² analysis (listed along with their isoforms in Table I). The rank based on the χ² analysis is listed in parentheses after the protein.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; protein number 43) was used as a negative control. GAPDH is a highly abundant, soluble protein commonly utilized as a loading control. Initial studies detected one and two total spectra matching GAPDH in spinophilin immunoprecipitates using goat and rabbit antibodies, respectively, with no peptide matches in the IgG control. Moreover, GAPDH matched five and four spectra in the goat and rabbit antibody immune complexes, respectively, from WT tissue with two and one peptides detected in corresponding complexes isolated from KO tissue (p = 0.27 and 0.38, respectively). These statistical analyses, the low number of total spectral matches, and the high abundance of GAPDH suggest that it may be a nonspecific interaction. Consistent with this possibility, we were unable to detect binding of GAPDH to any of the GST-Sp fusion proteins.
CaMKII isoforms are encoded by four genes (α, β, γ, and δ), which can be alternatively spliced and form mixed isoform dodecameric complexes. CaMKIIα is ~3-fold more abundant in the forebrain than CaMKIIβ (52), and these two isoforms together comprise ~0.7% of total striatal protein (53). CaMKII plays a key role in regulating multiple dendritic processes that are essential for normal synaptic plasticity (54). Initial studies matched four and eight total spectra in spinophilin immune complexes isolated using the goat and rabbit antibodies, respectively, to CaMKIIα with one and two total matching spectra in the IgG control. Furthermore, CaMKIIα (protein number 3) matched five and seven unique (28 and 22 total) peptides in spinophilin immune complexes from WT striatum when using the goat and rabbit antibodies, respectively; whereas zero and one peptides (two and two total spectra) were detected in complexes isolated from
Ex Vivo Spinophilin Complex Shotgun Proteomics

KO striatum ($p = 2.18e^{-07}$ and $1.15e^{-04}$, respectively). CaMKIIa (protein number 13) matched seven and four unique peptides (26 and 14 spectra) in spinophilin immune complexes from WT striatum when using the goat and rabbit antibodies, respectively, whereas six and two peptides (eight and two total spectra) were detected in complexes isolated from KO striatum ($p = 9.02e^{-04}$ and $9.59e^{-04}$, respectively). Both CaMKIIa and CaMKIIβ associated with the GST-Sp(817) fusion protein, which contains a C-terminal fragment of spinophilin, but not with GST alone (Fig. 5B). However, both CaMKII isoforms also associated somewhat more weakly with the GST-Sp(300) and GST-Sp(484) proteins. Thus, striatal CaMKII appears to specifically associate either directly or indirectly with multiple domains in spinophilin.

Myosin Va (protein number 4) is a PSD-enriched motor protein (55, 56). It was initially identified in our rabbit spinophilin proteomics (eight matching spectra) using IgG as a control (zero matching peptides) and confirmed in both the rabbit and goat immunoprecipitations using WT (31 and 20 spectral counts, respectively) and KO (five and one spectral counts, respectively) tissue ($p = 6.87e^{-05}$ and $5.95e^{-06}$, respectively). Myosin Va strongly associated with both GST-Sp(300) and GST-Sp(484) with no detectable binding to GST-Sp(817) or GST alone (Fig. 5B). Taken together, these data suggest that myosin Va directly or indirectly associates with N-terminal domains in spinophilin.

Multiple intermediate filament proteins were detected in our screen, including NF-L (protein number 14), neurofilament medium polypeptide (protein number 28), and α-internexin (protein number 16). NF-L also has been shown to bind PP1 (57). We detected four and 19 total spectra matching to NF-L in spinophilin immune complexes isolated using the goat and rabbit antibodies, respectively, with no spectra detected in the KO controls ($p = 0.054$ and $1.63e^{-05}$, respectively). NF-L selectively associated with GST-Sp(300), with much reduced binding to GST-Sp(484) and GST-Sp(817), and with no detectable binding to GST alone (Fig. 5B). Taken together, these data suggest that NF-L directly or indirectly associates with N-terminal domains in spinophilin.

PSD-95 (protein number 36) is an abundant scaffolding protein that is commonly used as a PSD marker. We specifi-

### Table I—continued

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<th>#</th>
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<th>p-value (KO)</th>
<th>p-value (IgG)</th>
<th>Unique peptides</th>
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<th>p-value (KO)</th>
<th>p-value (IgG)</th>
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1 Calcium/calmodulin-dependent protein kinase type II β (IPI00649778); 68-kDa protein (IPI00875723).
2212-kDa protein (IPI00928007); myosin Va (IPI00928350).
3 Myosin-10 (IPI00515398); cDNA, RIKEN full-length enriched library, clone MSC1080G13, product: myosin heavy chain 10, non-muscle, full insert sequence (IPI00757312).
4 Protein phosphatase 2 catalytic subunit (IPI00227773).
5 Isoform 4 (IPI00406790); isoform 2 (IPI475044); isoform 3 (IPI00828919); adult male olfactory brain cDNA, RIKEN full-length enriched library, clone 643057P05, product: calcium/calmodulin-dependent protein kinase II, δ, full insert sequence (IPI00857865.1); calcium/calmodulin-dependent protein kinase type II δ isoform 1 (IPI00858128); 55-kDa protein (IPI00858144).
6 Isoform 2 (IPI00228044); isoform 3 (IPI00228045).
7 Actin, cytoplasmic 2 (IPI00874482).
8 Isoform α CaMKII of calcium/calmodulin-dependent protein type II α chain (IPI00621806).
9 Actin, α cardiac muscle 1 (IPI0114593); actin, aortic smooth muscle (IPI0117043); actin, γ enteric smooth muscle (IPI00404804); 14-, 17-day embryo heart cDNA, RIKEN full-length enriched library, clone 3200002L22, product: actin, α, cardiac, full insert sequence (IPI00480406); 17-day embryo heart cDNA, RIKEN full-length enriched library, clone I20058J24, product: actin, α, cardiac, full insert sequence (IPI00653007); 17-day embryo heart cDNA, RIKEN full-length enriched library, clone I20072D16, product: actin, α, cardiac, full insert sequence (IPI00654242).
10 Isoform 1 (IPI00622720.1); isoform 3 (IPI00626797).
11 Leucine-rich repeat-containing 7 (IPI00875695).
matched seven and six spectra in complexes isolated using the goat and rabbit antibodies, respectively, with only one peptide being detected in the KO (Table I). Moreover, unidentified α-actinin isoforms were present in complexes isolated from striatal extracts using GST-Sp(300) and GST-Sp(484) but not using GST-Sp(817) or GST alone (Fig. 5B).

We then further characterized the interaction of α-actinin with spinophilin using additional biochemical approaches. Spinophilin was specifically detected in α-actinin immune complexes isolated from mouse striatum (Fig. 6B). Moreover, spinophilin in a detergent-soluble forebrain extract associated with purified GST-tagged full-length α-actinin-1 or α-actinin-2 but not with GST alone (Fig. 6C). Deletion of the N-terminal actin binding domain (residues 1–254) from GST-α-actinin-2 (ΔABD) substantially reduced the interaction with spinophilin in a brain extract (Fig. 6D). GST-Sp(300) and GST-Sp(484), but not GST-Sp(817), bound HA-α-actinin-2 from extracts of transfected HEK293 cells (Fig. 6E). Furthermore, GST-Sp(300) and GST-Sp(151–607), but not GST-Sp(286–390) or GST-Sp(427–470), bound to an α-actinin-2 mutant lacking the spectrin repeats (Fig. 6F). Given that both spinophilin and α-actinin bind to actin, we used purified proteins to determine whether spinophilin directly interacts with α-actinin-2. A full-length His-α-actinin-2 protein bound to both GST-Sp(300) and GST-Sp(484) but not to GST-Sp(817) or GST alone (Fig. 6G). In combination, these data suggest that an N-terminal domain of α-actinin-2 directly interacts with residues 151–300 of spinophilin.

**Probing Depth of Proteomics Data: Validation of Densin as SpAP—**A protein that is selectively detected with a low number of peptides, although a lower confidence hit, may still be a valid interacting protein. Densin (protein number 73) matched two peptides in the spinophilin immunoprecipitates using the goat spinophilin antibody and one peptide using the rabbit spinophilin antibody when KO tissue was used as a control (p = 0.233 and 1, respectively). Densin is an interesting candidate because it is a scaffolding protein (Fig. 7A) that can bind Shank1 and Shank3/ProSAP2 (64), which in turn interact with multiple proteins that regulate dendritic functions (for a review, see Ref. 65). Notably, multiple densin- or Shank-binding proteins also were detected in our SpAP screens, such as α-actinin, CaMKII, homer, and ProSAPip1 (66–70) (supplemental Table S2). Moreover, previous studies implicated densin in the modulation of cell-cell contacts and dendritic morphology (64, 65, 71). Thus, densin was selected for further study, allowing us to also gain insight into the useful depth of our proteomics data.

As a first step toward validating densin as an SpAP, manual inspection of one of the MS/MS spectra identified an intense y-5 ion peak corresponding to fragmentation N-terminal to a proline residue in the tryptic peptide (Fig. 7B), a predicted cleavage for this peptide sequence (72). We then confirmed by Western blotting that densin was present in spinophilin immune complexes isolated from striatal extracts (Fig. 7C). In
addition, densin in a striatal extract selectively interacted with GST-Sp(817) over GST-Sp(300), GST-Sp(484), or GST alone (Fig. 5B). Experiments using lysates of HEK293 cells expressing densin and/or spinophilin showed that densin was specifically detected in spinophilin immune complexes and that spinophilin was specifically detected in densin immune complexes (Fig. 7D). GST-densin fusion proteins containing the C-terminal domain (densin-CTA and densin-MPD) bound to full-length Myc-tagged spinophilin expressed in HEK cells (Fig. 7E). In addition, GST-Sp(817), but not GST alone or GST-Sp(484), strongly associated with GFP-tagged densin-FL expressed in HEK293 cells (Fig. 7F). The C-terminal truncation of densin to remove only the PDZ domain (densin-ΔPDZ) or both the PDZ and CaMII binding domains in a natural splice variant (densin-D43) (34) had little effect on binding to GST-Sp(817) (Fig. 7G). In contrast, we could not detect binding of a natural densin splice variant lacking a large central domain (densin-D23) to GST-Sp(817) (Fig. 7G). GST-Sp(817), but not GST alone or GST-Sp(300), was able to directly bind to a His-tagged C-terminal domain fragment of densin (densin-CTD) in vitro (Fig. 7H). Thus, the combined data show that the C-terminal domain of spinophilin interacts directly with a C-terminal domain of densin in vitro and in intact cells, demonstrating the useful depth of our proteomics data.

**Co-localization of Spinophilin, α-Actinin, and Densin**—We next compared the localization of spinophilin, α-actinin, and densin in a relevant cell system, STHdh+/Hdh− striatal cells, which can be induced to initiate a neuronal differentiation program, including extension of processes containing the dendritic marker MAP-2 (47). Using confocal microscopy, spinophilin predominantly localized to cortical regions of undifferentiated cells in contrast to diffuse cytosolic staining of the co-expressed mCherry marker (Fig. 8A). The differentiation mixture (see “Experimental Procedures”) induced formation of multiple processes containing GFP-spinophilin, which was often concentrated in the tips of the processes, whereas the mCherry marker was more diffusely localized in the processes and throughout the cell body (Fig. 8A and supplemental Fig. S1). Myc-tagged spinophilin partially co-localized with either HA-α-actinin-2 (Fig. 8B and supplemental Fig. S2) or full-length GFP-densin (Fig. 8C and supplemental Fig. S3) in both undifferentiated and differentiated cells. Using the image correlation analysis method of Li et al. (49) to quantify co-localization (Fig. 8D), there was little or no co-localization of spinophilin with mCherry in undifferentiated or differentiated cells (ICQs of 0.02 ± 0.004 and 0.003 ± 0.010, respectively). In contrast, α-actinin-2 and densin exhibited significantly more co-localization than mCherry with spinophilin in undifferentiated (ICQs of 0.15 ± 0.02 and 0.09 ± 0.01, respectively) and differentiated (ICQs of 0.13 ± 0.02 and 0.09 ± 0.02, respectively) cells. Co-localization of densin with spinophilin appeared modestly weaker than that of α-actinin-2 with spinophilin (p < 0.05 in undifferentiated cells only).

The STHdh+/Hdh− cells also were transfected to co-express Myc-spinophilin, HA-α-actinin-2, and GFP-densin-FL. Protein localization was then compared in both undifferentiated and differentiated cells, revealing striking differences in the ratios of the three proteins in different subcellular domains (Fig. 8E). Additional images of triple transfected cells in
supplemental Fig. S4 show the variability in cell morphology with some triple transfected, undifferentiated cells having “neurite”-like outgrowths. In general, there were few areas in which spinophilin was not co-localized with at least one of the other proteins. However, the co-localization of all three proteins was relatively restricted (white arrows), and spinophilin co-localized with either densin or α-actinin-2 alone in other regions (cyan and magenta arrows, respectively). In contrast,
densin and α-actinin-2 did not co-localize in many regions of the cells (yellow arrows), instead adopting quite distinct localizations (green and red arrows, respectively). There was a broad overlap of spinophilin and α-actinin-2 throughout the processes of differentiated cells (magenta arrows).

DISCUSSION

The identification of protein-protein interactions is key to understanding the specificity and fidelity of many physiological processes as well as many diseases (see the Introduction). For example, protein kinases and phosphatases are often assembled into complexes by scaffolding proteins, providing exquisite control of the phosphorylation state of co-assembled proteins (73). Systematic efforts to map protein interactomes using established methods, such as yeast two-hybrid screens or fusion protein pulldowns, have yielded substantial advances. However, these approaches may miss interactions that require post-translational modifications or that are specific to a particular biological context and also demand substantial follow-up to identify the appropriate biological context for each novel interaction. The methodological approach described here (Fig. 9), although initially somewhat time-consuming, circumvents some of these limitations by facilitating direct identification of proteins in an interactome within a defined biological context (e.g. native tissue or disease state) and limits the identification of false positives.

Our approach optimally requires a KO animal lacking the protein of interest and multiple antibodies to the native protein ideally raised to distinct epitopes in different species. Systematic efforts are underway to knock out all genes in the mouse (74) and rat. In addition, both commercial (e.g. Santa Cruz Biotechnology) and non-commercial (e.g. NeuroMab) efforts are underway to create or collect antibodies to all of the proteins encoded in the human and mouse genomes. Moreover, it is becoming increasingly cost-effective to reliably generate custom antibodies to protein epitopes of interest. The use of multiple antibodies to different epitopes in the protein of interest with KO tissue controls means that high quality (i.e. specificity and titer) antibodies are not strictly required.

Solubilization of the protein of interest without disrupting interactions with potential partners is a crucial first step in characterizing an interactome. As shown here, the ionic strength and nature of the detergent can have profoundly different effects on the solubilization of different proteins. Our use of a low ionic strength buffer for the proteomics studies tends to destabilize F-actin and its associated proteins, allowing us to efficiently solubilize spinophilin with only Triton X-100, a relatively mild detergent, hopefully with minimal disruption of the spinophilin interactome. However, it may prove interesting to compare spinophilin interactomes extracted under different conditions.

The usefulness of KO tissue to validate interacting proteins from brain and to identify major co-precipitating proteins that can be detected by staining polyacrylamide gels has been demonstrated previously (75). Given the non-selective interactions identified in our initial shotgun proteomics screen of rat tissue using a non-immune IgG control, we expanded this approach to perform a shotgun proteomics analysis of complexes isolated from both WT and KO mouse tissue using three spinophilin antibodies and analyzing entire gel lanes (excluding the IgG heavy and light chains) to allow for identification of proteins that may be present below the detection limit for protein staining. Optimization of protocols to cross-link antibodies to protein G-Sepharose may allow for detection of additional proteins that co-migrate with IgGs using mass spectrometry-based proteomics.

The use of multiple antibodies provided additional dimensions to the proteomics data, identifying novel SpAPs that co-precipitate from native tissue with high fidelity. Although there is a legitimate bias to focus on SpAPs identified using more than one antibody, it is possible that antibodies isolate distinct subpopulations from the total repertoire of spinophilin complexes (e.g. binding of an SpAP may conceal certain epitopes). In addition, antibodies can vary in the efficiency of immunoprecipitation, perhaps affecting the sensitivity for de-
tection of weakly interacting proteins or low abundance complexes. Thus, even proteins identified in a single immune complex and with few peptide matches may be true interac-
tors. For instance, PSD-95 was detected only using the goat antibody. Furthermore, densin had two peptide matches with the goat antibody but only one peptide match when using the rabbit antibody. Given our two-peptide cutoff, this protein may have been missed if only the rabbit antibody was used.

Also, although NF-L was detected using both the rabbit and goat antibodies, it had more spectral counts from immunoprecipitations using the rabbit antibody (19 spectral counts) compared with the goat antibody (four spectral counts). Although these studies were not quantitative, the number of spectra (spectral counts) identified in proteomics screens is related to the abundance of the protein in the sample. Therefore, in an initial effort to rank protein hits, we statistically analyzed each set of proteomics data based on the enrichment of spectral counts in experimental versus control samples. The χ² values from multiple immunoprecipitations were then combined to generate a χ² value (see “Experimental Procedures”). This technique provided an overall ranking of confidence that each protein is a legitimate SpAP based on the multiple repetitions using different antibodies and controls. Future studies may benefit from merging our methodologies with evolving quantitative technologies that allow for characterization of proteomes using stable isotope labeling (76, 77).

Our co-immunoprecipitation methodology has the advantage of identifying direct or indirect interactions with spinophilin that are relevant in a specific biological context. Given concerns about the specificity of co-immunoprecipitations, especially for abundant proteins, we used an independent GST-spinophilin co-sedimentation approach to test the validity of some of the proteomics data, focusing on proteins that were highly ranked from the χ² value and also proteins with interesting known biological roles that were not as highly ranked. Because we used three overlapping GST-spinophilin fragments that spanned the entire protein, this approach also provided an initial indication about specific regions/domains on spinophilin that were important for the interaction. Associations of myosin Va (protein number 4), CaMKIIβ and CaMKIIα (protein number 3 and protein number 13), NF-L (protein number 14), PSD-95 (protein number 36), α-actinin (protein number 7 and protein number 21), and densin (protein number 73) with one or more GST-spinophilin fragments were detected by Western blot (Fig. 5B). We then further validated the interactions of spinophilin with α-actinin-2 and densin using a combination of reciprocal co-immunoprecipitations from native rodent striatum and transfected cells, in vitro interactions of purified proteins, and co-localization studies. Taken together, these findings demonstrate the robustness of our methodology and the useful depth of the resulting data.

Although our data show that spinophilin can bind independently to densin and α-actinin, previous studies have shown that the C-terminal PDZ domain of densin can interact directly with the C terminus of α-actinin (67). Both densin and α-actinin function as scaffolding proteins, independently assembling complexes of several other neuronal/synaptic proteins, some of which were selectively detected in our spinophilin immunoprecipitations (Fig. 9F). Spinophilin and α-actinin are known to bind to actin, but the interaction of spinophilin with α-actinin is independent of actin because GST-Sp(300) and GST-Sp(484) directly precipitated purified α-actinin in vitro. Notably, several neurofilament proteins were also detected in spinophilin immune complexes in our proteomics studies, including α-internexin (protein number 16), NF-L (protein number 14), and neurofilament medium polypeptide (protein number 28). Although NF-L was previously shown to interact with PP1 (57), it is unlikely that PP1 is bridging the interaction with spinophilin because we showed that NF-L associates with GST-Sp(300), a spinophilin fragment that does not bind PP1 (78). Spinophilin, α-actinin, densin, and α-internexin have all been independently implicated in regulating cellular morphology and/or synaptic function (21, 63–65, 71, 79). A number of the other candidate SpAPs identified in our proteomics screen have also been implicated in regulating dendrite and/or dendritic spine morphology, including CaMKII isoforms, homer, ProSAPiP, SynGap, and synaptopodin (64, 65, 80–86). Moreover, multiple myosin motor proteins, including myosin Va (protein number 4) and myo-
sin-10 (protein number 5), were detected, and we confirmed that myosin Va associates with N-terminal domains of spinophi-
lin (Fig. 5B). These myosins are PSD-enriched motor proteins that have been implicated in regulating dendritic morphology (55, 56, 87). To our knowledge, this is the first study linking these actin-based motor proteins to spinophilin.

Alterations in dendritic morphology and/or the number of dendritic spines have been linked to numerous neurological disorders, including PD, Angelman syndrome, and fragile X syndrome (3–7), and the interaction between spinophilin and PP1γ is altered in an animal model of PD (17). Our data suggest that a novel spinophilin complex(es) associated with neurofilaments and/or motor proteins may be important for physiological and pathological regulation of dendritic morphology and other dendritic processes. It will be important to further investigate the biochemical basis for assembly of these complexes and their biological roles.

In summary, we used an ex vivo, co-immunoprecipitation approach, coupled to shotgun proteomics using tandem mass spectrometry, to identify putative, novel SpAPs that are known regulators of dendritic morphology. Importantly, this work demonstrates a powerful method that can be applied to novel protein targets to rapidly and selectively identify novel interactomes. We focused on native tissue to identify protein–protein interactions that are contextually important (i.e., proteins that reside in the striatum). Yates and co-workers (31) recently discussed potential pitfalls that may occur in proteomics studies, and our data expand this discussion by demonstrating the benefit of using multiple antibodies and the
necessity for a KO tissue control to reduce wastage of time and resources chasing false positives. The rapidly increasing availability of antibodies and KO animals suggests that this approach could be systematically applied to gain insight into the biological roles of poorly characterized proteins.

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** This article contains supplemental Tables S1-S3, Figs. S1-S4, File RatTissueMouseAntibody.xls, and File MouseTissueAllAntibodies.xls.

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