Cellular/Molecular

NOS1AP Associates with Scribble and Regulates Dendritic Spine Development

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The formation and function of the neuronal synapse is dependent on the asymmetric distribution of proteins both presynaptically and postsynaptically. Recently, proteins important in establishing cellular polarity have been implicated in the synapse. We therefore performed a proteomic screen with known polarity proteins and identified novel complexes involved in synaptic function. Specifically, we show that the tumor suppressor protein, Scribble, associates with neuronal nitric oxide synthase (nNOS) adaptor protein (NOS1AP) [also known as C-terminal PDZ ligand of nNOS (CAPON)] and is found both presynaptically and postsynaptically. The Scribble–NOS1AP association is direct and is mediated through the phosphotyrosine-binding (PTB) domain of NOS1AP and the fourth PDZ domain of Scribble. Further, we show that Scribble bridges NOS1AP to a β-Pix [β-p21-activated kinase (PAK)-interacting exchange factor]/Git1 (G-protein-coupled receptor kinase-interacting protein)/PAK complex. The overexpression of NOS1AP leads to an increase in dendritic protrusions, in a fashion that depends on the NOS1AP PTB domain. Consistent with these observations, both full-length NOS1AP and the NOS1AP PTB domain influence Rac activity. Together these data suggest that NOS1AP plays an important role in the mammalian synapse.

Introduction

Polarity complexes are important for axonal specification, dendrite development, and synaptic function by regulating actin dynamics through Rho GTPases (Arimura and Kaibuchi, 2007; Barnes and Polleux, 2009). Recently, three conserved protein complexes have been identified to be important for establishing cellular polarity, the Crumbs complex [Crumbs/Pals-associated tight junction protein (Patj)/protein associated with Lin7 (Pals)], the Partitioning defective (Par) complex [Par3/Par6/atypical protein kinase C (aPKC)] and the Scribble (herein Scrib) complex [Scrib/lethal giant larvae (Lgl)/discs large (Dlg)] (Wiggin et al., 2005). Scrib, Lgl, and Dlg are tumor suppressors that interact genetically (Bilder et al., 2000). Recent work shows that Scrib and Lgl2 interact biochemically (Kallay et al., 2006) and that Scrib influences Dlg localization in migrating astrocytes (Osmani et al., 2006). As well, Scrib forms a complex with β-p21-activated kinase (PAK)-interacting exchange factor (β-Pix), G-protein-coupled receptor kinase-interacting protein (Git1), and PAK (Audebert et al., 2004; Nola et al., 2008), three proteins implicated along with Scrib in RhoGTPase signaling (Osmani et al., 2006; Zhan et al., 2008).

Scrib contains 16 N-terminal leucine-rich repeats (LRRs) followed by four PDZ domains common to many scaffolding proteins (Bilder, 2003). What is known to date about the function of Scrib in the synapse comes largely from studies on *Drosophila*. *Scrib* mutant flies show a redistribution of synaptic vesicles from the presynaptic zone, with more synaptic vesicles localized within the reserve pool and fewer found in the active zone (Roche et al., 2002). These synapses show defects in vesicle recycling and in several forms of plasticity, including loss of facilitation and post-tetanic potentiation (Roche et al., 2002). In the mammalian synapse, Scrib is thought to regulate presynaptic vesicle localization downstream of β-catenin (Sun et al., 2009). Further, dominant-negative mutations in either Scrib or the Scrib-associating protein, β-Pix, a RhoGEF for Rac and Cdc42 (Bagrodia et al., 1998; Manser et al., 1998), inhibit Ca^{2+}-dependent exocytosis (Audebert et al., 2004). Interestingly, the Scrib-associating proteins β-Pix, Git1, and PAK function together to regulate dendritic spine development through myosin II regulatory light chain (MLC) (Zhang et al., 2005); whether Scrib also functions postsynaptically within a β-Pix, Git1, and PAK complex remains to be tested.

Received July 30, 2009; revised Feb. 19, 2010; accepted March 1, 2010.

This work was supported by grants from Natural Sciences and Engineering Research Council of Canada (NSERC) (J.F., S.K., W.S.T.); the EJLB Foundation (J.F.); Canadian Institutes of Health Research (CIHR) (W.S.T., T.P., J.F.); and National Cancer Institute of Canada, Genome Canada through the Ontario Genomics Institute, and the Ontario Research Fund (T.P.). L.R. was supported by an NSERC Sir Alexander Graham Bell studentship. L.C. and M.O. were funded in part by the Cancer Research Training Program Dalhousie University. W.S.T. is a Canada Research Chair (CRC) in Molecular Cell Biology. T.P. is a Distinguished Scientist of the CIHR. J.F. is a CRC in Brain Repair. We are grateful to Dr. Thomas Südhof (University of Texas Southwestern Medical Center, Dallas, TX) for the liprin-α and ERC1b antibodies, Dr. Albert Reynolds (Vanderbilt University, Nashville, TN) for the ARVCF antibody, and Dr. Samie Jaffrey (Cornell University, New York, NY) for the NOS1AP cDNA. We thank Rick Horwitz (University of Virginia, Charlottesville, VA) for the Git1 cDNA and antibody and Kelly Pike for kindly providing the Scrib shRNA construct. We acknowledge Paul O'Donnell for help with the proteomic screen.

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DOI:10.1523/JNEUROSCI.3726-09.2010

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In an effort to better understand the role of polarity proteins in the nervous system, we conducted a mass spectrometry (MS) screen with known polarity proteins to characterize their interacting partners. We identified a novel interaction between Scrib and the neuronal nitric oxide synthase (nNOS) adaptor protein (NOS1AP) [also known as C-terminal PDZ ligand of nNOS (CAPON)], a phosphotyrosine-binding (PTB) domain-containing protein implicated in schizophrenia (Brzustowicz et al., 2004) and long QT syndrome (Eijgelsheim et al., 2009). We show Scrib functions as a scaffold to link NOS1AP with the β-Pix, Git1, and PAK proteins, and that NOS1AP influences Rac activation, leading to changes in dendritic spine morphologies, an event dependent on the PTB domain of NOS1AP. We speculate that the complex plays an important role in the synapse by regulating actin dynamics.

Materials and Methods

Mass spectrometry. For the targeted proteomic screen, a number of cDNAs for known polarity proteins were engineered to contain an N-terminal Flag-tag. We next generated stable cell lines in either BOSC or MDCK cell lines expressing either Flag-tagged Scrib, Lin7C, Lgl-1, AMOTL1, or AMOTL2 or Flag alone. Individual stable cell lines expressing Flag-constructs were grown to 70% confluence on eight 15 cm culture dishes. Cells were washed in PBS, and then lysed in NP40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP40) with 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM PMSF, 20 mg/ml RNase, 1 mM DTT, and 1 mM orthovanadate. Lysates were spun at 100,000 × *g*, and precleared with a 10% slurry (1 ml) of mouse agarose (Sigma) for 30 min at 4°C. The lysates were incubated with M2 agarose (Sigma) at 4°C for 2 h or overnight. Beads were washed three times in NP40 lysis buffer and resuspended in sample buffer. Endogenous Scrib or NOS1AP proteins were immunoprecipitated from homogenized rat brain lysate. Here, between two and five adult rat brains were homogenized in a Dounce homogenizer in NP40 lysis buffer (see above), the lysates were clarified at 100,000 × *g* for 1 h at 4°C, then precleared with 500 μl of a 10% protein A Sepharose mixture for 30 min at 4°C. Three microliters of either preimmune or immune antibody were added, and the samples were incubated overnight. One hundred microliters of a 10% protein A Sepharose slurry were added for 2 h at 4°C, and the pellets were washed three times and then resuspended in sample buffer. All samples were resolved on a 10% SDS-PAGE gel and stained with Colloidal Coomassie (Bio-Rad). Excised protein bands were digested according to the standard protocol and analyzed by liquid chromatography–mass spectrometry [HP 1100 HPLC System (Agilent) and either an LCQ-Deca or LTQ Mass Spectrometer (Thermo Fisher)]. The resulting MS/MS spectra were searched using the MASCOT program version 1.0 against Ensembl Databases (Human—Ensembl release 41, Dog—Ensembl release 42, Rat—Ensembl release 42). The results were further processed using the PeptideProphet and ProteinProphet (Nesvizhskii et al., 2003) algorithms. Hits that had a protein probability score >80% were retained. This score corresponds to an average estimated sensitivity of 73% and an error rate of 1.5% (for all immunoprecipitates analyzed, the ProteinProphet predicted sensitivity and error rates were averaged for the score of 80% protein probability). All reported hits are expressed as the human gene name for consistency, although Ensembl identifications are shown in supplemental Table 1 (available at www.jneurosci.org as supplemental material).

Constructs. See supplemental Table 2 (available at www.jneurosci.org as supplemental material).

Antibodies. The liprin-α and ERC1b antibodies were a kind gift from Dr. Thomas Südhof (University of Texas Southwestern Medical Center, Dallas, TX). The armadillo repeat gene deleted in velo-cardio-facial syndrome (ARVCF) antibody was a kind gift from Dr. Albert Reynolds (Vanderbilt University, Nashville, TN). The NOS1AP rabbit polyclonal antibody was raised against a GST-fusion protein encompassing the C terminus of rat NOS1AP 5′ primer (TTTCCGAATTCCATGGGCTCCCAG) and 3′ primer (TTTCGAGTCGACCTACACGGCGATCTC). The Scrib rabbit polyclonal antibody was generated against a GST fusion of the C-terminal region of Scrib 5′ primer (TTTCCAGGAATTCCCCTTCTGGGCAGGCCCTCACCCGGC) and 3′ primer (TTTAGAGTCGACTGGTACCCTCTAGGAGGGCACAGGGCCCAG). The PAK1 polyclonal antibody was purchased from Cell Signaling Technology. β-Pix and nNOS antibodies were from BD Transduction Laboratories. The myc and Git1 antibodies were from Santa Cruz Biotechnology. The GFP polyclonal antibody was purchased from Abcam. The Flag M2 was purchased from Sigma. The Rac1 monoclonal antibody was contained in the Cell Biolabs Rac Activation Assay Kit.

Northern blot analysis. Total RNA (10 μg) was isolated from rat tissues using the TRIzol reagent and separated on a 1% formaldehyde-agarose gel. RNA was then transferred to nylon membrane. To generate a NOS1APc-specific probe, we subcloned the NOS1APc-specific region of rat NOS1APc into the pSPORT vector using the following primers: 5′, TTTTCGAATTCTATGTTTGAGAATTG; 3′, GCGGCCGCGCTACTCAAAGGACAG. A sense strand was labeled with [^{32}P]-dCTP using the Prime-It RmT random primer labeling kit (Stratagene). Hybridization was performed overnight at 65°C, with PerfectHyb Plus Hybridization Buffer (Sigma). Three posthybridization washes were subsequently performed at 65°C, using 2× SSC containing 0.5% SDS. The blot was exposed to X-ray film in the presence of an intensifying screen for 3 d at −80°C to visualize the bands.

Mixing experiments. The GST fusion construct of the PTB domain was generated using the 5′ primer TTTTCGGGGCGCGCCATGCCCAGC and the 3′ primer TTTTTAATTAATTAGTCAATGTCGGT and cloned into a modified pGEX-4T2 vector (Smith et al., 2006). Each of the PDZ domains of Scrib were subcloned in frame with EcoR1 and Sal1 restriction sites into either pGEX-4T3 or pPROEX HTc. GST or His fusion proteins were generated and purified using standard protocols. For mixing experiments with brain lysate, 2.5 μg of purified GST, GST-PTB, or GST-PDZ1-4 was mixed with 5 μg of total rat brain lysate. For the direct mixing experiments 2.5 μg of purified GST or GST-PTB domain was mixed with 5 μg of purified Scrib HIS-PDZ4, followed by three washes in NP40 lysis buffer. Proteins were then separated by SDS-PAGE, transferred, and probed with the appropriate antibody or stained with Coomassie.

Cell culture and transfections. HEK293T cells were grown in DMEM (Wisent) with sodium pyruvate, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Wisent). Cells were transfected using a standard PEI protocol. To generate the stable Scrib knockdown cell line either a Scrib knockdown vector or control vector was transfected into HEK293T cells followed by puromycin (2 μg/ml) selection. Scrib knockdown was confirmed by probing equal amounts of cell lysate with our Scrib antibody.

Immunoprecipitation. Mouse brains were homogenized in a Dounce homogenizer in NP40 lysis buffer at 1/10 (w/v) and supplemented with protease inhibitors. Lysates were then centrifuged at 100,000 × *g* for 1 h. Cleared lysates were then incubated with different antibodies as outlined in the text, followed by a 1 h incubation with 100 μl of a 10% slurry of protein A Sepharose or protein G Sepharose. The immunoprecipitations (IPs) were then extensively washed before the addition of SDS-containing sample buffer. Cultured cells were washed twice in cold PBS and suspended in 1 ml of NP40 lysis buffer, cleared, and immunoprecipitated as outlined above.

Hippocampal neuron culture and transfection. Hippocampal neurons were isolated from E18 rat embryos as previously reported (Krueger et al., 2003). Dissociated hippocampal neurons were plated in 35 mm dishes containing 12 mm glass coverslips precoated in 0.05% (w/v) poly-L-lysine (Peptides International). Dissociated cells were plated at 40,000 cells per dish and grown in 2% B27 Neurobasal medium (Invitrogen) containing 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Wisent) for 13–15 d *in vitro* (DIV) and then transfected using a $CaPO_4$ method (Krueger et al., 2003) with cDNA constructs encoding yellow fluorescent protein (YFP), YFP-NOS1AP, YFP-NOS1AP PTB, or YFP-NOS1APΔPTB.

Immunofluorescence and image quantification. For cell staining, cells were washed twice in PBS before fixation in 4% paraformaldehyde (PFA) containing 4% sucrose. Cells were then stained as outlined by Krueger et al. (2003). For imaging dendritic protrusions, cells were fixed in 4% PFA

containing 4% sucrose 24 h after transfection and imaged on an inverted Leica CTR6000 with a Hamamatsu camera using a 63× oil objective. Three different neurites were used for each neuron counted with a minimum of 17 neurons counted in each condition that included at least three replicate experiments. For each neurite, the number of processes extending from a 50 μm segment was counted. Any process extending at least 0.1 μm from the neurite was counted. Both datasets were pooled, and a *t* test was used to assess significance between the different treatments.

Rac activation assay. The Rac activation assay was performed according to the manufacturer's protocol (Cell Biolabs). Briefly, HEK293T cells were transfected as above, left overnight, and then incubated in serum-free medium for an additional 24 h. The serum-free medium was then changed to serum-containing medium for 10 min. A positive control GTPγS was performed, and the pull-down of activated Rac using the PBD domain of PAK1 was also performed using the manufacturer's protocol.

Results

Scrib protein complexes identified in mass spectrometry screen

Protein–protein complexes play an important role in cellular polarity, including neuronal polarity and, more specifically, the development of the synapse. To better characterize the nature of these protein–protein complexes, we conducted a proteomic screen with a number of known polarity proteins. For results of the full screen, see supplemental Figure 1 and Table 1 (available at www.jneurosci.org as supplemental material).

In our initial screen using BOSC cells stably expressing Flag-tagged Scrib, in which Scrib was precipitated and associating complexes were identified by MS (see Materials and Methods), we noted that Scrib associated with a number of proteins implicated in the nervous system (supplemental Fig. 1, Table 1, available at www.jneurosci.org as supplemental material). After this initial analysis, we performed pull-down experiments using rat brain lysate followed by MS. Combining the results from these two screens (Fig. 1*A*; supplemental Fig. 1, Table 1, available at www.jneurosci.org as supplemental material), a number of known Scrib-binding partners were identified, including ARHGEF7 (β-Pix), Git1 (Audebert et al., 2004), PAK1 (Nola et al., 2008), and ARVCF protein, a member of the p120 catenin family that participates in the formation of adherens junction complexes (Tonikian et al., 2008) (Fig. 1*A*). We also identified Git2, ARHGEF6 (α-Pix), PPFIA3 (liprin α-3), ERC1 [also known as ELKS-Rab6-interacting protein (Erc1b)], nitric oxide synthase (NOS1), and α-*N*-catenin (CTNNA2). In addition, we found a number of peptides for the NOS1 adaptor protein NOS1AP, also known as CAPON (herein NOS1AP) (Fig. 1*A*). We also identified a number of cytoskeleton proteins, as well as proteins implicated in regulating RNA, DNA, and protein synthesis, and cell cycle regulation (see supplemental Table 1, available at www.jneurosci.org as supplemental material).

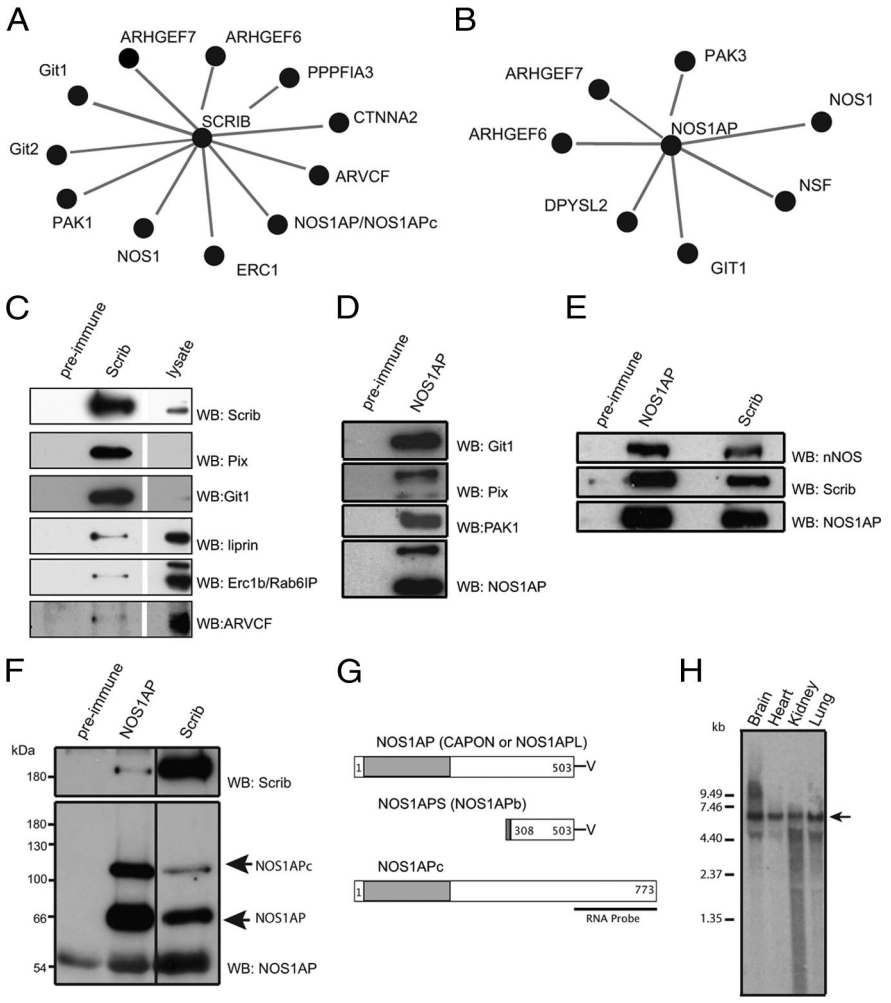

Figure 1. Identification of Scrib- and NOS1AP-associating proteins. ***A***, ***B***, Selected Scrib- (***A***) and NOS1AP- (***B***) associated proteins identified in a mass spectrometry screen (see Materials and Methods). ***C***, Rat brain lysate was immunoprecipitated with antibodies as indicated. The resulting blot was probed with the antibodies indicated. ***D***, NOS1AP precipitates β-Pix, Git1, and PAK confirming the results obtained from the mass spectrometry screen. ***E***, Endogenous NOS1AP and Scrib associated with nNOS. Rat brain lysate was immunoprecipitated with the antibodies indicated and then probed as indicated. ***F***, Endogenous Scrib and NOS1AP associate. Rat brain lysate was immunoprecipitated with the antibodies indicated. The resulting blot was probed with Scrib (upper panel) or NOS1AP (lower panel). Note a slower mobility band detected with our NOS1AP antibody (NOS1APc). ***G***, Schematic representing three known NOS1AP isoforms. Gray boxes represent the PTB domain, and -V indicates the PDZ binding motif. ***H***, Northern blot showing NOS1APc mRNA (arrow) is present in brain, heart, kidney, and lung.

NOS1AP binds Scrib-associated proteins and novel interacting proteins

One of the more interesting proteins identified in the Scrib screen was the nitric oxide synthase adaptor protein NOS1AP (Fig. 1*A*,*E*). NOS1AP has been identified as a susceptibility gene for schizophrenia (Brzustowicz et al., 2004), and more recently has been linked to long QT syndrome (Arking et al., 2006; Eijgelsheim et al., 2009), a congenital heart condition. NOS1AP contains an N-terminal PTB domain, which is important for its association with dexRAS (Fang et al., 2000), a Ras homolog, and the synaptic vesicle-associated protein, synapsin 1 (Jaffrey et al., 2002). Since Scrib and NOS1AP interact, and NOS1AP has previously been shown to play a role in the CNS (Jaffrey et al., 2002; Sun et al., 2009), we analyzed NOS1AP-associating proteins to gain insight into the functional connection between Scrib and NOS1AP. Therefore, we conducted a proteomic screen to identify NOS1AP-interacting proteins from rodent brain lysate, which identified the previously

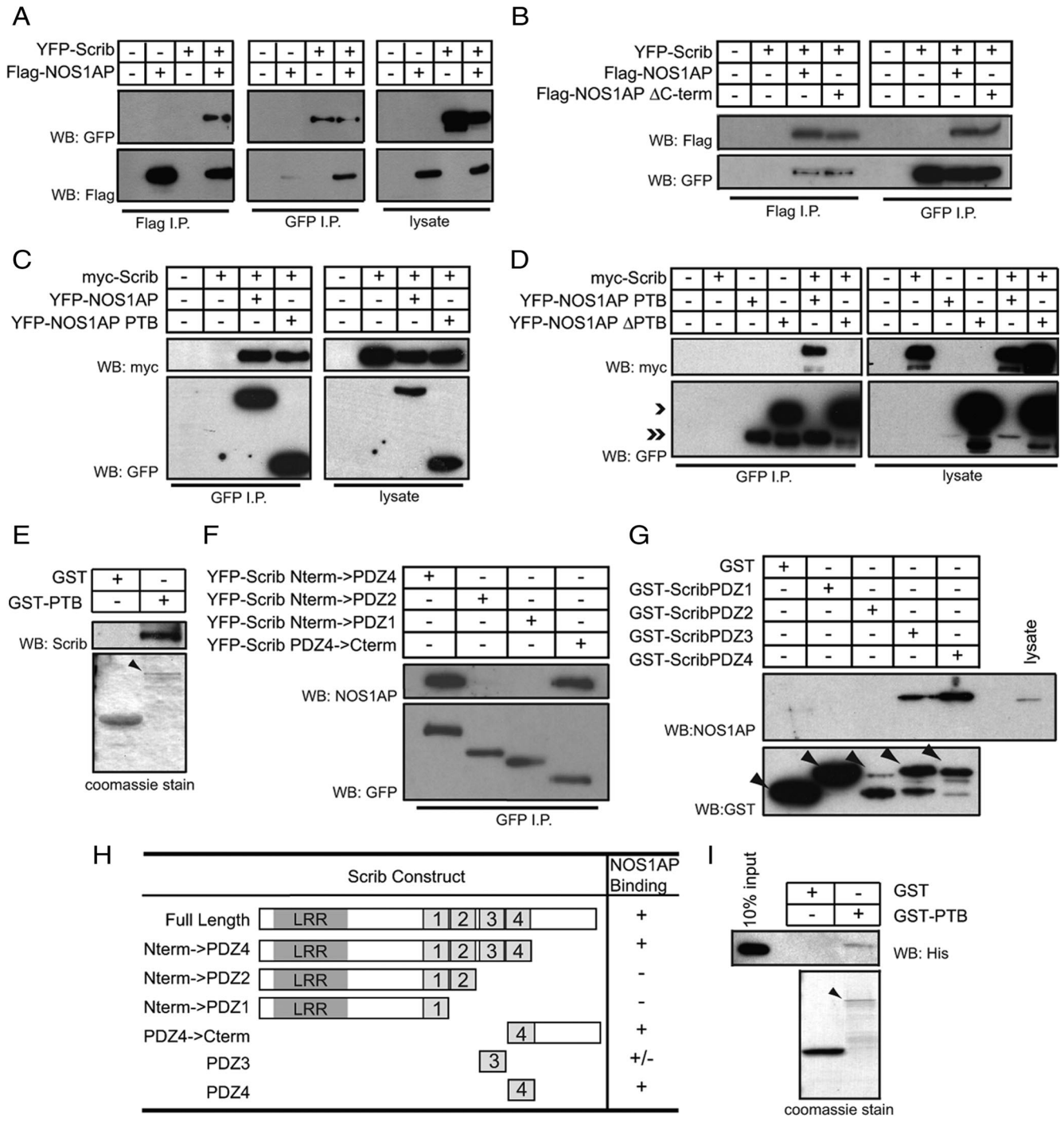

Figure 2. Scrib associates with NOS1AP through a unique PTB-PDZ interaction. ***A***, cDNA constructs encoding YFP-Scrib or Flag-NOS1AP were transfected into HEK293T cells as indicated. The resulting lysates were immunoprecipitated (I. P.) with anti-Flag (leftmost blot) or anti-GFP (middle blot) antibodies and probed with anti-GFP (upper panels in left, middle, and right blots) or anti-Flag (lower panels in left, middle, and right blots). ***B***, cDNA constructs encoding YFP-Scrib, Flag-NOS1AP, or Flag-NOS1AP ΔCterm were transfected into HEK293T cells as indicated. Lysates were immunoprecipitated as indicated, and the resulting blots were probed with either anti-Flag or anti-GFP as indicated. ***C***, cDNA constructs were transfected into HEK293T cells as indicated. The lysates were immunoprecipitated with anti-GFP and probed with anti-myc or anti-GFP as indicated. ***D***, cDNA constructs were transfected into HEK293T cells as indicated. The lysates were immunoprecipitated with anti-GFP and probed with anti-myc or anti-GFP as indicated. Double chevron, YFP-PTB NOS1AP; single chevron, YFP-ΔPTB NOS1AP. ***E***, Upper panel, GST-PTB is sufficient to precipitate endogenous Scrib. Lower panel, Coomassie stain showing loading of GST and GST-PTB (arrowhead). ***F***, HEK293T cells were transfected as indicated. Lysates from the cells were precipitated with an anti-GFP antibody. The resulting blots were probed with NOS1AP (upper panel) or GFP (lower panel) antibodies. ***G***, Purified GST fusion proteins of the individual PDZ domains of Scrib, and GST alone, were mixed with brain lysate and probed with NOS1AP antibody (upper panel). Lower panel shows levels of fusion protein used (arrowheads). ***H***, Summary of results from ***F*** and ***G***. ***I***, Purified GST or GST-PTB domain were mixed with purified His-PDZ4. The resulting pulldown was probed with anti-His antibody (upper gel). Lower panel, Coomassie stain showing loading of GST and GST-PTB (arrowhead).

known NOS1AP-associated protein, nNOS (also known as NOS1) (Jaffrey et al., 1998). Here we identified novel NOS1AP-associating proteins including ARHGEF6 (α-Pix), ARHGEF7 (β-Pix), Git1, PAK3, DPYSL2 (CRMP2), and NSF (Fig. 1*B*).

To confirm that particular Scrib- and NOS1AP-associating proteins identified by MS were both specific and reproducible, we precipitated either endogenous Scrib (Fig. 1*C*,*E*,*F*) or NOS1AP (Fig. 1*D–F*) and their associated proteins from rodent brain

lysate. The resulting IPs were probed with a number of antibodies specific for the proteins identified in the initial proteomics screen. We confirmed that endogenous β-Pix, Git1, Erc1b, liprin-α, ARVCF, nNOS, and NOS1AP were all Scrib-associated proteins (Fig. 1*C*,*E*,*F*). Further, we confirmed that endogenous β-Pix, Git1, and nNOS proteins were specifically precipitated with NOS1AP (Fig. 1*D*,*E*). Although we identified PAK3 peptides in the proteomic screen, we also determined that PAK1 could precipitate with NOS1AP using PAK1-specific antibodies (Fig. 1*D*), suggesting that more than one PAK protein can associate with NOS1AP. Together, these data support the results of the MS analysis.

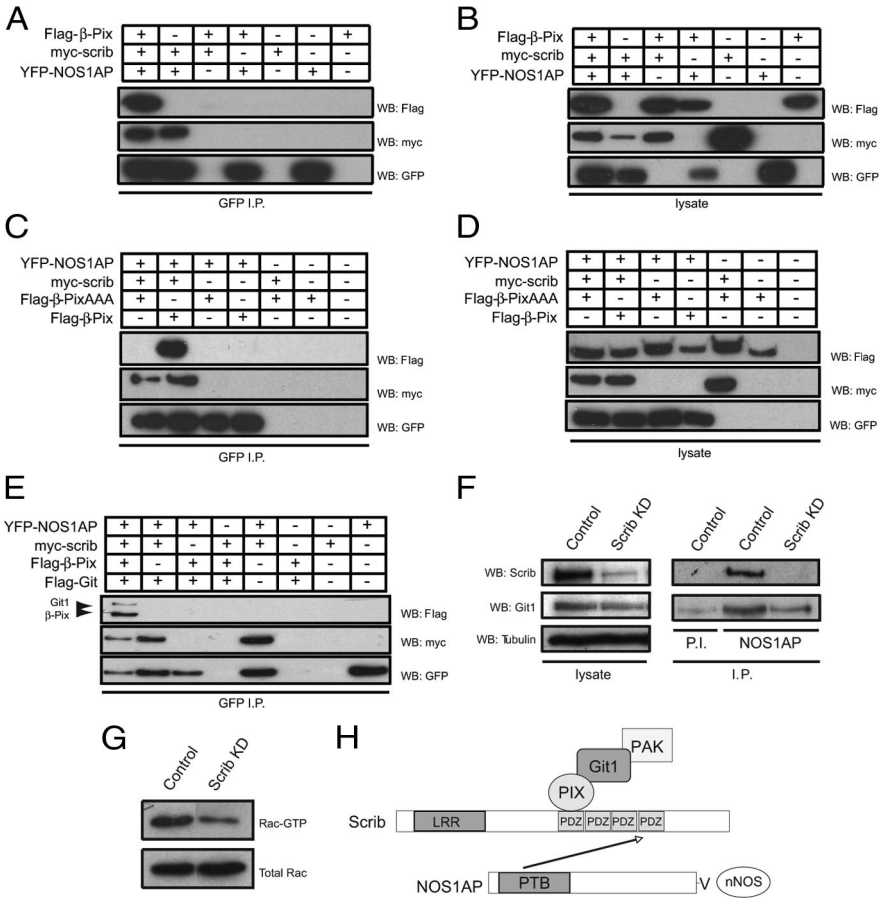

Figure 3. NOS1AP interacts with β-Pix through an interaction with Scrib. ***A***, ***B***, HEK293T cells were transfected with cDNA constructs as indicated. The resulting lysates were immunoprecipitated (I. P.) with anti-GFP antibody (***A***) and probed as indicated. ***B***, Lysate controls for ***A***. ***C***, ***D***, HEK293T cells were transfected with cDNA constructs as indicated. The resulting lysates were immunoprecipitated with anti-GFP antibody (***C***) and probed as indicated. ***D***, Lysate controls for ***C***. ***E***, HEK293T cells were transfected with cDNA constructs as indicated. The resulting lysates were immunoprecipitated with anti-GFP antibody and probed as indicated. ***F***, Left panel, Stable Cell lines generated with a Scrib shRNA knockdown vector or control vector show reduction in Scrib levels (upper panel) compared to Git1 (middle panel) or tubulin (lower panel). Right panel, Control or Scrib knockdown cell lines were immunoprecipitated with preimmune (P.I.) or NOS1AP-specific antibodies as indicated and then probed with anti-Scrib (upper panel) or anti-Git1 (lower panel). ***G***, Equal amounts of cleared lysate from the Scrib knockdown cell line or a control cell line were incubated with GST-PBD then probed with anti-Rac to determine the level of activated Rac (upper panel). Total Rac level (lower panel). ***H***, Summary of the interactions outlined in ***A–E***.

Scrib also associates with a novel NOS1AP isoform

We confirmed that endogenous NOS1AP and Scrib proteins coprecipitated from rat brain lysate (Fig. 1*F*). Of interest, in addition to the expected 70 kDa NOS1AP protein, the NOS1AP reprobe revealed a slower migrating band (~100 kDa) that was also present in the Scrib IP, suggesting either a cross-reacting band or a novel NOS1AP isoform (Fig. 1*F*, lower gel, upper band). We therefore reexamined the gel image from the Scrib IP used in the proteomic screen, and noted that the band identified as NOS1AP resolved at a molecular weight of ~100 kDa (data not shown). Based on the peptides identified in this band, the MASCOT search predicted two protein sequences, one being NOS1AP, which normally runs at 70 kDa, and the other a novel sequence for a 30 kDa protein. We noted that both protein sequences together could account for the 100 kDa sized protein. To test this possibility, we merged full-length *Rattus* NOS1AP and the novel 30 kDa predicted protein and subjected this novel protein to a BLAT search (http://genome.ucsc.edu/cgi-bin/hgBlat? command=start). We were able to generate a unique protein that mapped onto chromosome 13 within the appropriate genomic locus for *Rattus* NOS1AP. Further, we were able to identify exon and intron boundaries consistent with a novel NOS1AP isoform (herein NOS1APc) (Fig. 1*F*,*G*). To determine the sequence of the junction region between NOS1AP and the novel NOS1AP isoform, we PCR amplified a product from rat brain cDNA using a primer specific to the last exon in the existing NOS1AP isoform and a primer in the first exon of the novel NOS1AP isoform. We then sequenced this product and determined the sequence of the overlap region between NOS1AP and the novel region of the isoform; this confirmed the existence of this novel isoform (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) (NCBI accession #GU827476). To determine the expression of this isoform in different tissues, we performed a Northern blot using the unique region as a probe (Fig. 1*G*). A band migrating around 7 kb was seen in a number of tissues, including the brain (Fig. 1*H*). Together these data suggest that Scrib and NOS1AP interact, and that we have identified a novel NOS1AP isoform. We have chosen to name this novel isoform NOS1APc. To date, two isoforms of NOS1AP have been identified. The original isoform CAPON (Jaffrey et al., 1998) has also been termed NOS1AP or NOS1AP-L (Carrel et al., 2009). As well, a short isoform containing the last two exons of the NOS1AP gene with a unique 5′ sequence is known as NOS1AP-S (Xu et al., 2005). Since our isoform is longer than the originally identified NOS1AP/CAPON/NOS1AP-L isoform, and since it is the third isoform to be described, we have called it NOS1APc.

The fourth PDZ domain of Scrib directly associates with the PTB domain of NOS1AP

Since we found that endogenous Scrib and NOS1AP can form a complex, we investigated the precise nature of the interaction between these proteins. First we confirmed that Scrib and NOS1AP could interact by expressing tagged versions of both proteins in HEK293T cells (Fig. 2*A*). Next we generated deletion mutants of both NOS1AP and Scrib. Since Scrib contains four PDZ domains and NOS1AP contains a C-terminal PDZ binding motif, we explored whether this C-terminal region in NOS1AP is

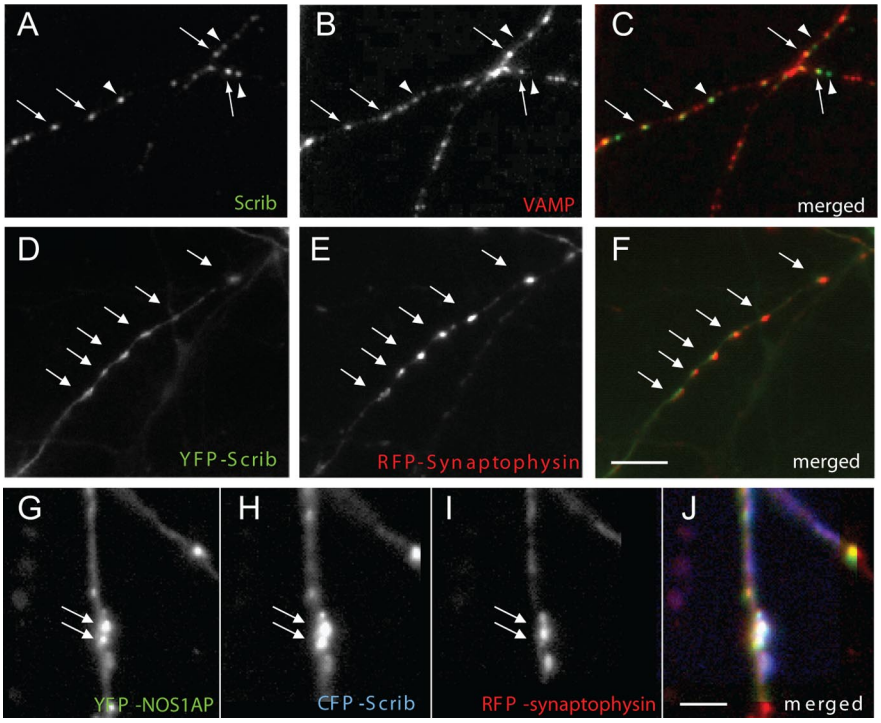

Figure 4. NOS1AP and Scrib colocalize to synaptophysin-positive presynaptic specializations in axons. ***A***, ***B***, Hippocampal neurons cultured for 16 DIV stained for Scrib (***A***) and the presynaptic marker VAMP (***B***). ***C***, Merged image of ***A*** and ***B***. Colocalization of endogenous Scrib with VAMP-positive puncta (arrows in ***A–C***). Some Scrib-positive puncta are not VAMP positive (arrowheads in ***A–C***). ***D***, ***E***, Images of primary hippocampal neurons following transfection with YFP-Scrib (***D***) and RFP-synaptophysin (***E***). ***F***, Merged image of ***D*** and ***E***. Scrib and synaptophysin colocalize in axons (arrows, ***D–F***). ***G–J***, NOS1AP (***G***) and Scrib (***H***) colocalize in synaptophysin (***I***)-positive puncta (arrows in ***G–J***). Scale bars: ***A–F***, 5 μm; ***G–J***, 2.5 μm.

required for its association with Scrib. To pursue this possibility, we destroyed the C-terminal PDZ binding motif of NOS1AP by deleting the last 5 aa, but found that this NOS1AP mutant was still able to interact with Scrib (Fig. 2*B*). Since the C-terminal PDZ binding motif of NOS1AP was not essential for its interaction with Scrib, we next focused on the other major protein-interaction domain identified in NOS1AP, the N-terminal PTB domain. We generated a construct of the N terminus of NOS1AP containing the PTB domain fused in frame with YFP (YFP-NOS1AP PTB), and then cotransfected this construct with myc-Scrib into HEK 293T cells. Using GFP antibody, we precipitated the PTB domain of NOS1AP and found that it coprecipitated with myc-Scrib (Fig. 2*C*). Conversely, a NOS1AP construct lacking the PTB domain (YFP-NOS1APΔPTB) was not able to interact with Scrib (Fig. 2*D*). To more directly test whether the PTB domain of NOS1AP was sufficient to precipitate Scrib, we generated a GST-fusion construct to the N-terminal region of NOS1AP containing the PTB domain, which we expressed in bacteria. The recombinant protein was then purified and mixed with rat brain lysate (Fig. 2*E*). Unlike GST alone, the GST-PTB fusion was sufficient to precipitate endogenous Scrib, indicating that the PTB domain of NOS1AP is indeed both necessary and sufficient to interact with Scrib.

To investigate the region of Scrib that interacts with NOS1AP, we generated Scrib deletion constructs, which lack the C-terminal region, various PDZ domains, and LRRs. Using these mutants, we found that the fourth Scrib PDZ domain was necessary for the interaction with NOS1AP (Fig. 2*F*,*H*), and that a construct lacking the LRRs and PDZ domains 1–3 was nonetheless sufficient for binding to NOS1AP (Fig. 2*F*). To more directly test whether the fourth PDZ domain of Scrib was sufficient to precipitate NOS1AP, we expressed the individual PDZ domains of Scrib as GST-fusion proteins. The recombinant proteins were then purified and mixed with rat brain lysate. Unlike GST, GST-PDZ1, or GST-PDZ2, GST-PDZ4 was sufficient to precipitate endogenous NOS1AP (Fig. 2*G*,*H*). We also noted some interaction with GST-PDZ3, suggesting that this domain may also interact with NOS1AP. Together this suggests that the fourth PDZ domain of Scrib is sufficient to bind NOS1AP.

Previously, a direct interaction has been reported between the PTB domain of the p72 kDa isoform of NUMB and the first PDZ domain of LNX (Nie et al., 2004). To test whether the PTB domain of NOS1AP could interact directly with the fourth PDZ domain of Scrib, we expressed the PTB domain as a purified GST fusion protein and mixed this with purified His-tagged PDZ4 of Scrib. Unlike GST alone, the GST-PTB domain was able to precipitate His-PDZ4, suggesting that these two proteins directly interact (Fig. 2*I*). Together these data suggest that NOS1AP and Scrib associate with one another, and that the PTB domain of NOS1AP and the fourth PDZ domain of Scrib mediate this interaction.

Since a recent publication reported that NOS1AP associates with carboxypeptidase E (CPE) (Carrel et al., 2009), we next wanted to test whether CPE was part of the NOS1AP-Scrib pathway. To test this, we coexpressed YFP-NOS1AP PTB along with Flag-CPE, then precipitated YFP-NOS1AP PTB and probed for Flag-tagged CPE. We found no association between the PTB domain of NOS1AP and CPE, suggesting that CPE likely functions in a separate pathway from the NOS1AP-Scrib pathway (supplemental Fig. 3, available at www.jneurosci.org as supplemental material).

NOS1AP associates with β-Pix and Git through Scrib

Since we identified β-Pix and Git1 in our NOS1AP MS screen, and β-Pix has been shown to bind directly to Scrib (Audebert et al., 2004), we next tested whether Scrib was important in bridging an interaction between NOS1AP on the one hand and β-Pix and Git1 on the other. To test this possibility, we cotransfected β-Pix and NOS1AP in the presence or absence of Scrib. Only in the presence of Scrib were we able to see an association between β-Pix and NOS1AP (Fig. 3*A*,*B*). Since β-Pix has been shown to bind Scrib directly through a PDZ binding motif (Zeniou-Meyer et al., 2005), we mutated the last 3 aa of β-Pix to alanines to test the effects of destroying this PDZ domain recognition sequence. The β-PixAAA mutant was not able to associate with NOS1AP even in the presence of Scrib, although we still detected an interaction between NOS1AP and Scrib in the same assay (Fig. 3*C*,*D*). This confirms the role of Scrib as a scaffolding protein that connects NOS1AP with β-Pix. To determine whether Git1 associates with NOS1AP, through both Scrib and β-Pix, we tested whether Git1 could precipitate with NOS1AP and Scrib in the absence of β-Pix. As predicted from this model, Git1 coimmunoprecipitated with NOS1AP only in the presence of both Scrib and β-Pix (Fig. 3*E*). To test the endogenous role of Scrib in bridging an interaction between NOS1AP and Git1, we used shRNA to reduce

endogenous Scrib levels in 293T cells. Following knockdown of endogenous Scrib levels, we immunoprecipitated NOS1AP and noted a reduction in Git1 association (Fig. 3*F*) compared to a control cell line. Finally, since Git1 and β-Pix have been shown to influence the RhoGTPase, Rac1 (Zhang et al., 2005), we tested whether Scrib knockdown would influence the activation of Rac1. To this end, we noted a reduction in activated Rac in Scrib knockdown cells (Fig. 3*G*), similar to results obtained in MCF-10A cells (Zhan et al., 2008). Together these data suggest that Scrib functions to bridge an interaction between NOS1AP and the β-Pix, Git1, PAK complex (Fig. 3*F*), and that Scrib plays a role in regulating RhoGTPase activity.

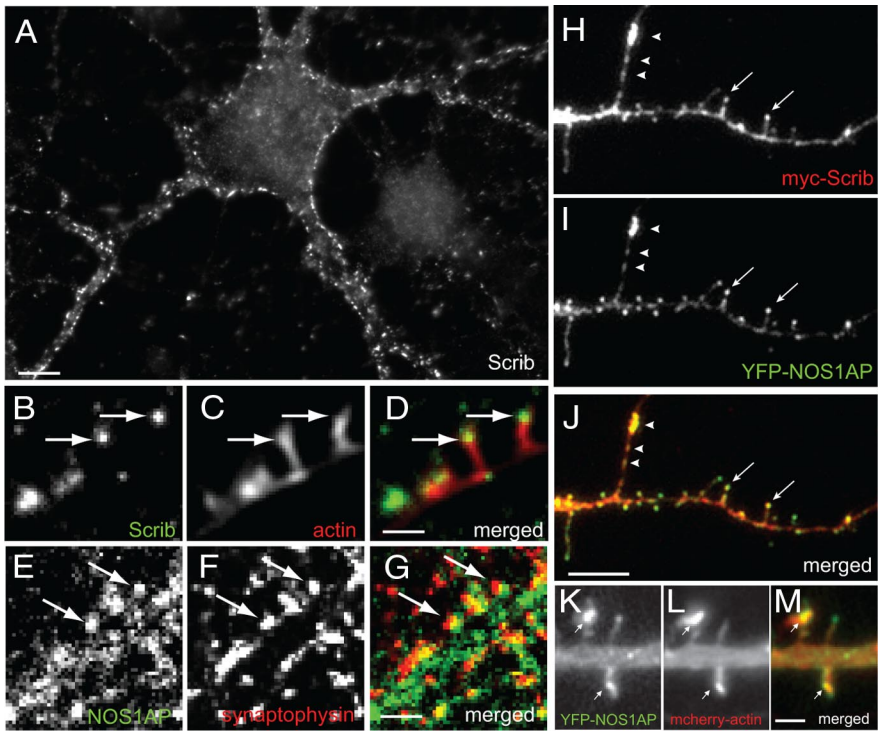

Figure 5. NOS1AP and Scrib colocalize in dendrites. ***A***, Fluorescent image of a dissociated hippocampal neuron cultured for 16 DIV and stained with a Scrib antibody. ***B***, ***C***, Dissociated hippocampal neurons 16 DIV stained for Scrib (***B***) and phalloidin (***C***). ***D***, Merged image of ***B*** and ***C***. Note the colocalization of endogenous Scrib in dendritic spines (arrows in ***B–D***). ***E***, ***F***, Dissociated hippocampal neurons 15 DIV stained for NOS1AP (***E***) and synaptophysin (***F***). ***G***, Merged image of ***E*** and ***F***. Note partial colocalization of endogenous NOS1AP and synaptophysin (arrows in ***E–G***). ***H***, ***I***, Dissociated hippocampal neurons 13 DIV were transfected with myc-Scrib (***H***) and YFP-NOS1AP (***I***). ***J***, Merged image of ***H*** and ***I***. Scrib and NOS1AP colocalize in dendritic shafts (arrowheads in ***H–J***) and in dendritic spines (***H–J***, arrows). ***K***, ***L***, NOS1AP (***K***) is seen at the tips of dendritic spines with actin (***L***) (arrows in ***K***, ***L***). ***M***, Merged image of ***K*** and ***L***. Scale bars: ***A***, 15 μm; ***B–G***, ***K–M***, 2.5 μm; ***H–J***, 10 μm.

NOS1AP and Scrib localize presynaptically

Previously, β-Pix and Git1 have been shown to precipitate with Scrib, and it has been suggested that this complex may play a functional role in vesicle dynamics and release in neurons (Audebert et al., 2004). However, the functional mechanism and significance of this complex remains unknown. Interestingly, our screen identified a number of Scrib-associating proteins that are important for synaptic vesicle dynamics, and the development of synaptic active zones, these include Git1, liprin-α, and Erc1b (Schoch and Gundelfinger, 2006). In addition, we identified a peptide in our Scrib IP for the presynaptic scaffold Piccolo (Kim et al., 2003), although this peptide was below the level of significance used in our screen (supplemental Table 1, available at www.jneurosci.org as supplemental material). Further work will determine whether Piccolo associates with Scrib. Nonetheless, Git1 has been shown to bind directly to liprin-α (Schoch and Gundelfinger, 2006) and Piccolo (Kim et al., 2003), while liprin-α in turn associates directly with Erc1b (Schoch and Gundelfinger, 2006). This complex of proteins is important for the development of the CAZ, a region implicated in defining neurotransmitter release sites and regulating synaptic vesicle release (Ko et al., 2003b; Schoch and Gundelfinger, 2006). As well, recent work has shown a role for Scrib downstream of β-catenin in clustering synaptic vesicles in the developing synapses (Sun et al., 2009). Consistent with these results, we see endogenous Scrib staining in punctate structures in axons (Fig. 4*A*) that partially colocalizes with the synaptic vesicle marker VAMP (Fig. 4*A–C*), indicating that Scrib associates with either synaptic vesicles or other transport vesicles, including Piccolo-Bassoon transport vesicles. To more directly test whether Scrib associates with a synaptic vesicle pool at presynaptic specializations, we cotransfected cDNA constructs encoding Scrib, fused in frame with YFP, and the presynaptic vesicle marker synaptophysin, fused in frame with red fluorescent protein (RFP), into dissociated hippocampal neurons that had previously been grown for 14 DIV. Analysis revealed colocalization between YFP-Scrib and RFP-synaptophysin (Fig. 4*D–F*, arrows). Together these data suggest that endogenous Scrib localizes to presynaptic vesicles, consistent with our endogenous Scrib staining [Fig. 4*A* and Sun et al. (2009)]. Since NOS1AP interacts with the synaptic vesicle-associating protein synapsin1 (Jaffrey et al., 2002), we investigated whether NOS1AP is distributed in presynaptic compartments along with Scrib. Since both our Scrib and NOS1AP antibodies were raised in rabbits, we turned to overexpression studies to determine whether Scrib and NOS1AP could colocalize presynaptically. We cotransfected dissociated rat hippocampal neurons with constructs encoding YFP-NOS1AP, CFP-Scrib, and RFP-synaptophysin. We saw colocalization of all three markers, supporting the notion that NOS1AP and Scrib could play a role presynaptically (Fig. 4*G–J*, arrows). Together with our results showing that Scrib associates with presynaptic proteins, these localization data suggest that NOS1AP localizes with Scrib to presynaptic specializations, where it may play a role in synaptic vesicle dynamics and active zone formation.

NOS1AP and Scrib localize in dendrites, dendritic spines, and synapses

We have identified a scaffolding complex for NOS1AP that includes Scrib, β-Pix, Git1, and PAK proteins. Of these, β-Pix, Git1, and PAK proteins are important in the postsynaptic region and in regulating dendritic spine development (Wiggin et al., 2005; Zhang et al., 2005). In addition, nNOS, a protein previously identified as a NOS1AP-associating protein, has been shown to localize in the postsynaptic region (Watanabe et al., 2003). Since these data implicate a potential postsynaptic role for NOS1AP and Scrib, we tested whether these proteins could localize to dendritic spines. To determine whether endogenous Scrib and NOS1AP

localize to dendritic spines, we stained 15–16 DIV dissociated hippocampal neurons with either an anti-Scrib antibody or anti-NOS1AP antibody. Interestingly, endogenous Scrib staining revealed, in addition to the punctate stain in axons (Fig. 4*A*), punctate staining surrounding cell bodies and dendrites (Fig. 5*A–D*). Since dendritic spines are enriched for actin, we costained our cultured neurons with phalloidin, a marker of actin. Here we saw clear colocalization between Scrib and actin in dendritic spines (Fig. 5*B–D*). Interestingly, a punctate stain was seen with our NOS1AP antibody. Here NOS1AP partially colocalized with the synaptic vesicle protein synaptophysin (Fig. 5*E–G*). To show colocalization in dendritic spines, we cotransfected mature hippocampal neurons with YFP-NOS1AP and myc-Scrib. We found a clear colocalization of these proteins in dendritic shafts (Fig. 5*H–J*, arrowheads), dendritic spines, and spine heads (Fig. 5*H–J*, arrows). To confirm the localization of NOS1AP in dendritic spines, we cotransfected our dissociated hippocampal neurons with YFP-NOS1AP and mCherry-actin, since actin is enriched in dendritic spine heads and colocalized with endogenous Scrib (Fig. 5*B–D*). This analysis showed colocalization of YFP-NOS1AP and actin in spine heads (Fig. 5*K–M*, arrows), supporting the notion that NOS1AP is found in dendritic spines. Together, these data suggest that NOS1AP and Scrib proteins can localize postsynaptically in dendritic spines.

NOS1AP overexpression leads to increases in dendritic protrusions and activates Rac through its PTB domain

Since endogenous Scrib and NOS1AP localize to dendritic spines, and we have linked NOS1AP with proteins important for regulating dendritic spine morphology, namely the RhoGEF β-Pix and its associated proteins Git1 and PAK through the Scrib scaffold, we next investigated whether NOS1AP had an effect on dendritic spine morphology. For this purpose, we overexpressed either YFP or YFP-NOS1AP in dissociated hippocampal neurons that had been cultured for 13 DIV (Fig. 6*A*,*B*). Twenty-four hours after transfection, we detected a 30% increase in the number of dendritic protrusions in the YFP-NOS1AP-transfected neurons (Fig. 6*E*).

β-Pix, Git1, and PAK function to regulate dendritic spine morphology through the Rac GTPase (Zhang et al., 2003, 2005). Since NOS1AP associates with β-Pix, Git1, and PAK through Scrib, we considered the possibility that NOS1AP could influence the activation of the Rho family GTPases. To test this possibility, we overexpressed NOS1AP in HEK293T cells and investigated the effect on the level of GTP-bound Rac. In YFP-NOS1AP-expressing cells, we saw an increase in active GTP-bound Rac compared to cells expressing YFP alone (Fig. 6*F*,*G*). Since we have shown that the N-terminal region containing the PTB domain of NOS1AP is sufficient for association with Scrib, and Scrib is important to connect NOS1AP with the RhoGEF protein β-Pix, we next tested whether expressing the PTB domain of NOS1AP could activate Rac, and found that this was the case (Fig. 6*F*,*G*), suggesting that this region plays an important role in regulating RhoGTPase activity. Further, expression of the PTB domain in dissociated hippocampal neurons showed a 35% increase in the number of dendritic protrusions, while a NOS1AP mutant lacking the PTB domain (YFP-NOS1APΔPTB) had no effect, suggesting that the PTB domain is sufficient to elicit a response (Fig. 6*C*,*D*). Together, these data suggest that the PTB domain of NOS1AP is sufficient to increase the number of dendritic protrusions and leads to an increase in Rac activity, likely through an effect on the Scrib, β-Pix, Git, and PAK complex.

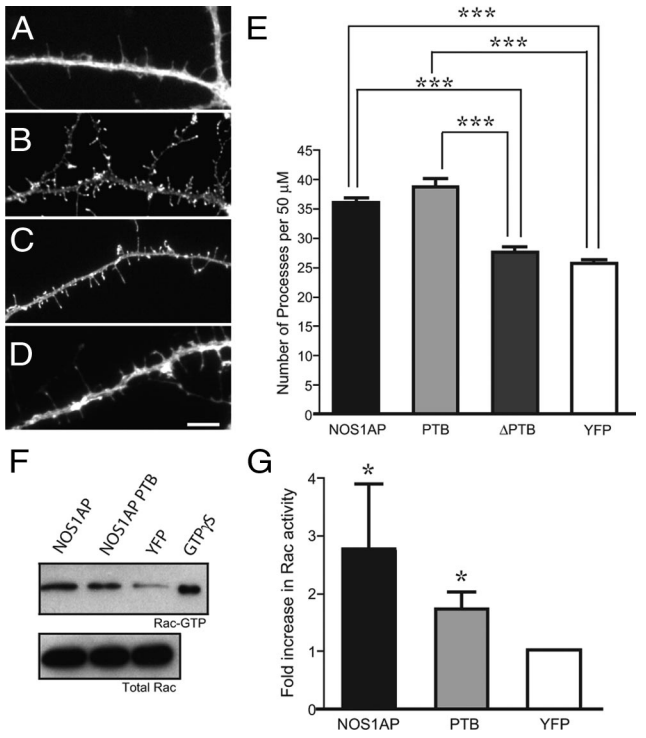

Figure 6. NOS1AP increases processes outgrowth and influences Rac activity. ***A–D***, Epifluorescent images of dissociated hippocampal neurons cultured for 13 DIV and then transfected with YFP (***A***), YFP-NOS1AP (***B***), YFP-NOS1AP PTB (***C***), or YFP-NOS1APΔPTB (***D***). Scale bar, 5 μm. ***E***, Quantification of the number of processes per 50 μm segment of dendrite in YFP ($n = 122$), YFP-NOS1AP ($n = 87$), YFP-NOS1AP PTB ($n = 17$), and YFP-NOS1APΔPTB ($n = 18$). ***F***, HEK293T cells were transfected as indicated. Cleared lysates were incubated with GST-PBD and then probed with anti-Rac to determine the amount of activated Rac (upper panel). GTPγS was included as a positive control. Whole-cell lysate from the transfected cells was probed with anti-Rac (lower panel). ***G***, Quantification of activated Rac. An increase in Rac activity was seen in YFP-NOS1AP ($n = 5$) and YFP-NOS1AP PTB ($n = 5$) relative to YFP alone ($n = 5$). $^{***}p < 0.0001$; $^{*}p < 0.05$.

Discussion

Our study indicates that NOS1AP influences Rac activation through a Scrib complex to regulate synapse formation. We identified a unique interaction between the NOS adaptor protein, NOS1AP, and the polarity protein Scrib through a targeted proteomic screen. Scrib contains a number of signaling domains, including LRR and four PDZ domains, and we show that the fourth PDZ domain of Scrib interacts with the PTB domain of NOS1AP. We note that the N-terminal region of NOS1AP containing the PTB domain was both sufficient and necessary to precipitate Scrib protein, implicating its importance for the NOS1AP-Scrib interaction. Further, we show a direct interaction between the PTB domain of NOS1AP with the fourth PDZ domain of Scrib. To our knowledge, this is the second description of this unique interaction between a PTB and PDZ domain. Previously, the PTB domains of the p72 and p66 isoforms of the cell fate determinant protein NUMB have been reported to be necessary for an interaction with the first PDZ domain of the E3 ubiquitin ligase protein LNX (Nie et al., 2004), supporting the notion that this type of interaction does occur. Interestingly, the PTB domain of NOS1AP is most similar to the PTB domain of mouse Numb (Jaffrey et al., 2002), suggesting that the PTB domain of NOS1AP may have a similar fold. The association of the p72 NUMB PTB domain with the PDZ domain of LNX is dependent on three critical lysine residues within an 11 aa insertion between

the α-helix A2 and β-strand B2 (Nie et al., 2004). Alignments of the NOS1AP PTB domain and the p72 NUMB PTB domain fail to show the 11 aa insert between the α-helix A2 and β-strand B2; however, there is an 11 aa insert in the NOS1AP PTB domain that contains a number of lysine residues between the β-strands B3 and B4. Whether this insert region is critical for the association between NOS1AP and Scrib remains to be determined. Nonetheless, the unique interaction identified here suggests that PTB-PDZ domain interactions may be more common than previously supposed, especially in the context of the polarity network.

In our proteomic screen, we identified β-Pix, Git1, and PAK as associating proteins with Scrib, supporting previous studies (Audebert et al., 2004; Nola et al., 2008), and with NOS1AP. Further, we show that Scrib functions as a bridge between NOS1AP and the β-Pix, Git1, and PAK complex. This is consistent with a role for Scrib as a multifaceted scaffolding protein. PDZ domain-containing proteins commonly function to scaffold multiple protein complexes (Tonikian et al., 2008), and in the nervous system, PDZ-containing proteins are especially important to scaffold complexes important for the development and function of the synapse (Feng and Zhang, 2009). In addition to PDZ domain-containing proteins, recent evidence suggests that LRR-containing proteins also function as important regulators of synaptic function (Ko and Kim, 2007; Linhoff et al., 2009; Woo et al., 2009). Here, we suggest that Scrib is an organizing protein that functions to localize NOS1AP, β-Pix, Git1, and PAK at the synapse. Consistent with a role for Scrib as a major presynaptic scaffold a number of proteins identified in our screen function in the CAZ (Schoch and Gundelfinger, 2006). These include β-Pix, Git1, liprin-α, and Erc1b (Zhen and Jin, 1999; Dai et al., 2006; Schoch and Gundelfinger, 2006). As well, a recent report has shown that Scrib functions downstream of β-catenin to localize synaptic vesicles in the developing synapse, implicating Scrib in the organization of the presynaptic region (Sun et al., 2009). Here we show that both NOS1AP and Scrib localize presynaptically (Fig. 4), moreover NOS1AP has been shown to bind synapsin1 (Jaffrey et al., 2002), an important phosphoprotein involved in linking synaptic vesicles and the actin cytoskeleton to regulate vesicle dynamics (Bloom et al., 2003). This suggests that NOS1AP may play an important role with Scrib in the development of the presynaptic region. In addition to the presynaptic localization, endogenous Scrib and NOS1AP are enriched in dendritic spines (Fig. 5), and many of the Scrib-associating proteins also function in the postsynaptic compartment. For example, liprin-α localizes postsynaptically and functions with Git1 (Ko et al., 2003a) in the trafficking of AMPA receptors (Wyszynski et al., 2002; Ko et al., 2003a). β-Pix, Git1, and PAK function together to regulate dendritic spine morphology through MLC (Zhang et al., 2003, 2005). Consistent with a scaffold function for Scrib, we find that both Scrib and its associating protein NOS1AP colocalize in both presynaptic and postsynaptic regions.

Functionally, many of the proteins identified in our proteomic screen as Scrib- and NOS1AP-associating proteins have been implicated in regulating dendritic spine morphology. Here we show that overexpression of NOS1AP leads to an increase in dendritic protrusions, a role dependent on the N-terminal region containing the PTB domain of NOS1AP. Recent evidence increasingly suggests that polarity proteins function in regulating spine morphology by regulating Rho GTPase signaling. For example, Par3 functions through the Rac GEF, Tiam1, to regulate the maturation of dendritic spines (Zhang and Macara, 2006), while Par6 and aPKC function through a Rho-dependent mechanism to regulate spine morphology independently of Par3 (Zhang and Macara, 2008). In our study, we show that overexpression of NOS1AP or the PTB domain in non-neuronal cells influences Rac activity. This raises the question of how NOS1AP might influence Rac activity to affect dendritic spine development. One possibility is that the NOS1AP-Scrib association may affect nitric oxide (NO) signaling to regulate spine development, since NO signaling is known to modify spine morphology (Nikonenko et al., 2008; Steinert et al., 2008). However, this possibility seems unlikely since the NOS1AP PTB domain, which is not coupled to NOS, is the critical region in NOS1AP affecting spine development. Another possibility is that NOS1AP functions with CPE to regulate dendritic spines, since CPE has been shown to regulate dendrite patterning in immature hippocampal neurons (Carrel et al., 2009). This is unlikely, however, since the region found to affect spine development in this study fails to associate with CPE. Rather, we speculate that NOS1AP induces or stabilizes a Scrib, β-Pix, Git1, PAK complex, leading to an increase in Rac activity. This is consistent with previous studies showing that a complex of Scrib, β-Pix, Git1, and PAK can be regulated, leading to an increase in Rac activity (Zhan et al., 2008). Further, *Circletailed* mutant mice, which contain a spontaneous mutation in Scrib, show a reduction in the number of dendritic spines found in the adult hippocampus, supporting the role of Scrib in dendritic spine development (M. Moreau and N. Sans, personal communication). Finally, we cannot rule out that NOS1AP may recruit an associating protein that can regulate the GEF activity of β-Pix. Indeed signaling complexes upstream of β-Pix, Git1, and PAK can regulate β-Pix GEF activity, leading to increased Rac activity and spine development (Saneyoshi et al., 2008).

In summary, we have conducted a proteomic screen with a number of known polarity proteins and identified several unique interactions, including a novel link between Scrib and a PTB-containing protein NOS1AP. Their association is dependent on the N-terminal region containing the PTB domain of NOS1AP and the fourth PDZ domain of Scrib. Our results suggest that NOS1AP and Scrib associate and are involved in the synapse, both presynaptically and postsynaptically. Interestingly, a NOS1AP isoform lacking the N-terminal region that includes the PTB domain, NOS1AP-S, is upregulated in families with bipolar disorder and schizophrenia (Xu et al., 2005). Since schizophrenia is a disease affecting the synapse (McGlashan and Hoffman, 2000), our data, combined with the effects on NOS1AP on dendritic patterning (Carrel et al., 2009), may shed new light on the role NOS1AP plays in disease states.

References

Arimura N, Kaibuchi K (2007) Neuronal polarity: from extracellular signals to intracelluar mechanisms. Nat Rev Neurosci 8:194–205.

Arking DE, Pfeufer A, Post W, Kao WH, Newton-Cheh C, Ikeda M, West K, Kashuk C, Akyol M, Perz S, Jalilzadeh S, Illig T, Gieger C, Guo CY, Larson MG, Wichmann HE, Marbán E, O'Donnell CJ, Hirschhorn JN, Kääb S, et al. (2006) A common genetic variant in the NOS1 regulator NOS1AP modulates cardiac repolarization. Nat Genet 38:644–651.

Audebert S, Navarro C, Nourry C, Chasserot-Golaz S, Lécine P, Bellaiche Y, Dupont JL, Premont RT, Sempéré C, Strub JM, Van Dorsselaer A, Vitale N, Borg JP (2004) Mammalian Scribble forms a tight complex with the betaPIX exchange factor. Curr Biol 14:987–995.

Bagrodia S, Taylor SJ, Jordon KA, Van Aelst L, Cerione RA (1998) A novel regulator of p21-activated kinases. J Biol Chem 273:23633–23636.

Barnes AP, Polleux F (2009) Establishment of axon-dendrite polarity in developing neurons. Annual review of neuroscience 32:347–381.

Bilder D (2003) PDZ domain polarity complexes. Curr Biol 13:R661–R662.

Bilder D, Li M, Perrimon N (2000) Cooperative regulation of cell polarity and growth by *Drosophila* tumor suppressors. Science 289:113–116.

Bloom O, Evergren E, Tomilin N, Kjaerulff O, Löw P, Brodin L, Pieribone VA, Greengard P, Shupliakov O (2003) Colocalization of synapsin and actin during synaptic vesicle recycling. J Cell Biol 161:737–747.

Brzustowicz LM, Simone J, Mohseni P, Hayter JE, Hodgkinson KA, Chow EW, Bassett AS (2004) Linkage disequilibrium mapping of schizophrenia susceptibility to the CAPON region of chromosome 1q22. Am J Hum Genet 74:1057–1063.

Carrel D, Du Y, Komlos D, Hadzimichalis NM, Kwon M, Wang B, Brzustowicz LM, Firestein BL (2009) NOS1AP regulates dendrite patterning of hippocampal neurons through a carboxypeptidase E-mediated pathway. J Neurosci 29:8248–8258.

Dai Y, Taru H, Deken SL, Grill B, Ackley B, Nonet ML, Jin Y (2006) SYD-2 Liprin-alpha organizes presynaptic active zone formation through ELKS. Nat Neurosci 9:1479–1487.

Eijgelsheim M, Aarnoudse AL, Rivadeneira F, Kors JA, Witteman JC, Hofman A, van Duijn CM, Uitterlinden AG, Stricker BH (2009) Identification of a common variant at the NOS1AP locus strongly associated to QT-interval duration. Hum Mol Genet 18:347–357.

Fang M, Jaffrey SR, Sawa A, Ye K, Luo X, Snyder SH (2000) Dexras1: a G protein specifically coupled to neuronal nitric oxide synthase via CAPON. Neuron 28:183–193.

Feng W, Zhang M (2009) Organization and dynamics of PDZ-domain-related supramodules in the postsynaptic density. Nat Rev Neurosci 10:87–99.

Jaffrey SR, Snowman AM, Eliasson MJ, Cohen NA, Snyder SH (1998) CAPON: a protein associated with neuronal nitric oxide synthase that regulates its interactions with PSD95. Neuron 20:115–124.

Jaffrey SR, Benfenati F, Snowman AM, Czernik AJ, Snyder SH (2002) Neuronal nitric-oxide synthase localization mediated by a ternary complex with synapsin and CAPON. Proc Natl Acad Sci U S A 99:3199–3204.

Kallay LM, McNickle A, Brennwald PJ, Hubbard AL, Braiterman LT (2006) Scribble associates with two polarity proteins, lgl2 and vangl2, via distinct molecular domains. J Cell Biochem 99:647–664.

Kim S, Ko J, Shin H, Lee JR, Lim C, Han JH, Altrock WD, Garner CC, Gundelfinger ED, Premont RT, Kaang BK, Kim E (2003) The GIT family of proteins forms multimers and associates with the presynaptic cytomatrix protein Piccolo. J Biol Chem 278:6291–6300.

Ko J, Kim E (2007) Leucine-rich repeat proteins of synapses. J Neurosci Res 85:2824–2832.

Ko J, Kim S, Valtschanoff JG, Shin H, Lee JR, Sheng M, Premont RT, Weinberg RJ, Kim E (2003a) Interaction between liprin-α and GIT1 is required for AMPA receptor targeting. J Neurosci 23:1667–1677.

Ko J, Na M, Kim S, Lee JR, Kim E (2003b) Interaction of the ERC family of RIM-binding proteins with the liprin-alpha family of multidomain proteins. J Biol Chem 278:42377–42385.

Krueger SR, Kolar A, Fitzsimonds RM (2003) The presynaptic release apparatus is functional in the absence of dendritic contact and highly mobile within isolated axons. Neuron 40:945–957.

Linhoff MW, Laurén J, Cassidy RM, Dobie FA, Takahashi H, Nygaard HB, Airaksinen MS, Strittmatter SM, Craig AM (2009) An unbiased expression screen for synaptogenic proteins identifies the LRRTM protein family as synaptic organizers. Neuron 61:734–749.

Manser E, Loo TH, Koh CG, Zhao ZS, Chen XQ, Tan L, Tan I, Leung T, Lim L (1998) PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol Cell 1:183–192.

McGlashan TH, Hoffman RE (2000) Schizophrenia as a disorder of developmentally reduced synaptic connectivity. Arch Gen Psychiatry 57:637–648.

Nesvizhskii AI, Keller A, Kolker E, Aebersold R (2003) A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem 75:4646–4658.

Nie J, Li SS, McGlade CJ (2004) A novel PTB-PDZ domain interaction mediates isoform-specific ubiquitylation of mammalian Numb. J Biol Chem 279:20807–20815.

Nikonenko I, Boda B, Steen S, Knott G, Welker E, Muller D (2008) PSD-95 promotes synaptogenesis and multiinnervated spine formation through nitric oxide signaling. J Cell Biol 183:1115–1127.

Nola S, Sebbagh M, Marchetto S, Osmani N, Nourry C, Audebert S, Navarro C, Rachel R, Montcouquiol M, Sans N, Etienne-Manneville S, Borg JP, Santoni MJ (2008) Scrib regulates PAK activity during the cell migration process. Hum Mol Genet 17:3552–3565.

Osmani N, Vitale N, Borg JP, Etienne-Manneville S (2006) Scrib controls Cdc42 localization and activity to promote cell polarization during astrocyte migration. Curr Biol 16:2395–2405.

Roche JP, Packard MC, Moeckel-Cole S, Budnik V (2002) Regulation of synaptic plasticity and synaptic vesicle dynamics by the PDZ protein Scribble. J Neurosci 22:6471–6479.

Saneyoshi T, Wayman G, Fortin D, Davare M, Hoshi N, Nozaki N, Natsume T, Soderling TR (2008) Activity-dependent synaptogenesis: regulation by a CaM-kinase kinase/CaM-kinase I/betaPIX signaling complex. Neuron 57:94–107.

Schoch S, Gundelfinger ED (2006) Molecular organization of the presynaptic active zone. Cell Tissue Res 326:379–391.

Smith MJ, Hardy WR, Murphy JM, Jones N, Pawson T (2006) Screening for PTB domain binding partners and ligand specificity using proteome-derived NPXY peptide arrays. Mol Cell Biol 26:8461–8474.

Steinert JR, Kopp-Scheinpflug C, Baker C, Challiss RA, Mistry R, Haustein MD, Griffin SJ, Tong H, Graham BP, Forsythe ID (2008) Nitric oxide is a volume transmitter regulating postsynaptic excitability at a glutamatergic synapse. Neuron 60:642–656.

Sun Y, Aiga M, Yoshida E, Humbert PO, Bamji SX (2009) Scribble interacts with beta-catenin to localize synaptic vesicles to synapses. Mol Biol Cell 20:3390–3400.

Tonikian R, Zhang Y, Sazinsky SL, Currell B, Yeh JH, Reva B, Held HA, Appleton BA, Evangelista M, Wu Y, Xin X, Chan AC, Seshagiri S, Lasky LA, Sander C, Boone C, Bader GD, Sidhu SS (2008) A specificity map for the PDZ domain family. PLoS Biol 6:e239.

Watanabe Y, Song T, Sugimoto K, Horii M, Araki N, Tokumitsu H, Tezuka T, Yamamoto T, Tokuda M (2003) Post-synaptic density-95 promotes calcium/calmodulin-dependent protein kinase II-mediated Ser847 phosphorylation of neuronal nitric oxide synthase. Biochem J 372:465–471.

Wiggin GR, Fawcett JP, Pawson T (2005) Polarity proteins in axon specification and synaptogenesis. Dev Cell 8:803–816.

Woo J, Kwon SK, Kim E (2009) The NGL family of leucine-rich repeat-containing synaptic adhesion molecules. Mol Cell Neurosci 42:1–10.

Wyszynski M, Kim E, Dunah AW, Passafaro M, Valtschanoff JG, Serra-Pagès C, Streuli M, Weinberg RJ, Sheng M (2002) Interaction between GRIP and liprin-alpha/SYD2 is required for AMPA receptor targeting. Neuron 34:39–52.

Xu B, Wratten N, Charych EI, Buyske S, Firestein BL, Brzustowicz LM (2005) Increased expression in dorsolateral prefrontal cortex of CAPON in schizophrenia and bipolar disorder. PLoS Med 2:e263.

Zeniou-Meyer M, Borg JP, Vitale N (2005) [The GIT-PIX protein complex: a hub to ARF and Rac/Cdc42 GTPases]. Med Sci (Paris) 21:849–853.

Zhan L, Rosenberg A, Bergami KC, Yu M, Xuan Z, Jaffe AB, Allred C, Muthuswamy SK (2008) Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. Cell 135:865–878.

Zhang H, Macara IG (2006) The polarity protein PAR-3 and TIAM1 cooperate in dendritic spine morphogenesis. Nat Cell Biol 8:227–237.

Zhang H, Macara IG (2008) The PAR-6 polarity protein regulates dendritic spine morphogenesis through p190 RhoGAP and the Rho GTPase. Dev Cell 14:216–226.

Zhang H, Webb DJ, Asmussen H, Horwitz AF (2003) Synapse formation is regulated by the signaling adaptor GIT1. J Cell Biol 161:131–142.

Zhang H, Webb DJ, Asmussen H, Niu S, Horwitz AF (2005) A GIT1/PIX/Rac/PAK signaling module regulates spine morphogenesis and synapse formation through MLC. J Neurosci 25:3379–3388.

Zhen M, Jin Y (1999) The liprin protein SYD-2 regulates the differentiation of presynaptic termini in *C. elegans*. Nature 401:371–375.