A PP2A Phosphatase High Density Interaction Network Identifies a Novel Striatin-interacting Phosphatase and Kinase Complex Linked to the Cerebral Cavernous Malformation 3 (CCM3) Protein*\(^a\)

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The serine/threonine protein phosphatases are targeted to specific subcellular locations and substrates in part via interactions with a wide variety of regulatory proteins. Understanding these interactions is thus critical to understanding phosphatase function. Using an iterative affinity purification/mass spectrometry approach, we generated a high density interaction map surrounding the protein phosphatase 2A catalytic subunit. This approach recapitulated the assembly of the PP2A catalytic subunit into many different trimeric complexes but also revealed several new protein-protein interactions. Here we define a novel large multiprotein assembly, referred to as the striatin-interacting phosphatase and kinase (STRIPAK) complex. STRIPAK contains the PP2A catalytic (PP2Ac) and scaffolding (PP2A A) subunits, the striatin (PP2A regulatory B” subunits), the striatin-associated protein Mob3, the novel proteins STRIP1 and STRIP2 (formerly FAM40A and FAM40B), the cerebral cavernous malformation 3 (CCM3) protein, and members of the germinal center kinase III family of Ste20 kinases. Although the function of the CCM3 protein is unknown, the CCM3 gene is mutated in familial cerebral cavernous malformations, a condition associated with seizures and strokes. Our proteomics survey indicates that a large portion of the CCM3 protein resides within the STRIPAK complex, opening the way for further studies of CCM3 biology. The STRIPAK assembly establishes mutually exclusive interactions with either the CTTNBP2 proteins (which interact with the cytoskeletal protein cortactin) or a second subcomplex consisting of the sarcolemmal membrane-associated protein (SLMAP) and the related coiled-coil proteins suppressor of IKK (SIKE) and FGFR1OP2. We have thus identified several novel PP2A-containing protein complexes, including a large assembly linking kinases and phosphatases to a gene mutated in human disease. Molecular & Cellular Proteomics 8:157–171, 2009.

Protein phosphatase 2A (PP2A)\(^1\) is a major eukaryotic serine/threonine phosphatase that has been implicated in the control of cell growth, proliferation, and differentiation (1–4). The catalytic subunit of PP2A is represented by two genes in humans (gene names are in the supplemental materials; gene products here are referred to as PP2Ac\(a\) and PP2Ac\(b\) sharing 97% identity at the protein level (5). Many mutually exclusive protein complexes containing the PP2A family catalytic subunits have been characterized biochemically (3, 6). The PP2A catalytic (PP2Ac) subunit binds directly to the PP2A A scaffolding (PP2A A) subunits, the striatin (PP2A regulatory B” subunits), the striatin-associated protein Mob3, the novel proteins STRIP1 and STRIP2 (formerly FAM40A and FAM40B), the cerebral cavernous malformation 3 (CCM3) protein, and members of the germinal center kinase III family of Ste20 kinases. Although the function of the CCM3 protein is unknown, the CCM3 gene is mutated in familial cerebral cavernous malformations, a condition associated with seizures and strokes. Our proteomics survey indicates that a large portion of the CCM3 protein resides within the STRIPAK complex, opening the way for further studies of CCM3 biology. The STRIPAK assembly establishes mutually exclusive interactions with either the CTTNBP2 proteins (which interact with the cytoskeletal protein cortactin) or a second subcomplex consisting of the sarcolemmal membrane-associated protein (SLMAP) and the related coiled-coil proteins suppressor of IKK (SIKE) and FGFR1OP2. We have thus identified several novel PP2A-containing protein complexes, including a large assembly linking kinases and phosphatases to a gene mutated in human disease. Molecular & Cellular Proteomics 8:157–171, 2009.

\(1\) The abbreviations used are: PP2A, protein phosphatase 2A; PP2Ac, PP2A catalytic; STRIPAK, striatin-interacting phosphatase and kinase; STRIP1, striatin-interacting protein 1 (formerly FAM40A); STRIP2, striatin-interacting protein 2 (formerly FAM40B); GCK-III, germinal center kinase, subgroup III; CCM, cerebral cavernous malformation; AP, affinity purification; TAP, tandem affinity purification; SIKE, suppressor of IKK; SLMAP, sarcolemmal membrane-associated protein; IP, immunoprecipitation; IPI, International Protein Index; HA, hemagglutinin; CCT, chaperonin-containing TCP1; FOP, fibroblast growth factor receptor 1 oncogenic partner; CTTNBP2, cortactin-binding protein 2; CTTNB2NL, cortactin-binding protein 2, N-terminal-like; aa, amino acid(s); FHA, forhead-associated; FGFR1, fibroblast growth factor receptor 1; CnMKIV, calcium-calmodulin-dependent kinase IV; STRN, striatin. See supplemental materials for gene names; LTQ, linear trap quadrupole; XML, eXtensible Markup Language.
High confidence interactors are in turn cloned, expressed, and identified using mass spectrometry (gel-free method). Interaction partners are purified, subjected to proteolytic digestion, and characterized using tandem affinity purification (TAP) and FLAG affinity purification to produce a high density interaction map surrounding the PP2A catalytic subunit (we previously reported on the same approach used to study PP4c (33)). Our map recapitulated previously characterized assemblies for PP2A but also allowed the discovery of a large complex surrounding the previously characterized PP2Ac–PP2A–a-striatin-Mob3 assembly. This complex, which we refer to as striatin-interacting phosphatase and kinase (STRIPAK), contains the uncharacterized proteins Fam40A/Fam40B, which we have renamed striatin-interacting proteins 1 and 2 (STRIP1 and STRIP2). In addition, STRIPAK contains all members of the GCK-III subfamily of Ste20 protein kinases (STK24, STK25, and STST4) as well as cerebral cavernous malformation 3 (CCM3; gene name PDCD10), a protein encoded by a gene mutated in familial cases of cerebral cavernous malformations (34, 35). In addition, we present evidence that STRIPAK can establish mutually exclusive interactions with the cortactin-binding proteins cortactin-binding protein 2 (CTTNBP2)/cortactin-binding protein 2, N-terminal-like (CTTNBP2NL) or with an assembly of sarcocelmal membrane-associated protein (SLMAP) and the related proteins SIKE/FGFR1OP2.

Given the relevance of the composition of protein complexes in the biological functions of PP2A, it is important to devise approaches to characterize the many PP2A-containing molecular assemblies. Affinity purification coupled to mass spectrometry (AP-MS) is a powerful method to identify and characterize interaction partners (21–26). However, although a single AP-MS can successfully identify multiple interactors, this method is uninformative regarding the relationships between components of multiple multiprotein complexes containing a protein of interest. For example, AP-MS of PP2Ac or PP2A A would yield identification of both B and B’ proteins in the same purification, yet B and B’ proteins are mutually exclusive binding partners of the PP2Ac–PP2A A core and are never found in the same complex (2). Generating a high density interaction map in which most, and ideally all, interacting partners are purified in parallel experiments consisting of reciprocal purifications can help to resolve the components of individual complexes. The strategy utilized here is depicted in Fig. 1A. Briefly, the protein of interest is epitope-tagged and stably expressed in human cells. The protein and its interacting partners are purified, subjected to proteolytic digestion, and identified using mass spectrometry (gel-free method). High confidence interactors are in turn cloned, expressed, and purified, and their interacting partners are identified. The process may be repeated in an iterative fashion to generate high density local interactomes centered around proteins of interest. The high density of the interactions allows for a more precise understanding of the multitude of alternative complexes containing a particular protein of interest, a problem not always addressed in lower density global interaction studies (27–30).

Here we utilized tandem affinity purification (TAP) and FLAG affinity purification to produce a high density interaction map surrounding the PP2A catalytic subunit (we previously reported on the same approach used to study PP4c (33)). Our map recapitulated previously characterized assemblies for PP2A but also allowed the discovery of a large complex surrounding the previously characterized PP2Ac–PP2A–a-striatin-Mob3 assembly. This complex, which we refer to as striatin-interacting phosphatase and kinase (STRIPAK), contains the uncharacterized proteins Fam40A/Fam40B, which we have renamed striatin-interacting proteins 1 and 2 (STRIP1 and STRIP2). In addition, STRIPAK contains all members of the GCK-III subfamily of Ste20 protein kinases (STK24, STK25, and STST4) as well as cerebral cavernous malformation 3 (CCM3; gene name PDCD10), a protein encoded by a gene mutated in familial cases of cerebral cavernous malformations (34, 35). In addition, we present evidence that STRIPAK can establish mutually exclusive interactions with the cortactin-binding proteins cortactin-binding protein 2 (CTTNBP2)/cortactin-binding protein 2, N-terminal-like (CTTNBP2NL) or with an assembly of sarcocelmal membrane-associated protein (SLMAP) and the related proteins SIKE/FGFR1OP2.

**EXPERIMENTAL PROCEDURES**

cDNA and Stable Cell Lines— Constructs used in this study are described in supplemental Table S2; all inserts were sequenced in their entirety. Briefly, coding sequences for proteins of interest were amplified by PCR using Phusion Ultra (or other high fidelity polymerases) from a HeLa cell cDNA library (Stratagene) or cDNA clones from the mammalian gene collection or IMAGE collection. Whenever human cDNAs were available they were used; in some cases, the mouse ORF was utilized instead. Our original clone for mouse Fam40A/STRIP1 lacked the initiator methionine and a glutamic acid codon as compared with the longest mouse cDNA (GenBank accession number NM_153563). A full-length construct of mFam40A/STRIP1 was therefore generated by adding coding sequences for methionine and glutamic acid in the PCR primer. The resulting sequence corresponds to GenBank accession number NM_153563 (coding sequence). Inserts were cloned in-frame into pcDNA3-FLAG, pcDNA3–3HA, or pcDNA3–NTAP vectors. Constructs were sequenced and found to match the sequence of the original cDNAs with the exception of MST4, which carried a single amino acid substitution (Ile-356 to Thr; no wild type clones were obtained). These constructs were stably expressed in low passage HEK293 cells as described previously (33), and pools of selected cell lines were generated. Expression was monitored by Western blotting using normal rabbit serum (for TAP-tagged constructs) or an antisera directed against the FLAG epitope, respectively. To determine the approximate percentage of the FLAG-tagged proteins solubilized in our extraction pro-
procedure, we separately analyzed the pellet and supernatant following lysis; in each case, at least half of the protein was extracted, consistent with the previous annotation of many of the complex components as distributed in both membrane-associated and soluble compartments.

**Antibodies**—Rabbit anti-GST-alpha4 (rabbit 2972, a kind gift from Dr. N. Sonenberg) recognizes a single major band in HEK293 cell extract (not shown). Commercial antibodies were as follows (catalog numbers are in parentheses): anti-PP2Acα (610555), anti-striatin (610838), and anti-MST4 (612684) were from BD Transduction Laboratories; anti-PP2A A was from Upstate Biotechnology (67-250); anti-SG2NA (STRN3) was from Cell Signaling Technology (S68); anti-PP4R2 was from Bethyl Laboratories (A300-838A), anti-FLAG was from Sigma (F3165); and anti-HA was from Covance Research Products (MMS-101P). Secondary antibodies for immunoblotting were donkey anti-mouse IgG and donkey anti-rabbit IgG, both conjugated to horseradish peroxidase (GE Healthcare). For the IP/Western of MST4 in Fig. 3A, the anti-mouse IgG TrueBlot Ultra horseradish peroxidase conjugate from eBioscience (88-8817-31) was used as a secondary antibody.

**Tandem Affinity Purification and Mass Spectrometric Analysis**—Tandem affinity purification was performed essentially as described previously (33), and proteins were eluted by incubation in 25 mM EGTA in 50 mM ammonium bicarbonate, pH 8. Sequencing grade modified trypsin (0.5–1 μg; Promega) was added directly to the eluate. Digestion was performed overnight at 37 °C. Following digestion, the sample was lyophilized and then resuspended in reversed-phase HPLC buffer A1 (20 μl; 0.4% AcOH, 0.005% heptafluorobutyric anhydride in H2O). Prior to loading onto the reversed-phase column, the sample was centrifuged at 13,000 rpm for 10 min, and the supernatant was transferred to a fresh tube. Microcapillary reversed-phase columns (75-μm inner diameter, 363-μm outer diameter; Polymicro Technology) were cut to a final length of 15–20 cm, and spray tips were prepared as above. Each IP/Western of MST4 in Fig. 3A, the anti-mouse IgG TrueBlot Ultra horseradish peroxidase conjugate from eBioscience (88-8817-31) was used as a secondary antibody.

**FLAG Affinity Purification, Immunoprecipitation Using Antibodies to Endogenous Proteins, and Mass Spectrometric Analysis**—FLAG affinity purification was performed essentially as described previously (32) with the following modifications. Detergent concentration in the lysis buffer was 0.5% Nonidet P-40, the lysis buffer was added at 4 ml/g of wet cell pellet, and cells were subjected to passive lysis (30 min) followed by one freeze-thaw cycle. For the AP-MS of endogenous proteins, 0.3 mg of cell pellet from untransfected HEK293 cells was resuspended in 2.7 ml of lysis buffer, and cell extract was prepared as above. For each IP, 7 mg of crude lysate was used. The lysate was subjected to two preclearing steps: one on 20 μl of packed protein G beads for 1 h and one on 20 μl of packed protein G beads preincubated with 4 μl of anti-HA antibody for 1 h. IP was performed by adding anti-MST4 (16 μl), anti-striatin (8 μl), or anti-FLAG (8 μl) antibodies and G-Sepharose (25 μl of packed beads) to the preclarified lysate and incubating for 2 h. Beads were washed three times in lysis buffer and three times in 50 mM ammonium bicarbonate. Samples (from the FLAG or endogenous immunoprecipitations) were eluted with ammonium hydroxide, lyophilized in a SpeedVac, resuspended in 50 mM ammonium bicarbonate (pH 8–8.3), and incubated at 37 °C with trypsin overnight. The ammonium bicarbonate was evaporated, and the samples were resuspended in HPLC buffer A2 (2% acetonitrile, 0.1% formic acid) and then directly loaded onto capillary columns packed in house with Magic 5 μm, 100 Å, C18 AQ. MS/MS data were acquired in data-dependent mode (over a 2-h acetonitrile 2–40% gradient) on a ThermoFinnigan LTQ or a ThermoFinnigan LTQ-Orbitrap equipped with a Proxeon NanoSource and an Agilent 1100 capillary pump. mzXML files were generated from ThermoFinnigan *.RAW files using the ReAdW tool available in the TransProteomic Pipeline platform. The searched database contained the human IPI fasta sequence file (version 3.38; 70,856 sequences) and an Agilent 1100 capillary pump. mzXML files were generated with X!Tandem (39) with k-score plug-in (40) using the following parameters: b- and y-ion partials, trypsin digestion, allowing for one missed cleavage site, and methionine oxidation and N-terminal acetylation specified as variable modifications. The fragment mass tolerance was 0.8 Da (monoisotopic mass), and the mass window for the precursor was from −1 to 4 Da (average mass) in the case of LTQ data and from −100 to 100 ppm (monoisotopic) in the case of LTQ-Orbitrap data.

Search results were converted into pepXML format using Tandem2XML. PeptideProphet (37) was run on each result set, generating probability scores for each search result that are added to the pepXML documents. In the case of Orbitrap data, the high mass accuracy option was used. The resulting lists of peptides were assembled into proteins using ProteinProphet (38). ProteinProphet files were parsed and exported into a local Mysql database for further analysis and extraction of spectral count information.

Data were exported into Excel files and manually curated. Biologically replicates were analyzed for FLAG-PP2Ac (n = 3) and FLAG alone (n = 3) to verify the reproducibility of the procedure (not shown). To generate lists of high confidence specific interactors, proteins detected in any of the FLAG alone samples were subtracted from the final list. Only those proteins that were detected with spectral counts >20 with at least two of the peptides were reported. The error rate is 0% as measured by both ProteinProphet and using decoy scores. Supplemental Table S5 contains the experimental data; supplemental Tables S6 and S7 list the proteins identified on the basis of a single peptide together with additional evidence for the interaction. Supplemental Fig. S7 presents the annotated spectra of those proteins identified by a single unique peptide.

**STRIPAK Links Phosphatases and Kinases to CCM3**

- **Molecular & Cellular Proteomics 8.1**
High confidence interactions reported here were submitted to the BioGrid (41).

Immunoprecipitation/Western Blot—Lysates from stable cell lines (prepared as above) were incubated with FLAG M2-agarose beads for 1.5 h, washed as above, and directly eluted by boiling in Laemmli or Criterion XT sample buffer. Alternatively lysates from untransfected HEK293 cells were incubated with protein G-Sepharose (GE Healthcare) and antibodies to endogenous striatin (5 μl), striatin3 (2.5 μl), MST4 (2.5 μl), PP4R2 (5 μl), or the FLAG epitope (2.5 μl) for 2 h and washed three times in lysis buffer and once in PBS prior to elution in 25 μl of 2× Laemmli sample buffer. Precipitated proteins were separated via SDS-PAGE, transferred to nitrocellulose, and detected with antibodies directed against the FLAG or HA epitopes or endogenous proteins.

**RESULTS**

A PP2A High Density Interaction Network—The catalytic subunits of PP2A (PP2Acα and PP2Acβ) were cloned in-frame C-terminal to a TAP cassette and stably expressed in HEK293 cells. Cells were lysed, and proteins recovered following dual purification were analyzed by mass spectrometry as described previously (33). After background subtraction (as in Ref. 33), we identified high confidence interactors for these catalytic subunits (Table I). Several of the interactors (alpha4 and the multisubunit CCT chaperonin complex) were detected previously with PP4c (note that we previously detected PP2Ac, PP4c, and PP6c peptides in immunoprecipitates from another protein, hTip41, but that we have not detected pep-



These results indicate that our approach can successfully identify complexes described previously by biochemical approaches and that this type of analysis can be extended to identify potentially novel interactions.

In addition to the classical PP2A family trimers, a number of additional proteins were also detected in our network. For example, several peptides for liprin α (PPFIA1) were identified in PP2A Aα purifications (and a few in PP2Ac purifications). Liprins are multidomain scaffolding proteins first identified as interactors of the leukocyte antigen-related (protein tyrosine phosphatase, receptor type F) tyrosine phosphatase (40) linked to synaptic functions in several organisms (40, 44). A recent publication reported the interaction of liprin α with the Bγ PP2A regulatory subunit (45). Another interaction partner for PP2Acβ is FOP (also see supplemental Table S5), a protein associated with CAP350, required for microtubule anchoring to the centrosome (46). FOP was initially discovered as a fusion partner for fibroblast growth factor receptor 1 (FGFR1) in stem cell myeloproliferative disorders and confers dimerization (and constitutive activation) to FGFR1 (47–49). Other than the liprins and FOP, all other new PP2A interactors co-purified with striatin4 and Mob3; these interactors are described in more detail below. FOP and liprins did not associate with Mob3 and striatin4 and will be the topic of a separate follow-up study.

PP2A, Striatins, and Mob3 Stably Associate with Novel Components—We were particularly intrigued by an apparent higher order complex containing the catalytic and scaffolding PP2A subunits, the regulatory Bζ molecule striatin4, and the Mob3 protein. Three striatin proteins exist in humans (gene names STRN, STRN3, and STRN4). Striatin expression levels are highest in brain where it has been implicated in synaptic functions (50–54). Earlier co-precipitation studies (16) identified the small Mob3 protein (referred to in the initial publications as phoecin or hMob1, gene name MOBKL3 (15, 55)) as a striatin-interacting partner. Multiple Mob-like proteins exist in higher eukaryotes that appear to regulate the related nuclear DumbBell forming (Dbf)2-related and large tumor suppressor family of kinases through direct binding (56). Other striatin interactors detected in these earlier studies by co-immunoprecipitation and two-dimensional gel electrophoresis remained uncharacterized (16).

Our TAP tagging and AP-MS analysis of striatin4 and Mob3 (as well as PP2Ac and PP2A A) yielded many peptides corresponding to two different protein families: 1) FAM40A/FAM40B, which we have renamed STRIP1 and STRIP2 (also see below), and 2) CTTNBP2NL/CTTNBP2 (Fig. 1, B and D; lower amounts of peptides for additional unrelated proteins were also detected). STRIP1 and STRIP2 are highly related proteins (68% identity; supplemental Fig. S1) of unknown function with no significant homology to other proteins in the human genome and no recognizable sequence motifs. However, the STRIP1/2 proteins bear 13% identity with the yeast
protein Far11, which has been linked to pheromone signaling, and 21% identity with the *Neurospora crassa* HAM-2 gene product, which is implicated in hyphal fusion (supplemental Fig. S1). CTTNBP2 and CTTNBP2NL are also related; the N terminus of CTTNBP2 is 38% identical and 53% similar to CTTNBP2NL over 649 aa (supplemental Fig. S2). This N-terminal portion is predicted to contain a ~200-aa stretch of coiled-coil motifs. A shorter variant of CTTNBP2 (also known as CBP90), corresponding to the N-terminal 630 aa, was first identified as a protein interacting with the Src homology 3 domain of cortactin (cortical actin-binding protein) in an overlay assay and GST pulldown (57). This interaction is apparently mediated by a proline-rich region at the C terminus of CTTNBP2 that is conserved in CTTNBP2NL (supplemental Fig. S2). Note that we did not detect cortactin in any of our interaction studies.

### Table 1

**Human PP2A-type phosphatase interaction network**

Common names for the bait and prey proteins are listed alongside the spectral counts (PeptideProphet probability value >0.9; PEPT) and sequence coverage (% COVER) for the prey proteins. Results were sorted according to decreasing prey protein coverage. Preys in bold are STRIP1 and STRIP2 as well as CTTNBP2 and CTTNBP2NL, which are studied further in Fig. 2. For identifications based on a single unique peptide, a list of the experimental evidence is presented in supplemental Tables S6 and S7; annotated spectra are shown in supplemental Fig. S7.

| BAIT   | PREY   | PEPT | % COVER | BAIT   | PREY   | PEPT | % COVER | BAIT   | PREY   | PEPT | % COVER |
|--------|--------|------|---------|--------|--------|------|---------|--------|--------|------|---------|
| PP2Ac  | PP2Ac  | 112  | 63.2    | PP2Ac  | PP2Ac  | 124  | 55.0    | Mob3   | dynemin| 6    | 62.9    |
| PP2Acβ | PP2Acβ | 77   | 55.3    | PP2Acβ | PP2Acβ | 39   | 45.5    | Mob3   | 27    | 50.3   |
| PP2Acα | PP2Acα | 64   | 55.1    | PP2Acα | PP2Acα | 33   | 44.9    | striatin| 63    | 46.4   |
| alpha4 | 37     | 51.3 |         | PP2Acαβ| PP2Acαβ| 36   | 42.7    | striatin| 63    | 46.4   |
| PP2Acβδ| 29     | 37.0 |         | STRIP1  | 27   | 27.2  |
| PP2Acβδ| 11     | 27.5 |         | STRIP1  | 27   | 27.2  |
| Mob3   | 4      | 22.8 |         | STRIP1  | 27   | 27.2  |
| striatin3| 9     | 15.8 |         | striatin| 63    | 46.4   |
| PP2Acβ | 10     | 14.6 |         | striatin| 63    | 46.4   |
| PP2Acβ | 11     | 14.4 |         | striatin| 63    | 46.4   |
| STRIP1 | 4      | 12.3 |         | striatin| 63    | 46.4   |
| PP2Acβ | 4      | 12.2 |         | striatin| 63    | 46.4   |
| CCT2   | 2      | 7.9  |         | striatin| 63    | 46.4   |
| TCP1   | 2      | 5.6  |         | TCP1   | 56    | 54.1  |
| striatin| 2     | 4.5  |         | striatin| 63    | 46.4   |
| PP2Acβα| 2      | 3.7  |         | striatin| 63    | 46.4   |
| striatin4| 4     | 2.5  |         | striatin| 63    | 46.4   |
| PP2Acβ | 69     | 46.3 |         | PP2Acβ | 56    | 54.1  |
| PP2Acβ | 60     | 45.6 |         | PP2Acβ | 56    | 54.1  |
| PP2Acβ | 60     | 45.6 |         | PP2Acβ | 56    | 54.1  |
| striatin| 55     | 39.7 |         | striatin| 63    | 46.4   |
| STRIP1 | 41     | 38.8 |         | striatin| 63    | 46.4   |
| striatin| 41     | 38.8 |         | striatin| 63    | 46.4   |
| striatin3| 22    | 34.3 |         | striatin| 63    | 46.4   |
| striatin4| 30    | 33.9 |         | striatin| 63    | 46.4   |
| TCP1   | 22     | 31.1 |         | TCP1   | 56    | 54.1  |
| CCT2   | 20     | 30.5 |         | CCT7   | 39    | 53.8  |
| alpha4 | 12     | 28.6 |         | CCT4   | 35    | 47.4  |
| PP2Acβ | 10     | 26.7 |         | CCT3   | 32    | 47.1  |
| Mob3   | 12     | 22.8 |         | CCT5   | 41    | 46.0  |
| CCT4   | 8      | 22.6 |         | PP2Acβ | 51    | 44.7  |
| CCT3   | 12     | 21.3 |         | PP2Acβ | 44    | 44.4  |
| CCT6A  | 14     | 19.8 |         | CCT8   | 33    | 39.4  |
| CCT5   | 12     | 19.6 |         | CCT6   | 42    | 37.2  |
| CCT8   | 9      | 16.6 |         | PP2Acβ | 16    | 28.8  |
| CTTNB2NL| 14    | 15.2 |         | PP2Acβ | 10    | 9.1   |
| FOP    | 3      | 4.2  |         | PP2Acβ | 10    | 9.1   |
| PP2Acβδ| 2      | 9.1  |         | PP2Acβ | 10    | 9.1   |
Because we obtained many more peptides for STRIP1 (FAM40A) and CTTNBP2NL than for their respective paralogs in the TAP experiments, we used these two proteins for subsequent IP/Western validation studies. STRIP1 and CTTNBP2NL were cloned into the pcDNA3-FLAG vector (see “Experimental Procedures”) and stably expressed in HEK293 cells. Lysates were subjected to immunopurification using FLAG M2-agarose beads followed by immunoblotting using antibodies directed against endogenous striatin and PP2Ac (the interaction of STRIP1 with PP2A Aα is shown in supplemental Fig. S3). As a control, cells were transfected with the FLAG vector alone or FLAG-alpha4 (according to our MS results, alpha4 interacts with PP2Ac but not with striatin). As shown in Fig. 2 (and despite the low expression of FLAG-CTTNBP2NL) STRIP1 and CTTNBP2NL co-precipitated striatin and PP2Ac, although the percentage of PP2Ac (and PP2A A; supplemental Fig. S3) co-precipitated was low. This is consistent with our MS results in which the number of STRIP1 and CTTNBP2NL peptides was highest in striatin4 and Mob3 purifications. The relatively low abundance of STRIP1 and CTTNBP2NL peptides in PP2Ac and PP2A A purifications (13 each with PP2Ac) may be accounted for by alternate complexes containing the same PP2Ac and PP2A A subunits (2–4).
**Interaction Partners for STRIP1 and CTTNB2NL**—We next focused on the characterization of proteins associating with STRIP1 and CTTNB2NL. To increase throughput and potentially enrich for weaker interactors, we utilized a purification strategy using a single N-terminal FLAG epitope. Although this approach yielded a significant increase in background contaminants (32, 58), it also allowed us to obtain significantly increased peptide coverage for each of the proteins in our complex. FLAG-tagged STRIP1 and CTTNB2NL stably expressed in HEK293 cells (as in Fig. 2) were used for AP-MS, and contaminant proteins were subtracted as described under “Experimental Procedures” to generate an annotated list of interactors (Table II and supplemental Table S5).

As shown in Table II, purification of the FLAG-tagged mouse STRIP1 protein efficiently recovered PP2A catalytic and scaffolding subunits as well as all three striatins and Mob3, confirming these interactions (Fig. 1). CTTNB2NL and CTTNB2 were also detected in STRIP1 AP-MS, indicating that these proteins can be present within the same complex as STRIP1. Additional proteins were also confidently identified by several peptides (Table III and text below for a brief description of these proteins), including SLMAP; two small related proteins, SIKE and FGFR1OP2; members of the GCK-III subfamily of the Ste20 kinases (STK24, STK25, and MST4); and the CCM3 protein. Purification of CTTNB2NL also recovered all of these proteins (including STRIP1/FAM40A and STRIP2/FAM40B) with the exception of SLMAP, FGFR1OP2, and SIKE (Figs. 3 and 4). A brief description of each of these interactors follows.

SLMAP was initially identified as a component of the cardiac sarcolemma involved in myoblast fusion and muscle contraction (59–61). Four SLMAP splicing variants have been studied; all encode a putative transmembrane domain toward the C terminus as well as two putative leucine zippers but vary in their N termini: one of the longest isoforms encodes an N-terminal FHA domain and localizes to the centrosome (62).

SIKE is a small coiled-coil-containing protein first characterized as an interaction partner for the kinases TBK1 and IKKe, and as a negative regulator of the interferon pathway (63). SIKE is 50% identical to another co-precipitating protein, FGFR1OP2. SIKE and FGFR1OP2 orthologs have been detected throughout metazoan, but no functions have been ascribed to these proteins in model organisms (supplemental Fig. S4). Interestingly FGFR1OP2 is implicated in the 8p11 myeloproliferative syndrome, a rare and aggressive...
hematological malignancy characterized by the fusion of unrelated genes to the FGFR1, resulting in the dimerization and activation of FGFR1 in a ligand-independent manner. The FGFR1OP2 fusion involves two coiled-coil domains from FGFR1OP2 that likely promote dimerization of the FGFR1OP2-FGFR1 protein (64).

The CCM3 protein is encoded by the gene PDCD10, mutations of which are found in families with cerebral cavernous malformations (34, 35, 65). Cerebral cavernous malformations are a type of angioma affecting 0.5–1% of the population (66–68). Although most individuals are asymptomatic, roughly 30% will develop symptoms ranging from headaches to seizures, strokes, and even death. The CCM3 protein was demonstrated previously to interact with the GCK-III sub-group of Ste20 family kinases both in yeast two-hybrid assays (where the interaction was first reported) and by co-immunoprecipitation followed by Western blotting (69, 70). Consistent with this, several peptides for these three Ste20 kinases (STK24, STK25, and MST4) were found in association with STRIP1 and CTTNB2NL in our study. These three kinases define a subgroup of the germinal center kinases (GCK-III) characterized by an N-terminal kinase domain followed by a C-terminal tail of unknown function. The GCK-III kinases have been linked to proliferation, apoptosis, and stress response, but the substrates and molecular mechanism of action remain largely unknown (71–77).

The interaction of STRIP1 and CTTNB2NL with endogenous MST4 was confirmed by immunoprecipitation/immunoblotting (Fig. 3A); FLAG-alpha4, which was used as a negative control, failed to co-precipitate MST4. We also monitored the interactions among endogenous proteins. Antisera directed against MST4, striatin, and striatin3 (but not anti-PP4R2 or anti-FLAG) all recovered striatin and striatin3 from untransfected HEK293 cells (Fig. 3B), indicating that the interaction detected between the MST4 kinase and striatins is not due to the presence of an epitope tag or mild overexpression. We also subjected the anti-MST4 and anti-striatin3 immunoprecipitates to mass spectrometry and were able to recover many of the binding partners also seen in FLAG AP-MS (supplemental Table S8).

### Table III

| Protein or paralogy group | %ID | Protein name | Structural motifs | Biological role |
|---------------------------|-----|-------------|------------------|----------------|
| 1 PP2Ac | 97 | PP2Acα | Phosphatase catalytic, PP2A family | Protein dephosphorylation; multiple substrates |
|                |     | PP2Acβ | Phosphatase catalytic, PP2A family | Protein dephosphorylation; multiple substrates |
| 2 PP2AAα | 85 | PP2AAα | HEAT repeats, PP2A scaffold | Phosphatase scaffold; multiple substrates |
|                |     | PP2AAβ | HEAT repeats, PP2A scaffold | Phosphatase scaffold; multiple substrates |
| 3 striatin | 46 | striatin | WD40, coiled-coil, PP2A regulatory | Protein binding; calcium signalling; synaptic functions |
|                |     | striatin3 | WD40, coiled-coil, PP2A regulatory | Protein binding; calcium signalling; synaptic functions |
|                |     | striatin4 | WD40, coiled-coil, PP2A regulatory | Protein binding; calcium signalling; synaptic functions |
| 4 N/A | Mob3 | Mob3 | None detected | Suggested roles in trafficking, synaptic functions |
| 5 68 | STRIP1 | STRIP1 | None detected | Not determined |
|                |     | STRIP2 | None detected | Not determined |
| 6 36 | CTTNB2NL | CTTNB2NL | Coiled-coil, Proline-rich | Not determined |
|                |     | CTTNB2 | Coiled-coil, Proline-rich, ankyrin repeats | Binds the cytoskeletal protein cortactin |
| 7 20 | SLMAP | SLMAP | Coiled-coil, FHA, leucine zipper, hydrophobic membrane anchor | Binds Traf3; activates stress pathway |
|                |     | TRAF3IP3 | Coiled-coil | Binds TRAF3; activates stress pathway |
| 8 47 | SIKE | SIKE | Coiled-coil | Binds IKKε, TBK1; negatively regulates interferon pathway |
|                |     | FGFR1O2 | Coiled-coil | Fusion partner for FGFR1 in myeloproliferative syndrome |
| 9 N/A | CCM3 | CCM3 | None detected | Gene mutated in cerebral cavernous malformations |
| 10 66 | STK24 | STK24 | Ste20 kinase, GCK-III subgroup | Protein phosphorylation; putative involvement in proliferation, apoptosis, stress response |
|                |     | STK25 | Ste20 kinase, GCK-III subgroup | Protein phosphorylation; putative involvement in proliferation, apoptosis, stress response |
|                |     | MST4 | Ste20 kinase, GCK-III subgroup | Protein phosphorylation; putative involvement in proliferation, apoptosis, stress response |
In summary, AP-MS of STRIP1 and CTTNBP2NL confirmed the interactions with PP2Ac, PP2A A, striatins, and Mob3 but also revealed that these proteins are capable of interacting with each other. In addition, SLMAP, SIKE, FGFR1OP2, CCM3, and the GCK-III subfamily of kinases were able to interact with these proteins and are putative components of this complex (Figs. 3 and 4).

**Reciprocal Interactions among Complex Components**—To further characterize the many newly detected protein-protein interactions, FLAG-PP2Aα, FLAG-PP2Acβ, FLAG-PP2A αα, FLAG-striatin, FLAG-striatin3, FLAG-STK24, FLAG-STK25, FLAG-CTTNBP2, and FLAG-SIKE were stably expressed in HEK293 cells for a second round of interaction studies. We could not express SLMAP in HEK293 cells (supplemental Fig. S6 and data not shown) and therefore cloned and expressed the only related family member of SLMAP, namely TRAF3IP3 (supplemental Fig. S5). SLMAP and TRAF3IP3 share 22% identity at the amino acid level (a stretch of 343 amino acids exhibits 28% identity and 50% similarity), and both proteins contain predicted coiled-coil regions. TRAF3IP3, initially cloned as a TRAF3 interactor involved in signaling via the c-Jun N-terminal kinase (JNK) pathway, is normally restricted to cells of lymphoid origin and is not endogenously expressed in HEK293 cells. At the onset of these studies (and because the similarity between SLMAP and TRAF3IP3 is fairly low) we did not know whether TRAF3IP3 would interact with the same partners as SLMAP. With the inclusion of FLAG-TRAF3IP3, the list of baits represents at least one member for each of the paralogous protein families detected in our STRIP1 and CTTNBP2NL AP-MS analyses.
The interaction of each of these proteins with endogenous striatins was confirmed by IP/Western from each of the FLAG-tagged stable cell lines (supplemental Fig. S6 and data not shown). AP-MS for each of the FLAG-tagged proteins was also performed to detect their associations with each other (Table II; a complete list of the MS data is in supplemental Table S5; interactions of some of these proteins with STK24 were reported previously by Ewing et al. (78), but the low density of the data did not allow determination of complex composition). Striatins as well as the Mob3 protein, the PP2A scaffolding subunit, CCM3, and the Ste20 kinases of the GCK-III group co-precipitated every other protein in this large group, indicating that these proteins have the ability to form interactions with each member of the complex. In addition, as shown above and as reported previously, we observed that the striatins can associate with each other; striatin brought down unique peptides for striatin3 and striatin4 and vice versa. The PP2Ac/PP2A and striatin-Mob3-STRIP-CCM3-GCK-III proteins thus form a large multiprotein complex in vivo that we refer to as the STRIPAK complex.

Interestingly AP of CTTNBP2 (as with CTTNBP2NL above) did not contain detectable amounts of SLMAP, SIKE, or FGFR1OP2. Conversely immunoprecipitates of the SLMAP-related protein TRAF3IP3 did not yield detectable amounts of CTTNBP2 or CTTNBP2NL but did yield large quantities of SIKE and FGFR1OP2. SIKE immunoprecipitates contained a large number of SLMAP and FGFR1OP2 peptides but no detectable CTTNBP2 or CTTNBP2NL peptides. These data suggest that the association of CTTNBP2NL or CTTNBP2 with the remainder of the STRIPAK complex may preclude the association of SLMAP/TRAF3IP3 and SIKE/FGFR1OP2 and...
vice versa; i.e., these interactions appear to be mutually exclusive. To validate these findings, transient transfections of either an HA-tagged version of CTTNBP2NL or of TRAF3IP3 with a panel of FLAG-tagged proteins (SIKE, Mob3, striatin3, STRIP1, STK24, CTTNBP2NL, TRAF3IP3, PP4R2, or FLAG alone) was performed. Recombinant FLAG-tagged proteins were immunoprecipitated, and the co-precipitation of the HA-tagged proteins was detected by immunoblotting with an antibody against the HA epitope. As expected, both HA-CTTNBP2NL and HA-TRAF3IP3 were efficiently recovered in Mob3, striatin3, STRIP1, and STK24 pulldowns but not in the negative controls (FLAG alone and PP4R2; Fig. 4, A and B). In agreement with our mass spectrometry results, however, HA-CTTNBP2NL was not detectable in SIKE or TRAF3IP3 pulldowns (Fig. 4A, top panel, lanes 1 and 2). Conversely HA-TRAF3IP3 was not detectable in CTTNBP2NL pulldowns but was easily detected in SIKE pulldowns (Fig. 4B, lanes 1 and 2) consistent with the notion that SIKE/FGFR1OP2 and SLMAP/TRAF3IP3 can be present in the same complex but do not co-exist with CTTNBP2NL/CTTNBP2.

Taken together, our data highlight a stable, core complex containing PP2A catalytic and scaffolding subunits, striatin(s), Mob3, and STRIP (Fig. 4, C and D). Because we did not detect CCM3 and the Ste20 kinases in our TAP experiments, they are not likely to be necessary for the formation of this core complex and may be associated with it more loosely to form the STRIPAK complex. The STRIPAK assembly appears to associate either with a member of the CTTNBP2 family or with SLMAP/TRAF3IP3 in combination with SIKE/FGFR1OP2.

Discussion

CCM3 and Its GCK-III Kinase Subfamily Partners Are Physically Associated with PP2A Complexes—Here we report the identification of a large molecular assembly (STRIPAK) that links the PDCD10 gene product CCM3 and associated GCK-III kinases to a PP2A-striatin-Mob3-STRIP complex. Mutations in the CCM1, CCM2, and CCM3 genes account for nearly all cases of familial cerebral cavernous malformations (68). Previous proteomics studies detected an interaction between CCM3, CCM1, and CCM2, suggesting a common mode of action for all three proteins (70, 79). However, here we demonstrate that (at least in HEK293 cells) CCM3 preferentially associates with GCK-III kinases and our newly defined PP2A-striatin-Mob3-STRIP complex. No CCM1 or CCM2 peptides were detected in our analyses either with CCM3 or with other STRIPAK components. However, we did obtain evidence for an interaction between CCM3 and CCM2 when performing AP-MS using CCM2 (rather than CCM3) as a bait. In that case, FLAG-tagged CCM2 pulldowns yielded numerous peptides for CCM1; yet a small number of CCM3 peptides were also detected (not shown). These data suggest that CCM1 and CCM2 form an abundant complex that likely associates with CCM3 in sub-stoichiometric amounts, whereas a large portion of CCM3 is associated with the STRIPAK complex. Hence we posit that members of the PP2A-striatin-Mob3-STRIP complex should be analyzed for their involvement in non-familial cases of CCM where the genetic basis for the disease is unknown (mutations in CCM1–3 only account for 60% of sporadic cases (68)).

Interactions between GCK-III Kinases and PP2A—It is intriguing that the STRIPAK complex contains both phosphatase and kinase components. Associations between PP2A and several other kinases have been reported previously. Casein kinase II, the ribosomal S6 protein kinase (S6K1), the calcium-calmodulin-dependent kinase IV (CaMKIV), and the Raf protein were all described as interactors for the catalytic subunit of PP2A (80–83) but were not identified as interaction partners for PP2A in this study. This apparent discrepancy may be due to differences in the biological system used (for example the initial identification of the CaMKIV-PP2A interaction was performed in T cells (80)) or more likely due to the differential sensitivity of the methods of analysis used. Previous studies detected interactions by affinity purification of one component (usually the kinase) followed by immunoblotting for the other factor (PP2A). By contrast, we relied on immunoprecipitation of the PP2A phosphatase coupled to direct MS analysis. Because PP2A is a highly abundant protein and is capable of interacting with multiple binding partners, the fraction of PP2A complexed with a specific protein may be below the detection limits of our method. In this regard, even the interactions of PP2A with the Ste20 kinases were not detected in every analysis; co-precipitation of the GCK-III kinases was much more readily detected when starting from striatin or other complex components that reside predominantly in STRIPAK.

The presence of the GCK-III kinases and the PP2A phosphatase within a single complex raises the intriguing question of the enzyme/substrate relationships. Are the kinases regulating the phosphatases or vice versa? Are the substrates for these two enzymes the other STRIPAK components, or are the substrates yet to be identified? Interestingly many of the STRIPAK components are phosphoproteins (16, 70, 76, 84, 85); known phosphopeptides are displayed in supplemental Figs. S1, S2, and S4. In addition, the striatins and the Mob3 protein were all described as interactors for the catalytic subunit of PP2A (80–83) but were not identified as interaction partners for PP2A in this study. This apparent discrepancy may be due to differences in the biological system used (for example the initial identification of the CaMKIV-PP2A interaction was performed in T cells (80)) or more likely due to the differential sensitivity of the methods of analysis used. Previous studies detected interactions by affinity purification of one component (usually the kinase) followed by immunoblotting for the other factor (PP2A). By contrast, we relied on immunoprecipitation of the PP2A phosphatase coupled to direct MS analysis. Because PP2A is a highly abundant protein and is capable of interacting with multiple binding partners, the fraction of PP2A complexed with a specific protein may be below the detection limits of our method. In this regard, even the interactions of PP2A with the Ste20 kinases were not detected in every analysis; co-precipitation of the GCK-III kinases was much more readily detected when starting from striatin or other complex components that reside predominantly in STRIPAK.

What Is the Function of STRIPAK?—Although our study allowed us to define a macromolecular complex(es) in which a large fraction of the CCM3 protein resides, the function of this complex remains to be elucidated. Several of the complex
components are localized to both the cytosol and intracellular membranes, and reports have linked them to intracellular trafficking in mammalian cells. For example, Mob3 (p60c) shares limited homology to clathrin adaptors and can interact with Eps15 (a protein involved in clathrin-mediated endocytosis) and the nucleoside-diphosphate kinase (whose product, GTP, is required for dynamin-dependent synaptic vesicle recycling) (91, 92). Similarly an IP/MS experiment with a peripheral endosome protein, GIPC, recently identified both STRN and STRN3 as binding partners (93). This is significant as GIPC regulates endocytosis and trafficking of the nerve growth factor receptor TrkA.

Interestingly STRIP1/2 orthologs are conserved from yeasts to humans; the budding yeast STRIP ortholog, Far11 (pheromone arrest 11) is a component of a protein complex (Far3-11) implicated in cell cycle arrest following pheromone signaling (94). Some sequence similarity can be detected between an additional Far protein, Far8, and the striatins (although the WD domain is not present in Far8). Limited sequence similarity is also detected between Far9/Vps64 and Far10 with SLMAP. Finally a link between the PP2A A ortholog (Tpd3) and Far11 was detected by both yeast two-hybrid assay (95) and TAP-MS,2 indicating that portions of the STRIPAK assembly are conserved throughout evolution.

Two of the STRIPAK components (STRIP and striatin) have orthologs that have been isolated independently by forward genetics screens in filamentous fungi. The only _Sordaria macrospora_ striatin ortholog, PRO11 regulates fungal cell differentiation and is required for fertility and fruiting body formation (96). The human striatin cDNA can functionally replace the PRO11 gene product, indicating functional conservation (96). The only STRIP ortholog in _Neurospora crassa_, HAM-2 has also been cloned from a genetic screen: HAM-2 mutants are female sterile, are slow growing, have short hyphae, and have a pronounced defect in hyphal fusion (97). Importantly directed mutations in the striatin homolog _HAM-3_ and the SLMAP/VPS64 homolog _HAM-4_, which has an FHA domain, result in strains with a fusion phenotype identical to that of _HAM-2_ mutants.3 These results indicate that components of the STRIPAK complex share similar functions in mediating hyphal fusion. As hyphal fusion may be a model system to study membrane fusion events, it is tempting to speculate that the STRIPAK complex may affect cell fusion in other systems. Consistent with this possibility, overexpression of SLMAP prevents the myoblast to myotube transition in C2C12 cells without precluding the expression of differentiation markers (98).

Although this study highlighted the composition of STRIPAK, the topology of this large multiprotein complex remains to be defined. For example, the striatins were shown to dimerize (or perhaps multimerize) (Ref. 16 and Tables I and II), raising the possibility that the STRIPAK complex may be asymmetric. The presence of so many paralogous protein species may indicate that subcomplexes containing different paralogs perform slightly different functions. For example, the functions of the three Ste20 kinases found in STRIPAK are likely very different: STK25 and to a lesser extent MST4 but not STK24 have been found associated with the Golgi apparatus and are required for Golgi integrity (84) An in-depth analysis will be required to further elucidate the function of the various paralogs within this complex.

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