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Tandem Affinity Purification in Transgenic Mouse Embryonic Stem Cells Identifies DDOST as a Novel PPP1CC2 Interacting Protein

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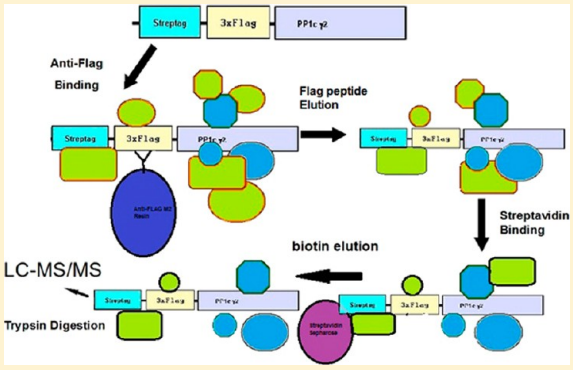
Supporting Information

ABSTRACT: Members of the PP1 family of protein phosphatases achieve functional diversity through numerous and varied protein−protein interactions. In mammals, there are four PP1 isoforms, the ubiquitously expressed PPP1CA, PPP1CB, and PPP1CC1, and the testis specific splice isoform PPP1CC2. When the mouse *Ppp1cc* gene is deleted, the only phenotypic consequence is a failure of spermatogenesis in homozygous males. To elucidate the function of the *Ppp1cc* gene, we sought to identify novel protein−protein interactions. To this end, we have created SBP-3XFLAG-PPP1CC1 and SBP-3XFLAG-PPP1CC2 knock-in mouse embryonic stem cell lines using a gene-trap-based system. Tandem affinity purification using our knock-in cell lines identified 11 significant protein−protein interactions, including nine known PP1 interacting proteins and two additional proteins (ATP5C1 and DDOST). Reciprocal in vitro sedimentation assays confirmed the interaction between PPP1CC2 and DDOST that may have physiological implications in spermatogenesis. Immunolocalization studies revealed that DDOST localized to the nuclear envelope in dissociated spermatogenic cells and persists throughout spermatogenesis. The knock-in system described in this paper can be applied in creating tandem affinity-tagged knock-in embryonic stem cell lines with any gene for which a compatible gene-trap line is available.



The current consensus is that members of the PP1 family of protein phosphatases achieve functional diversity via interaction with a large number of regulatory proteins termed PP1 interacting proteins (PIPs). To date, approximately 200 PIPs have been identified, but emerging evidence suggests that there could be many more, perhaps as many as 650.[1] Therefore, to fully understand the role of a PP1 isoform, such as PPP1CC2 (serine/threonine-protein phosphatase PP1-γ catalytic subunit, isoform 2), it is important to determine to the fullest extent possible the interactome of that protein. This can be a challenge for PIPs, as they are generally unrelated and difficult to identify on the basis of sequence alone. Previous attempts to identify PIPs computationally based on the presence of the RVxf motif that is present in many known interactors have been moderately successful;[2] however, they cannot identify the complete interactome, as not all PIPs contain an RVxf motif.

The difficulty of identifying PIPs in silico means that to fully describe the interactome of PP1s, experimental approaches are necessary. One powerful system of identifying protein−protein interactions is tandem affinity purification (TAP).[3] The use of two affinity purification steps significantly reduces the amount of contaminating proteins in the final product, making the technique more sensitive for the identification of legitimate interactors. The use of LC−MS/MS in protein identification aids in this sensitivity, allowing for the simultaneous identification of many interactors, even those at a relatively low stoichiometry. Additional advantages of TAP over other methods such as the yeast two-hybrid method include the ability to identify protein complexes that have more than two members and the ability to target expression of the bait protein to a variety of locations within the cell. Numerous TAP systems that feature improved expression in mammalian systems have been developed, employing combinations of affinity tags such as the FLAG tag and streptavidin-binding peptide (SBP).[4,5] In addition to being more readily expressed in mammalian cells, these tags are also smaller in size and thus less likely to interfere with the function of the tagged protein. TAP has now been used in many studies, most frequently using a cell culture with transiently expressed TAP-tagged proteins. Such techniques are able to give great insight into the interactomes of many proteins, but they do have limitations based on the artificial nature of the system, including false-positive interactions based on over- or misexpression of the bait protein. In addition, not all cell types or tissues can be modeled in culture, and thus cell or tissue specific interactions may be missed. In an effort to combat these limitations, some groups have begun to utilize

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TAP in transgenic mice. One such example utilized FLAG and HA tandem affinity-tagged Cyclin D1 knock-in mice to identify Cyclin D1 interacting proteins in multiple mouse tissues, including brain and eyes.[6]

PPP1CC2 is a testis specific splice isoform of the PP1 family member *Ppp1cc* that is essential for spermatogenesis.[7] Deletion of *Ppp1cc* results in a wide range of morphological abnormalities in the testis, most prominently a severe depletion of developing germ cells. However, the precise mechanism by which deletion of the *Ppp1cc* gene contributes to the failure of spermatogenesis remains unknown. In an effort to gain insight into the role of PPP1CC2 in spermatogenesis, our aim is to characterize its interactome. It is currently not possible to model the testis in cell culture because of its complex architecture, and the fact that most of the relevant cells are postmeiotic. Therefore, we have sought to generate a system for performing PPP1CC2 TAP in transgenic mouse cells. In an effort to recapitulate the wild-type expression pattern of the bait protein, and avoid artifacts associated with overexpression, we have relied on gene-trap technology to ensure that expression is under the control of the endogenous promoter. In addition to PPP1CC2, this system is amenable to any gene for which a gene-trap ES cell line is available, making it a powerful tool for assessing protein−protein interactions in a transgenic setting.

EXPERIMENTAL PROCEDURES

Knock-In Vector Construction. The streptavidin-binding peptide (SBP)-3XFLAG knock-in vector (Figure 1A) was built using pFloxin as a backbone.[8] The pFloxin plasmid contains a β-actin promoter sequence followed by a *lox66* site and was generously provided by B. Skarnes (Wellcome Trust Sanger Institute). Cre-mediated recombination between the *lox66* site in the knock-in vector and *lox71* in the pGT0lxf gene-trap vector creates a double mutant Lox site that shows a low affinity for Cre-recombinase and thus increases the efficiency of Cre-mediated insertion of the knock-in vector.[9] The Engrailed2 (*En2*) splice-acceptor sequence was cloned from pGT0lxf gene-trap line RRR804 (International Gene Trap Consortium) and inserted 3′ of the *lox66* site. The internal ribosomal entry site (IRES) of the encephalomyocarditis virus (ECMV) was cloned from the pGLUE plasmid[4] that was generously provided by S. Angers (Faculty of Pharmacy, University of Toronto). Immediately 3′ of the IRES, the SBP and 3XFLAG tandem affinity tags were inserted, having been cloned from the pGLUE and pTFHW plasmids, respectively. The SBP and 3XFLAG affinity tags were separated by a spacer sequence encoding amino acids GGSPGGT. Following the 3XFLAG affinity tag, an oligonucleotide sequence that included the coding region for the spacer peptide sequence GGSPGG, as well as SphI and AflII restriction sites, was inserted. Finally, a bovine growth hormone polyA signal fragment was cloned from the pGLUE plasmid and inserted 3′ of the multiple cloning site.

The coding sequences for *Ppp1cc1* and *Ppp1cc2* were amplified via PCR from pGEM7z vectors containing their respective full-length cDNA sequences[10] to generate sticky-ended PCR products according to the protocol of Walker et al.[11] Primer sequences are as follows: for *Ppp1cc1*, forward reaction 1, 5′-CATGGCGGATATCGACAAACTC-3′ (primer A); reverse reaction 1, 5′-TTAACTATTTCTTTGCTTGCTTTGTGATC-3′ (primer B); forward reaction 2, 5′-GCGATATCGACAAACTCAAC-3′ (primer C); reverse reaction 2, 5′-CTATTTCTTTGCTTGCTTTGTGATC-3′ (primer D); for *Ppp1cc2*, forward reaction 1, primer A; reverse reaction 1, 5′-TTAATCGGTCACTCGTAAGGA-3′ (primer E); forward reaction 2, primer C; reverse reaction 2, 5′-TCGGTCCACTCGTATAGGA-3′ (primer F). The sticky-ended PCR products were then ligated into the SphI (5′) and AflII (3′) sites of the SBP-3XFLAG knock-in vector. The vector sequence was verified by sequencing.

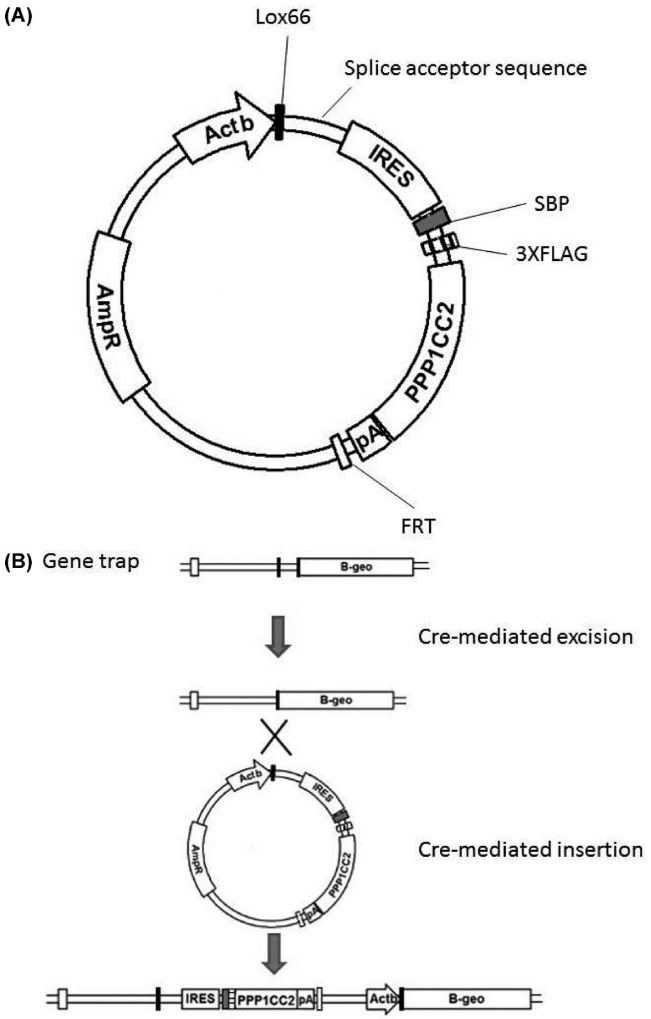

Figure 1. Strategy for the generation of SBP-3XFLAG-PPP1CC knock-in embryonic stem cells. (A) Schematic diagram of the knock-in vector used in this study. Abbreviations: Actb, β-actin promoter; pA, bovine growth hormone polyadenylation signal; AmpR, ampicillin resistance gene; SBP, streptavidin-binding peptide; SA, splice acceptor sequence; FRT, Flp recombinase site. (B) Two-step strategy for integration of the knock-in plasmid into pGTl0xf gene-trap lines.

Cell Culture. ES cells were cultured on 0.1% gelatin in high-glucose DMEM supplemented with 15% FBS, nonessential amino acids, 1 mM sodium pyruvate, 2 mM glutamine, penicillin-streptomycin, 10 μM β-mercaptoethanol, and 8 μg/mL leukemia inhibitory factor.

Cre-Mediated Excision. RRR804 (BayGenomics) ES cells were grown to ~70% confluence in a single well of a gelatinized 24-well tissue culture dish and subsequently transfected with 800 ng of pCMV-Cre-EGFP using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. The pCMV-Cre-EGFP plasmid encodes a Cre-EGFP fusion protein and was a generous gift from S. Cordes (Samuel Lunenfeld Research Institute, University of Toronto). Successful transfection was verified by viewing EGFP

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fluorescence. Proper Cre-mediated excision eliminates expression of the β-geo reporter sequence, resulting in G418 sensitivity. Thus, to aid in the selection of cells that have undergone Cre-mediated excision, we sorted pCMV-Cre-EGFP-treated RRR804 ES cells into GFP+ and GFP− fractions via fluorescence-activated cell sorting (FACS). Transfected cells were harvested and resuspended in 1× PBS, 5 mM EDTA, 25 mM HEPES, and 1% FBS, followed by filtration through a 40 μm nylon mesh cell strainer. FACS was conducted using a FACS Aria machine at the Faculty of Medicine Flow Cytometry Facility, University of Toronto. Sorted cells were collected in DMEM with 50% FBS, and the GFP+ fraction was plated on 10 cm tissue culture dishes at a low density in standard culture medium. Cells were grown for approximately 6 days until ES colonies became visible and were subsequently picked.

Cre-Mediated Insertion. RRR804-SA ES cells were grown to approximately 70% confluence in 10 cm tissue culture plates and were then trypsinized, harvested, and washed in PBS. After being resuspended in PBS, cells were counted using a hemacytometer. The cell concentration was then adjusted to approximately 10^7 cells per 800 μL of PBS; 10^7 RRR804-SA cells were added to a chilled electroporation cuvette along with 60 μg each of pCX-nlsCre and either PPP1CC1 or PPP1CC2 knock-in vector. pCX-nlsCre expresses Cre-recombinase with a nuclear localization signal, under the control of a β-actin promoter, and was a generous gift from A. Nagy (Samuel Lunenfeld Research Institute, University of Toronto). Cells were electroporated at 240 V and 500 μF using a Bio-Rad GenePulser. After electroporation, cells were incubated at room temperature for approximately 20 min and then plated evenly among six 10 cm tissue culture plates. G418 selection was started on the second day after electroporation and was conducted at a concentration of 300 μg/mL. Colonies were picked between days 8 and 12 of G418 selection.

RT-PCR. RNA extraction and cDNA synthesis were performed using standard protocols. The following primer sequences were utilized: 1F, 5′-CGCACAGTCTAGGTGGG-TATTGC-3′; 1R, 5′-CTGCAAAGGGTCGCTACAGACG-3′; 2F, 5′-ACGTGGTGGAGGGCCTGG-3′; 2R, 5′-GTGGTGT-GACAGGTCTCGTG-3′; 3F, 5′-GTTGCCTTTTATGGCT-CGAGCGG-3′; 3R, 5′-TAACCGTGCATCTGCCAGTTTG-AGG-3′; 4F, 5′-TGAGAGGGACAGGCCACCAAGG-3′; IRES forward primer, 5′-GGACGTGGTTTTCCTTTGAA-3′; *Ppp1cc* coding sequence 3′-region, 5′-TGTGACAGGTCTCG-TGGC-3′.

Western Blotting. SDS−PAGE and Western blotting were performed using standard protocols. The PPP1CC antibody (N-19, Santa Cruz Biotechnology) was used at a dilution of 1:500, with the donkey anti-goat HRP secondary antibody (Santa Cruz Biotechnology) at a dilution of 1:5000. The anti-FLAG M2 antibody (Sigma) was used at a dilution of 1:5000, with the goat anti-mouse HRP secondary antibody (Zymed) at a dilution of 1:5000.

Tandem Affinity Purification. The TAP protocol was adapted from Chen and Gingras[5] and Angers et al.[4] SBP-3XFLAG-PPP1CC knock-in ES cells were harvested by trypsinization and washed once with PBS. Cells were pelleted and flash-frozen in liquid nitrogen and stored at −80 °C until they were processed. Cells were thawed and resuspended in approximately 9 volumes of TAP lysis buffer [10% glycerol, 50 mM HEPES-NaOH (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.1% NP-40, 1 mM DTT, 10 mM NaF, 0.25 sodium orthovanadate, 50 mM β-glycerophosphate, 1 mM PMSF, and 1× sigma protease inhibitor] and incubated at 4 °C for 15 min while being rocked. The lysate was then frozen in liquid nitrogen and thawed at 4 °C, followed by centrifugation for 10 min at 10000*g* to remove insoluble material. Cleared lysate was then added to washed (3× with TAP lysis buffer) anti-FLAG-M2 resin (Sigma) at a ratio of approximately 2.5 μL of packed resin for each 10 cm plate of ES cells harvested. The FLAG pull-down fraction was incubated overnight at 4 °C with rocking and then washed three times with TAP lysis buffer. Protein was eluted from anti-FLAG-M2 resin using 3XFLAG peptide (Sigma) at a concentration of 100 μg/mL in 400 μL of TAP lysis buffer for 30 min at 4 °C with rocking. This elution step was repeated one additional time, and eluates were pooled. The FLAG eluate was then added to 50 μL of packed Streptavidin-Sepharose 4B (U.S. Biological) that had been washed three times in TAP lysis buffer and incubated overnight at 4 °C while being rocked. Streptavidin-Sepharose resin and bound proteins were washed three times with TAP lysis buffer and twice with streptavidin wash buffer [10 mM β-mercaptoethanol, 50 mM HEPES-NaOH (pH 8.0), 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 0.1% NP-40, and 2 mM $CaCl_2$]. Bound proteins were eluted from Streptavidin-Sepharose using streptavidin wash buffer supplemented with 50 mM D-biotin. Elution was performed with gentle vortex agitation at 4 °C, twice in 200 μL of elution buffer and once in 100 μL with all elutions pooled. Eluted proteins were precipitated via treatment with 30% trichloroacetic acid for 30 min on ice followed by centrifugation at ∼16000*g* for 30 min at 4 °C. The protein pellet was then washed twice with 500 μL of ice-cold acetone. The protein pellet was resuspended in 6 M urea and then reduced with 10 mM DTT in a final volume of 100 μL of 50 mM ammonium bicarbonate for 45 min at 50 °C. Next, to alkylate the sample, iodoacetamide was added to a concentration of 33.3 mM and the mixture incubated at room temperature for 1 h in the dark. To digest the sample, 1.2 μg of proteomics grade trypsin (Sigma) was added, and incubation at 37 °C was performed overnight. Trypsin was inactivated with 1% formic acid, and the sample was lyophilized.

Chimera Production. SBP-3XFLAG-PPP1CC2 knock-in ES cell clones were submitted to the Transgenic Core Facility at the Toronto Centre for Phenogenomics (TCP, http://www.phenogenomics.ca) for chimera generation via blastocyst microinjection with C57BL/6 hosts. ES cells were tested for both ploidy and the presence of contaminating viruses and bacteria (mycoplasma) before submission to TCP. Chimeric mice were identified via coat color. Chimeric mice were bred with both C57BL6 and CD1 mice, and offspring were identified by coat color.

LC−MS/MS Analysis and Database Searching for TAP Experiments. Peptide mixtures were subjected to LC−MS/MS analysis using a nanoLC system (Eksigent) attached to an LTQ-Orbitrap mass spectrometer (ThermoFisher), which was performed by the Centre for the Analysis of Genome Evolution and Function. LC−MS/MS data files were processed into peak lists using Mascot Daemon and submitted to Mascot version 2.3.02 (Matrix Science, London, U.K.) for database searching. SwissProt mouse protein database 57.15 (515203 entries) was queried, using the following parameters: carboxymethyl (C) set as a fixed modification, and oxidation (M) as a variable modification, fragment ion mass tolerance of 0.80 Da, and parent ion tolerance of 7 ppm.

His Tag Sedimentation Assay. A recombinant His-tagged DDOST fragment (Abcam), corresponding to amino acids

144−360 of human DDOST, was bound to Ni-NTA resin. The His-DDOST resin was incubated with 1 mg of mouse testis lysate in TAP lysis buffer for 2 h at 4 °C with rocking, followed by three washes with TAP lysis buffer. His-DDOST resin was boiled in 1× SDS−PAGE sample loading buffer and subjected to SDS−PAGE followed by Western blotting.

Immunohistochemistry. Wild-type and *Ppp1cc* mutant testes were harvested and placed in dMEM after the tunica albuginea had been removed and homogenized using a razor blade to release germ cells from the seminiferous tubules. To enhance germ cell release, tubules were squeezed repeatedly with square-tipped forceps. The resultant homogenate was mixed and allowed to settle for several minutes. The developing germ cells were then pelleted by centrifugation at 2000 rpm for 2 min. Following removal of the supernatant, the germ cells were resuspended in PBS and dropped onto poly-L-lysine-coated slides and fixed in 4% paraformaldehyde. Slides containing sections and dissociated cells were permeabilized with 0.01% Triton-X in PBS and blocked in 10% goat serum, 1% BSA, 0.01% Tween 20, and PBS solution, followed by a second blocking step in 0.1 mg/mL AffiniPure $F(ab')_2$ fragment goat anti-mouse IgG (Jackson ImmunoResearch). Primary antibody incubations were performed in antibody dilution buffer (5% goat serum, 1% BSA, 0.01% Tween 20, and PBS) overnight. Anti-DDOST (Santa Cruz Biotechnology, H-300) was used at a dilution of 1:250, and anti-MAb414 (Covance) was used at a dilution of 1:250. Secondary antibody incubations were performed in antibody dilution buffer for 2 h in the dark using Cy3-conjugated secondary antibodies, either AffiniPure goat anti-rabbit IgG (DDOST, 1:2000) or Dylight 488 goat anti-mouse (Abcam). Slides were then stained with DAPI and mounted in 50% glycerol for viewing with an Olympus BX60 microscope using the appropriate filters. Images were captured using Cool Snap and a CCD camera (RSPhotometrics). Images were merged using ImagePro Plus version 4.1 and adjusted for brightness and contrast using Photoshop 6.0 (Adobe).

Phosphopeptide Enrichment and Identification. Adult mouse testes were decapsulated and lysed in 400 μL of 7 M urea, 2 M thiourea, 4% CHAPS, and 40 mM Tris, reduced with 20 mM DTT for 1 h at room temperature, and alkylated with 40 mM iodoacetamide for 35 min at room temperature in the dark. Testis proteins were precipitated with acetone and dried using a speedvac, followed by resuspension in a 1 M urea/50 mM ammonium bicarbonate solution containing 10 μg of proteomics grade trypsin. Digestion was conducted overnight at 37 °C, and upon completion, the solution was acidified with 1% formic acid and centrifuged to remove insoluble material; 500 μg of testis protein was used for phosphopeptide enrichment via sequential elution from IMAC (SIMAC) using the protocol of Thingholm et al.,[12] but with 50 μL of TiO_2Mag Sepharose (GE Healthcare) substituted for TiO_2 phosphopeptide enrichment steps.

LC−MS/MS analysis and phosphopeptide identification were performed by the Advanced Protein Technology Centre (Toronto, ON). All MS/MS samples were analyzed using Sequest version 1.3.0.339 (Thermo Fisher Scientific, San Jose, CA) and X! Tandem version CYCLONE (2010.12.01.1) (The GPM, http://thegpm.org). Sequest was set up to search MOUSE-Uniprot-Sep-05-12.fasta (55250 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search the MOUSE-Uniprot-Sep-05-12 database (55270 entries) also assuming trypsin. Sequest and X! Tandem were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 ppm. The iodoacetamide derivative of cysteine was specified in Sequest and X! Tandem as a fixed modification. Oxidation of methionine and phosphorylation of serine, threonine, and tyrosine were specified in Sequest and X! Tandem as variable modifications. For protein identification, Scaffold version Scaffold_3.4.3 (Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they exceeded specific database search engine thresholds. Sequest identifications required deltaCn scores of at least >0.10 and XCorr scores of >1.8, >2.5, >3.5, and >3.5 for singly, doubly, triply, and quadruply charged peptides, respectively. X! Tandem identifications required at least −log(expect scores) scores of >2.0. Protein identifications were accepted if they contained at least one identified peptide. Proteins that contained similar peptides and could not be differentiated on the basis of MS/MS analysis alone were grouped to satisfy the principles of parsimony. Statistical analyses of phosphosite localizations were performed using ScaffoldPTM version 2.0.0 (Proteome Software Inc.).

RESULTS

Generation of SBP-3XFLAG-PPP1CC1 and SBP-3XFLAG-PPP1CC2 Knock-In ES Cell Lines. In an effort to identify interactors of PPP1CC isoforms from transgenic mouse cells, we created mouse embryonic stem (ES) cell lines carrying knock-in alleles of either PPP1CC1 or PPP1CC2 with N-terminal streptavidin-binding peptide (SBP) and 3XFLAG tandem affinity tags. A two-step strategy based on that originally proposed by Hardouin and Nagy[13] and also utilized by Singla et al.[8] was used (Figure 1B). The first step is the Cre-mediated excision of the additional loxP site in the pGT0lxf-based gene-trap line. pGT0lxf gene-trap ES cell lines feature a gene-trap cassette containing an *En2* splice acceptor sequence flanked by Lox71 (5′) and LoxP (3′) sites. Following the floxed splice acceptor is the coding sequence for the β-geo reporter gene. Recombination between the lox71 and loxP sites results in the removal of the splice acceptor sequence and should in theory restore the expression of the endogenous *Ppp1cc* transcript(s).

Ppp1cc gene-trap ES cell line RRR804, obtained through the International Gene Trap Consortium[14] and Mutant Mouse Regional Resource Centres, contains a gene-trap insertion at nucleotide 458 of intron 2, as verified by genomic DNA sequencing. RRR804 ES cells were transiently transfected with pCMV-Cre-EGFP plasmid that expresses a Cre-recombinase-EGFP fusion protein. Transfected cells were subjected to FACS, and the GFP-positive fraction was isolated. To assay for successful Cre-mediated excision, genomic DNA was isolated from GFP-positive colonies, followed by PCR using primers flanking the lox71-splice acceptor-loxP region. All GFP-positive colonies screened had successfully undergone Cre-mediated excision, as evidenced by the smaller PCR product in Figure 2. If the FACS step was omitted, the percentage of colonies that showed successful Cre-mediated excision was 25%. RRR804 cell lines with an excised splice acceptor region were designated RRR804-SA.

The second step is the integration of the knock-in plasmid at the remaining lox71 site. This was accomplished via Cre-mediated insertion resulting from recombination between the gene-trap lox71 site and a lox66 site in our knock-in plasmid. The backbone for our knock-in vector is the pFloxin plasmid

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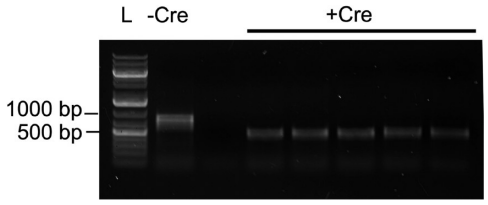

Figure 2. Cre-mediated excision of the second loxP site in pGT0lxf gene-trap line RRR804. Depicted is the genomic DNA PCR product amplified using primers flanking the lox71 and loxP sites in the gene-trap cassette. −Cre and +Cre indicate samples prior to and after treatment with Cre-recombinase, respectively; L indicates the DNA ladder. The expected size of the original genomic DNA fragment is 861 bp, while fragments having undergone Cre-mediated excision are expected to have 570 bp.

that was used in the generation of similar constructs by Singla et al.[8] To this backbone we added an *En2* splice acceptor sequence, followed by an IRES. Immediately following the IRES, the coding sequences for a SBP, a 3XFLAG tag, and either PPP1CC1 or PPP1CC2, as well as a BGH polyadenylation signal, were added (Figure 1A). Proper integration of the knock-in vector should lead to expression of a N-terminal SBP-3XFLAG-tagged version of the relevant transgene, under the control of the natural promoter for the gene-trap locus. This vector was designed such that a tandem affinity-tagged version of any transgene can be integrated into any pGT0lxf gene-trap line. RRR804-SA ES cells were simultaneously electroporated with both a Cre-recombinase-expressing plasmid (pCX-nls-Cre) and either the PP1CC1 or PPP1CC2 SBP-3XFLAG knock-in vector. To select for knock-in positive ES cells, transformants were grown in the presence of G418, as proper integration of the knock-in plasmid will expose the β-geo reporter gene to a β-actin promotor. A total of 48 G418 resistant ES cell colonies were isolated (19 PPP1CC1 and 29 PPP1CC2).

Verification of SBP-3XFLAG-PPP1CC1 and SBP-3XFLAG-PPP1CC2 Knock-In ES Cell Lines. To verify the presence of the knock-in allele, genomic DNA of 15 randomly selected G418 resistant colonies was assayed via PCR using a series of primer pairs (Figure 3). All but one of the 15 assayed G418 resistant ES cell colonies was positive for the presence of the knock-in construct. To verify the expression of the SBP-3XFLAG-*Ppp1cc* transcripts, G418 resistant clones were subjected to RT-PCR using a forward primer in the IRES sequence and a reverse primer in a 3′-region common to the *Ppp1cc1* and *Ppp1cc2* transcripts (Figure 4A). Expression of the SBP-3XFLAG-PPP1CC1 and SBP-3XFLAG-PPP1CC2 transgenes was detectable in respective knock-in ES cell lines at a size consistent with the predicted sequence. Finally, to verify the effective translation of the knock-in constructs, ES cell lysates were subjected to Western blotting with an antibody against the FLAG epitope. As seen in Figure 4B, both SBP-3XFLAG-PPP1CC1 and SBP-3XFLAG-PPP1CC2 knock-in ES cell lines showed a robust FLAG signal, while no signal was detected in the lysate from the original gene-trap ES cell line. Curiously, Western blotting with anti-PPP1CC showed only the <40 kb band of the endogenous PPP1CC1 signal, and no band with the expected molecular weight shift corresponding to the TAP tag. This could possibly be due to the fact that the PPP1CC antibody used is directed against the N-terminus, which may be sterically hidden by the N-terminal TAP tag.

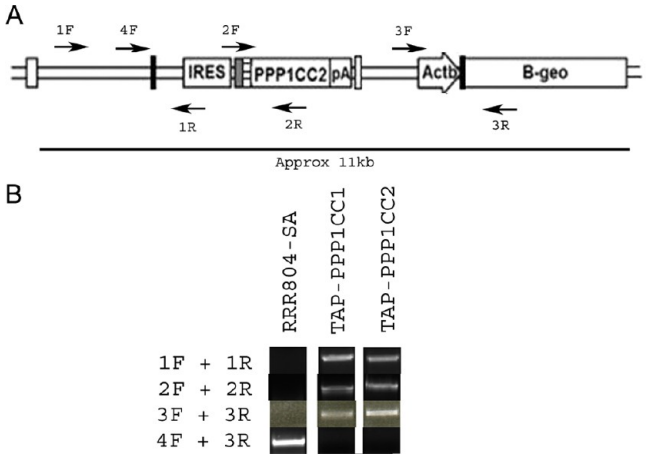

Figure 3. Verification of Cre-mediated insertion of the transgene into the RRR804-SA ES cell genome. (A) Schematic diagram depicting the genomic DNA sequence features of a transgene-positive clone at the locus of insertion. Sequence features are colored as in Figure 1. The location and direction of forward (F) and reverse (R) primers used in the following section are shown. (B) Genomic DNA PCR product of four different PCRs using the primer pairs indicated on the left in RRR804-SA gene-trap ES cells, and representative SBP-3XFLAG-PPP1CC1 and SBP-3XFLAG-PPP1CC2 knock-in clones.

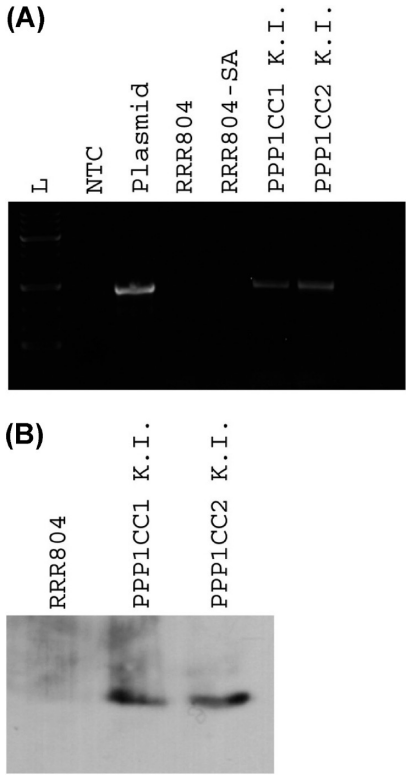

Figure 4. Expression of the SBP-3XFLAG-PPP1CC1 and -2 transgenes in knock-in ES cells at the cDNA and protein level. (A) Product from RT-PCR performed on ES cell RNA using a forward primer that anneals to the IRES sequence and a reverse primer that anneals to a region in the *Ppp1cc* coding sequence common to *Ppp1cc1* and *Ppp1cc2*. "Plasmid" indicates the use of the SBP-3XFLAG-PPP1CC knock-in plasmid as a template for the PCR, set to serve as a positive control. (B) Western blot of ES cell proteins using the anti-FLAG-M2 antibody (Sigma).

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Table 1. Protein−Protein Interactions Detected via Tandem Affinity Purification

gene symbol	Uniprot acession number	known PIP?	RVxF motif?	SBP-3XFLAG PPP1CC1 TAP[a]	SBP- 3XFLAG PPP1CC2 TAP[a]	best MASCOT protein score	no. of unique peptides	spectral count	% coverage
PPP1R7	Q3UM45	yes[34]	no	X	X	681	17	126	61.5
PPP1R8	Q8R3G1	yes[35]	yes	X	X	110	8	20	37.9
PPP1R2	Q9DCL8	yes[36]	yes	X	X	151	5	35	24.3
WDR82[b]	Q8BFQ4	yes[37]	no	X	X	68	4	8	16.3
DDOST	O54734	no	yes		X	56	3	3	6.3
PPP1R10	Q80W00	yes[38]	yes	X		43	3	5	6.1
PPP1R11	Q8K1L5	yes[39]	yes	X	X	75	2	11	39.7
RRP1B	Q91YK2	yes[40]	yes	X		29	3	8	6.9
ATP2A2	O55143	yes[42]	yes		X	85	2	3	3.8
ATP5C1	Q91VR2	no	no		X	41	2	2	7.4
LMTK2	Q3TYD6	yes[41]	yes	X	X	48	2	13	4.6
PPP1R42[c]	Q8R1Z4	yes[43]	yes		X	25	1	1	2

[a]X indicates that the protein was identified in a tandem affinity purification experiment with the indicated bait protein. [b]A protein shown to interact indirectly with PP1, via an intermediate protein. [c]An interaction that did not meet our requirement of two unique peptide hits but was included as it is a known PPP1CC2 interacting protein in the testis.

PPP1CC1 and PPP1CC2 Tandem Affinity Purification in ES Cells. To verify the functionality of the TAP tag in our SBP-3XFLAG-PPP1CC knock-in ES cells, TAP experiments were conducted in ES cell culture. Optimization of our ES cell TAP protocol resulted in a recovery of approximately 30% of our bait protein. This recovery is comparable with those of other mammalian TAP systems.[15] One note of particular interest is that initial experiments that utilized Streptavidin-Sepharose as the first affinity purification step yielded very little capture of the bait protein. Conversely, the efficiency of binding of the bait protein to the Streptavidin-Sepharose resin after elution from the anti-FLAG-M2 affinity resin was nearly 100%.

After optimization, TAP studies were performed on the cell lysate from SBP-3XFLAG-PP1CC ES cells, and the isolated proteins were digested with trypsin and subjected to LC−MS/MS. The resultant mass spectra were searched against the SwissProt mouse protein database using the MASCOT search tool, and unique peptides exceeding the threshold score calculated by MASCOT ($p < 0.05$; absolute value calculated independently in each experiment) were recorded. In total, approximately 60 tissue culture dishes (10 cm) each of SBP-3XFLAG-PPP1CC1 and SBP-3XFLAG-PPP1CC2 ES cells were subjected to TAP throughout three to five replicate experiments. Additionally, four negative control experiments were conducted using ES cells that did not express our knock-in construct. Lists of proteins identified in our TAP experiments as potential PPP1CC interacting proteins were subjected to a number of filtering criteria, designed to eliminate contaminants and identify bona fide interactors. First, proteins that were identified in our negative control experiments were discounted, as they were likely to be contaminants. Remaining proteins were then compared to three lists of likely contaminant proteins generated by other laboratories: a list of common background contaminants from FLAG IPs,[5] a data set from a series of FLAG IPs conducted in HEK293T cells expressing the FLAG tag (A. C. Gingras, unpublished data), and a TAP data set from experiments using the GS tag (includes SBP) that identifies nonspecific interactors.[16]

The top protein hit in all runs was identified as PPP1CC (average MASCOT protein score of 807), indicating that our transgenic lines were in fact expressing the expected bait protein, despite the lack of anti-PPP1CC signal in Western blotting. After application of the above-mentioned filtering parameters, significant unique peptides corresponding to 76 different proteins were identified (Table S1 of the Supporting Information). However, identification of a protein via a single unique peptide can result in a high incidence of false-positive interactions. Eleven proteins in our data set were identified by at least two unique and nonoverlapping peptides, representing a more confident set of PPP1CC interacting proteins (see Table 1). Among the identified PPP1CC interacting proteins, nine were known PP1 interacting proteins, thus validating the correct folding of our SBP-3XFLAG-PPP1CC fusion proteins in our knock-in system. Two of the known PP1 interacting proteins, WDR82 and LMTK2, had previously not been shown to bind to the PPP1CC2 isoform.

Aside from known PP1 interacting proteins, we have identified two novel PPP1CC interactors in our ES cell TAP experiments. However, one of these proteins, ATP5C1, is a member of the ATP synthase, H^+-transporting, mitochondrial F1 complex, to which three other proteins found in negative control pull-down data sets belong, calling its assignment as a legitimate PPP1CC interacting protein into question. The remaining putative PPP1CC interacting protein identified was dolichyl-diphosphooligosaccharide−protein glycosyltransferase 48 kDa subunit (DDOST). Examination of the amino acid sequences of this protein revealed the presence of the classical PP1 binding RVxF motif. DDOST contains the RVIF sequence as amino acids 239−242.

While identification of protein−protein interactions based on a single unique peptide is challenging and has a high rate of false positives, it is still possible to glean useful information from these cases. This is particularly true of low-abundance interactions that may be masked by the high abundance of previously described PP1 interaction partners such as PPP1R7. In fact, one known PPP1CC2 interacting protein, PPP1R42, was found in our list of proteins identified by a single unique peptide. One indication that there may be more legitimate PPP1CC interacting proteins among the low-confidence hit is the prevalence of the classical PP1 binding RVxF motif ([KR]-X(0,1)-[VI]-{P}-[FW]), found in up to 25% of all proteins[17] but found in 38% (25 of 65) in our single-peptide list, a significant enrichment ($p < 0.025$ via χ^2). One RVxF-motif containing protein that stands out as a candidate for PPP1CC2 interaction is BRWD1 (Bromodomain and WD repeat

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containing 1), which when deleted results in a male infertility phenotype very similar to that of the *Ppp1cc* knockout.[18]

Validation of PPP1CC2−DDOST Interaction in Testis. To validate DDOST as a PPP1CC2 interacting protein, we conducted a sedimentation assay using a bacterially expressed His-tagged DDOST construct (Figure 5). The assay was conducted in mouse testis lysate, as this is the only tissue in which both proteins are abundantly expressed. The His-DDOST construct utilized corresponds to amino acids 144−360 of human DDOST with an N-terminal His tag, which contains the PP1 docking motif; this region is highly conserved between human and mouse, with only five amino acid substitutions in 217 residues. His-DDOST, bound to Ni-NTA resin, was able to precipitate PPP1CC2 from mouse testis lysate, while the Ni-NTA resin alone was not. This reciprocal pull-down experiment validates the interaction between DDOST and PPP1CC2.

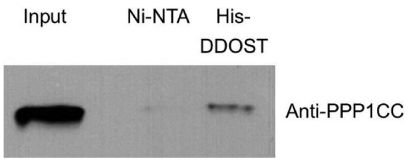

Figure 5. Validation of PPP1CC2−DDOST interaction in mouse testis lysate. Western blot using an anti-PPP1CC probe on eluates from His-DDOST and mock pull-downs. The input represents roughly 5% of the total testis protein used.

Immunolocalization of DDOST in Mouse Spermatogenic Cells. DDOST is a member of the ER membrane localized oligosaccharyltransferase (OST) complex. To the best of our knowledge, the distribution of DDOST throughout spermatogenesis has not been characterized. Therefore, immunolocalization studies of dissociated mouse spermatogenic cells were performed. Anti-DDOST staining displayed a punctate pattern in a range of germ cell types (Figure 6) that was not visible upon omission of the primary antibody (Figure 6C). While DDOST puncta were visible throughout the cytoplasm, the staining was primarily concentrated around the nucleus, most likely on the nuclear envelope as puncta were visible just outside the chromatin stained region (Figure 6A, arrows). This staining pattern was especially prevalent in spermatocytes, whereas round spermatids displayed an increase in the level of cytoplasmic staining (Figure 6A). In elongating spermatids, the level of nuclear envelope staining was reduced significantly especially in the dorsal and apical regions (Figure 6A). In a small subset of elongating spermatids, a large degree of cytoplasmic accumulation of the DDOST signal into a region distant from the nucleus was observed, which likely corresponds to the residual body in which excess cytoplasm and organelles are sequestered for elimination from the spermatid (Figure 6A, asterisk). The observed DDOST staining pattern was preserved in *Ppp1cc* knockout spermatogenic cells, from spermatocytes through the small number of morphologically abnormal elongating spermatids present in these mutants (Figure 6B). However, we did not observe the residual body-like staining pattern seen in a small number of wild-type elongating spermatids, in any *Ppp1cc* spermatids. This is likely due to the small number of these cells present in the mutants, as the amount of DDOST present in later spermatids appears to be similar to the amount in wild-type cells (Figure 6A,B, bottom rows). These results suggest that PPP1CC2 is not required for the proper localization of DDOST in the testis.

To confirm that DDOST localized to the nuclear envelope, we stained spermatogenic cells MAb414, which recognizes a family of nuclear pore complex (NPC) proteins on the nuclear envelope. MAb414 and DDOST stained the same region of the cell around the nucleus, confirming localization of DDOST to the nuclear envelope region (Figure 6C). Closer examination of DDOST and MAb414 puncta shows DDOST puncta are next to but not completely colocalized with MAb414 puncta (Figure 6C, inset), indicating that while localized to the nuclear envelope region, DDOST does not localize to the NPC.

Phosphorylation of OST Complex Member STT3B in the Mouse Testis. Localization of DDOST in the testis does not appear to be dependent on PPP1CC2 activity. This fact and the presence of the RVxF motif common to PP1 regulators suggest the possibility that DDOST serves to recruit PPP1CC2 to its subcellular locale during spermatogenesis, allowing for the dephosphorylation of similarly localized substrates. As DDOST is a member of the multiprotein OST complex, we sought to determine if any of the other members of the OST complex are Ser/Thr phosphorylated in the testis, making them potential PPP1CC2 substrates. To test this, we performed a phosphoproteomic analysis of the adult mouse testis, using SIMAC phosphopeptide enrichment. Among the recovered peptides we identified two different overlapping doubly phosphorylated peptides, ENPPVEDpSpSDEDDKR and ENPPVEDpSpSDEDDKRNPGNLYDK, corresponding to the STT3B protein, a member of the OST complex. The phosphorylated serine residues map to S495 and S496 of mouse STT3B. The localization probability of both phosphosites is 100% according to ScaffoldPTM, with Ascores of 1000 being assigned to each phosphosite. In total, these two peptides were identified 13 times throughout three SIMAC fractions, further strengthening our confidence in the phosphosite localization (see the Supporting Information for complete statistical data, annotated spectra, and fragmentation tables).

Generation of SBP-3XFLAG-PPP1CC2 Chimeric Mice. The original aim of the experiment was to identify PPP1CC2 interactions in a more biologically relevant setting, the testis, by creating SBP-3XFLAG-PPP1CC2 knock-in mice, using our engineered ES cells. The production of chimeras was performed by the Toronto Centre for Phenogenomics, using microinjection of blastocysts into C57BL6 host embryos. Two male and five female chimeras, all with a weak contribution from the knock-in cell lineage (evidenced by coat color), were generated from two different cell lines. One male chimera died before the commencement of breeding, and the second died after producing several litters. Breeding of the male chimera resulted in the production of 11 offspring derived from the ES cells, as judged by coat color, indicating that the ES cells were able to populate the germline; however, all were wild type, and none carried the SBP-3XFLAG-PPP1CC2 allele.

■ DISCUSSION

To truly understand the function of a protein in a given tissue, it is critical to define its interactome, the full complement of protein−protein interactions. Because of the recent advancements in proteomic technologies, high-throughput studies are making great inroads into defining the interactomes of many proteins. However, one major drawback of these studies is the fact that the vast majority are conducted in cell lines that may not be physiologically relevant to the question at hand and as

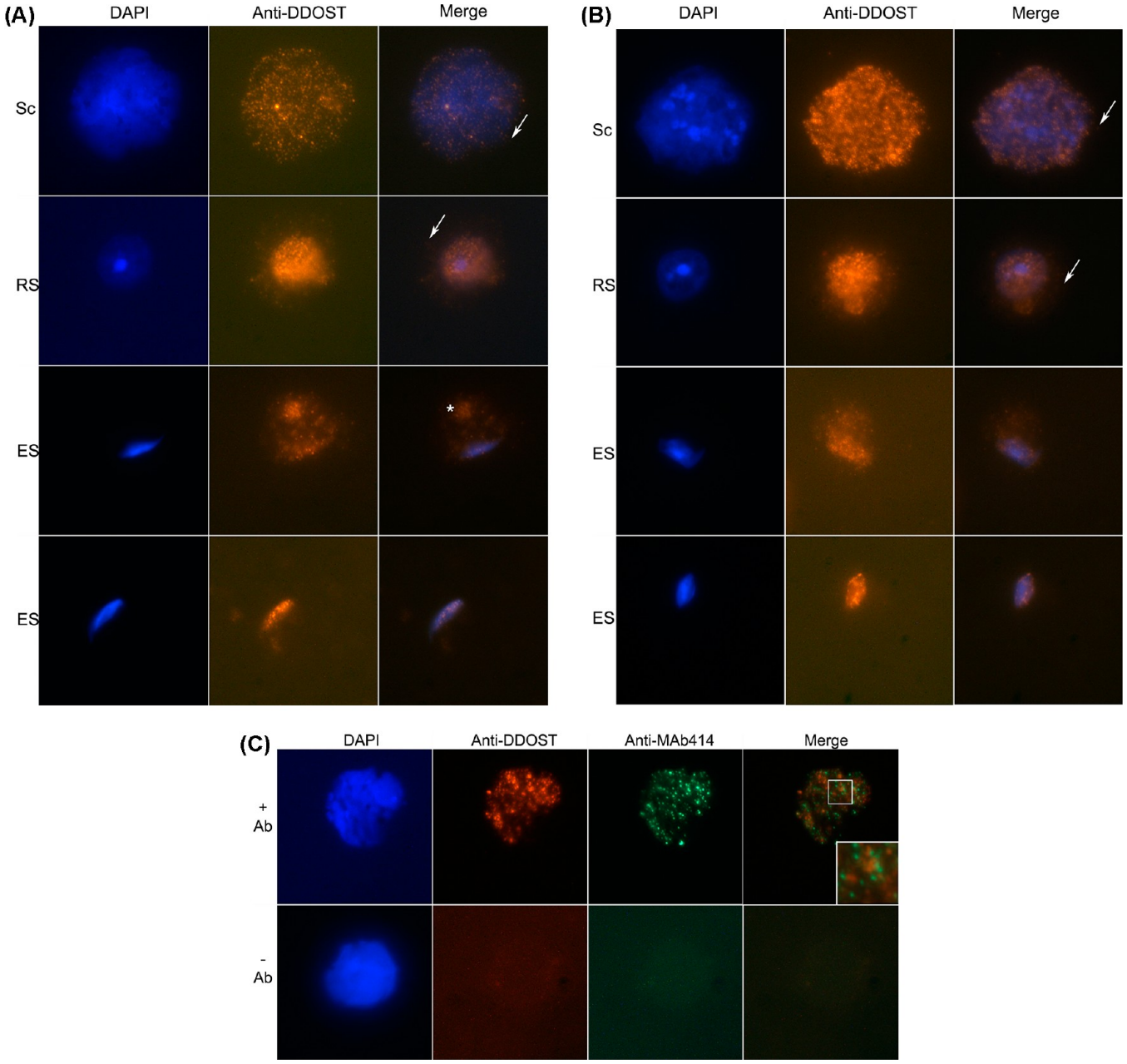

Figure 6. Localization of DDOST in dissociated spermatogenic cells. Shown are spermatocytes (Sc), round spermatids (RS), and elongating spermatids (ES). Nuclei are indicated using DAPI. Anti-DDOST was visualized with goat anti-rabbit Cy3 in wild-type (A) and *Ppp1cc* knockout (B) spermatogenic cells. Arrows indicate DDOST puncta falling outside the nuclear stained region. The asterisk indicates cytoplasmic accumulation of DDOST in the residual body. (C) Localization of DDOST to the nuclear envelope in wild-type spermatocytes using colocalization with NPC marker MAb414. +Ab indicates inclusion of primary antibodies, and −Ab indicates no primary antibody was used.

such cannot be directly translated to a protein's interactome in a specific cell type or tissue. One example of such a tissue is the testis, which cannot be modeled in tissue culture because of its complex architecture and its complement of postmeiotic cells. For this reason, we undertook to produce a system that would be useful in generating affinity-tagged knock-in ES cells that could theoretically be used to generate transgenic mice. The SBP-3XFLAG knock-in vector described in this paper, based on the floxin system, will allow for the creation of transgenic tandem affinity-tagged versions of any gene for which a pGT0lxf gene-trap line exists (more than 24000 different lines are currently available[8]).

PP1 isoform PPP1CC2 is a testis specific splice isoform that is essential in mouse spermatogenesis.[7] While approximately 200 PP1 interacting proteins have been identified,[19] the interactome of PPP1CC2 in the testis requires further study. Previous studies from our lab have sought to identify PPP1CC2 interacting proteins via approaches such as the yeast two-hybrid method.[20,21] While these approaches allowed us to identify several novel PPP1CC2 specific interacting proteins, to gain a complete picture of the PPP1CC2 interactome, we require a means of performing experiments directly in the testis. As shown above, we were able to successfully produce mouse ES cell lines expressing functional tandem affinity-tagged versions of our genes of interest. The recombinant proteins expressed by

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our transgenic ES cell lines not only expressed SBP-3XFLAG-PPP1CC1 and SBP-3XFLAG-PPP1CC2 but also were able to interact with a number of known PP1 interacting proteins. This demonstrates the utility of our system in creating such modified ES cell lines, which can easily be extended to many other genes by varying only a single cloning step. Our attempt to generate a line of transgenic mice expressing SBP-3XFLAG-PPP1CC2 was foreshortened by the early demise of the founder male. However, the fact the chimera sired 11 ES-derived offspring (all wild type), based on coat color, indicates that the ES line is capable of populating the germline. Singla et al.[8] utilized the same vector backbone and knock-in strategy and found that pluripotency was not affected by the process. Although the numbers are too low to draw any definitive conclusions, the possibility that the TAP-tagged PPP1CC2 has dominant-negative properties cannot be ruled out.

In ES cell TAP experiments, we have identified and validated a novel PP1 interacting protein, DDOST. This protein contains the classical PP1 docking motif that is found in approximately 90% of all identified PP1 binding proteins.[2] This study has demonstrated that DDOST can bind to PPP1CC2; however, its ability to bind to other PP1 isoforms has yet to be tested. DDOST is a member of the oligosaccharyltransferase complex and catalyzes the transfer of high-mannose oligosaccharides to nascent polypeptide chains across the ER membrane.[22] DDOST plays a noncatalytic role and is essential for the assembly of the OST complex.[23] One recent clinical study found that a patient's congenital disorder of glycosylation, with symptoms including severe dysfunction of multiple organs and cognitive impairment, was found to be due to a 22 bp deletion and missense mutation in DDOST.[24] In the rat testis, DDOST has been shown to be expressed throughout spermatogenesis, with its highest level of expression found at a time point corresponding to the beginning of spermatid formation.[25] Additionally, DDOST has recently been identified in the detergent resistant membrane fraction of both human and mouse spermatozoa and has a putative role in sperm−oocyte interaction and/or cell adhesion[26−28] as are other members of the OST complex RPN1 and RPN2. Despite these expression data, the question of whether DDOST plays a critical role in spermatogenesis remains unanswered.

Our results show that DDOST is expressed in a range of spermatogenic cell types and shows a prominent punctate localization to the nuclear envelope that is especially prevalent in spermatocytes. This is consistent with the known rough endoplasmic reticulum (RER) localization of DDOST, as the nuclear envelope is a specific domain of the ER[29] and spermatocyte nuclei are known to be covered with a mantle of RER that can cover up to 50% of the nuclear surface.[30] Later in spermatogenesis, the extent of localization of DDOST to the nuclear envelope appears to be reduced, with an accumulation in the cytoplasm evident, most likely corresponding to removal via the residual body, along with other excess cytoplasmic organelles and components being shed by the maturing spermatid. However, even in late elongating spermatids, a significant amount of DDOST remains present around the spermatid nucleus, and even in mature spermatozoa,[26−28] suggesting a potential role in late spermatogenesis, or fertility.

The presence of a PP1 docking motif, its membrane-bound status and the fact that its localization is unaffected by loss of PPP1CC2 in the testis, suggests the possibility that DDOST functions as a substrate targeting PIP. By sequestering a pool of PPP1CC2 to a specific region of the nuclear envelope, DDOST may bring the phosphatase into regions of close contact with potential substrates. A number of other PIPs with similar functions have been identified, including another ER protein PPP1R15A (GADD34).[31] If this is the case, obvious candidate substrates for PPP1CC2 dephosphorylation are the other members of the OST complex, such as STT3B, which we showed to be phosphorylated in the testis and has also been reported by another study of testis phosphoproteins.[32] However, at this stage, the experiments detailed in this study are preliminary in nature, and further inquiry is necessary to determine if DDOST plays an essential role in spermatogenesis and to determine the biological nature of the PPP1CC2−DDOST interaction.

In addition to the identification of DDOST as a novel PPP1CC2 interacting protein, our data indicate a significant enrichment of the classical PP1 RVxF docking motif among proteins identified by a single unique peptide in our TAP studies (see the Supporting Information). While at least two unique and nonoverlapping peptides are generally required for confident identification of a protein in such data sets, the enrichment of the RVxF motif suggests there are likely more legitimate PP1 interacting proteins within this data set. However, as a caveat, this subset of proteins will contain a high rate of false positives and any candidate interactors require validation by other means. A total of four genes within this data set are known to be required for fertility in males: *Brwd1*, *Fancl*, *Gapdhs*, and *Tial1*. All of these proteins contain the classical PP1 docking RVxF motif. *Brwd1* stands out as a particularly interesting candidate as its knockout phenotype includes a decrease in postmeiotic cell numbers, malformed sperm heads, acrosomal defects, and aberrant chromatin condensation, all of which are also seen in *Ppp1cc* mutants.[7,18,33]

The large number of proteins identified in our data set by single unique peptides could be a reflection of several factors. First, many other affinity purification−mass spectrometry (AP−MS) studies utilize vector-based expression systems for affinity-tagged genes, which often results in a significant overexpression of the bait protein and thus a larger number of interactions detected. Our system utilized the *Ppp1cc* promoter coupled to an IRES that will result in a lower level of expression of our bait construct. However, this should reflect a more endogenous expression pattern, and interactions that are detected are less likely to be due to ectopic gene expression. Another potential contributing factor is our utilization of a tandem affinity purification strategy. The use of two purification steps results in fewer contaminant proteins being identified, but at a cost of fewer legitimate interactors being identified, as weaker and less abundant interactions may be lost during the additional purification step.[5] A more physiological explanation could lie in the nature of ES cells. Being primed for differentiation, ES cells are thought to express more proteins, but at a lower abundance than differentiated cell types. This could highlight an interesting utility for ES cells in an interactome study, and comparison of the relative abundance of protein−protein interactions between different cell types could yield considerable insight into gene function.

■ ASSOCIATED CONTENT

Supporting Information

List of all proteins identified in this study by at least one significant peptide, excluding contaminant proteins, including summary results of LC−MS/MS data and the presence of the RVxF motif and known infertility KO phenotypes (Table S1);

further data on single-unique peptide LC−MS/MS hits (Table S2); annotated MS/MS spectrum and fragment assignments of known PPP1CC2 interacting protein PPP1R42, which was identified by a single unique peptide (Table S3); annotated MS/MS spectrum and fragment assignments of BRWD1 protein, which was identified by a single unique peptide (Table S4); ScaffoldPTM protein report displaying statistical information regarding STT3B phosphosite localization (Table S5), and annotated MS/MS spectra and representative fragment assignments for STT3B phosphopeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

PIPs, PP1 interacting proteins; TAP, tandem affinity purification; LC−MS/MS, liquid chromatography and tandem mass spectrometry; PBS, phosphate-buffered saline; SBP, streptavidin-binding peptide; HA, human influenza hemagglutinin; ES, embryonic stem; IRES, internal ribosomal entry site; ECMV, encephalomyocarditis virus; PCR, polymerase chain reaction; Cre, Cre-recombinase; (E)GFP, (enhanced) green fluorescent protein; cDNA, complementary DNA; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcriptase polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; PMSF, phenylmethanesulfonyl fluoride; *En2*, Engrailed2; BGH, bovine growth hormone; IP, immunoprecipitation; AP−MS, affinity purification and mass spectrometry; FRT, Flp recombinase site; AMPR, ampicillin resistance gene; pA, polyadenylation signal.

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